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Frequency of Undetected *CYP2D6* Hybrid Genes in Clinical Samples: Impact on Phenotype Prediction

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ABSTRACT:

Cytochrome P450 2D6 (*CYP2D6*) is highly polymorphic. *CYP2D6-2D7* hybrid genes can be present in samples containing *CYP2D6*4* and *CYP2D6*10* alleles. *CYP2D7-2D6* hybrid genes can be present in samples with duplication signals and in samples with homozygous genotyping results. The frequency of hybrid genes in clinical samples is unknown. We evaluated 1390 samples for undetected hybrid genes by polymerase chain reaction (PCR) amplification, PCR fragment analysis, TaqMan copy number assays, DNA sequencing, and allele-specific primer extension assay. Of 508 *CYP2D6*4*-containing samples, 109 (21.5%) harbored *CYP2D6*68 + *4*-like, whereas 9 (1.8%) harbored *CYP2D6*4N + *4*-like. Of 209 *CYP2D6*10*-containing samples, 44 (21.1%) were found to have *CYP2D6*36 + *10.* Of 332 homozygous samples, 4 (1.2%) harbored a single *CYP2D7-2D6* hybrid, and of 341 samples with duplication signals, 25 (7.3%) harbored an undetected *CYP2D7-2D6* hybrid. Phenotype before and after accurate genotyping was predicted using a method in clinical use. The presence of hybrid genes had no effect on the phenotype prediction of *CYP2D6*4*- and *CYP2D6*10*-containing samples. Four of four (100%) homozygous samples containing a *CYP2D7-2D6* gene had a change in predicted phenotype, and 23 of 25 (92%) samples with a duplication signal and a *CYP2D7-2D6* gene had a change in predicted phenotype. Four novel genes were identified (*CYP2D6*13A1 variants 1* and *2*, *CYP2D6*13G1*, and *CYP2D6*13G2*), and two novel hybrid tandem structures consisting of *CYP2D6*13B + *68×2 + *4*-like and *CYP2D6*13A1 variant 2 + *1×N* were observed.

Introduction

Cytochrome P450 2D6 (*CYP2D6*) encodes an enzyme clinically relevant to the use of tamoxifen (Goetz et al., 2005, 2007) and the selection of psychiatric drugs (Black et al., 2007). *CYP2D6* testing is sometimes done before tamoxifen treatment of breast cancer because patients who fail to metabolize tamoxifen to endoxifen via the *CYP2D6* pathway may be at increased risk of breast cancer relapse (Goetz et al., 2007). Genotyping is also used to select psychotropic medications (Topic et al., 2000; Murphy et al., 2003; Kirchheiner et al., 2004; Malhotra et al., 2004).

The *CYP2D* locus contains the *CYP2D8* and *CYP2D7* pseudogenes 5′ to the *CYP2D6* gene (Fig. 1A). *CYP2D6* genetic complexity is due to single nucleotide polymorphisms (SNPs), duplications and multiplications (Fig. 1B), deletions (Fig. 1C), and recombination events with the *CYP2D7* pseudogene (Fig. 2, A–F). The allelic nomenclature of *CYP2D6* is based upon the "star" (i.e., *) nomenclature method whereby alleles are named sequentially by the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee (http://www.cypalleles.ki.se/index.htm). Subfamilies of alleles are given the same number, but members of the family are given different suffixes. For example, *CYP2D6*4* is a subfamily with A to N members, and each has different SNPs associated with the defining SNP for *CYP2D6*4*: 1846G>A. *CYP2D6*4N* has a gene conversion to *CYP2D7* in exon 9, whereas *CYP2D6*4*-like, which is mentioned in this article, is different from *CYP2D6*4N* by the fact that it lacks a conversion to the *CYP2D7* sequence in exon 9 (Kramer et al., 2009). Genotyping platforms are not designed to detect the exact allele present in the *CYP2D6*4* subfamily so the suffix of A to N is often omitted in clinical genotyping results.

The recombinant events that occur with the *CYP2D7* pseudogene may give rise to hybrid genes, which occur singly or in hybrid tandem arrangements. Single hybrids can be of the *CYP2D6-2D7* variety, such as *CYP2D6*61*, which switches to the *CYP2D7* sequence from intron 7 onward. As an alternative, single hybrids can be of the *CYP2D7-2D6* variety. The home page of the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee has reclassified *CYP2D7-2D6* hybrid genes that contain a *CYP2D7*-derived exon 1 (see http://www.cypalleles.ki.se/cyp2d6.htm for details), and this article uses this new classification for the first time (S. C. Sim, personal communication). A key to conversion to the new nomenclature is described under *Materials and Methods*. An example of a *CYP2D7-*

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ABBREVIATIONS: SNP, single nucleotide polymorphism; ASPE, allele-specific primer extension assay; PCR, polymerase chain reaction; rt, real-time; UM, ultrarapid metabolizer; EM, extensive metabolizer; IM, intermediate metabolizer; kb, kilobase; C_T, the cycle number at which fluorescence crosses an arbitrary line in rtPCR.

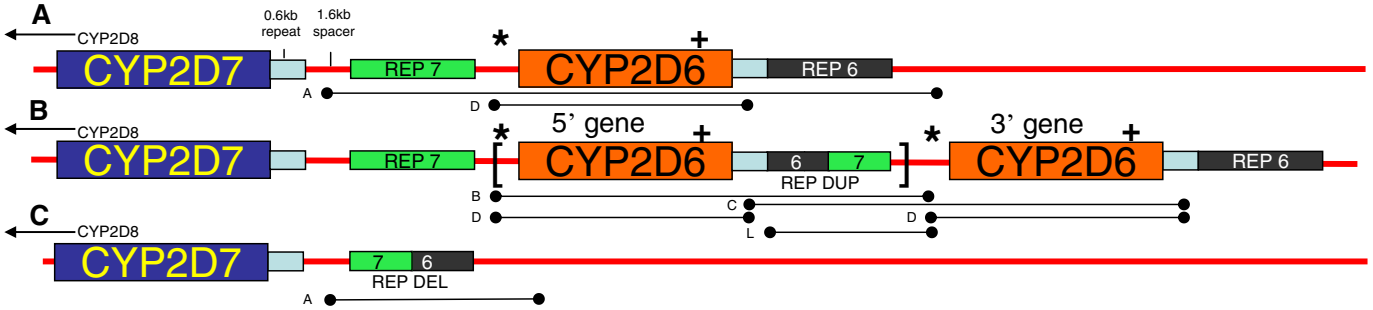

FIG. 1. *CYP2D* locus structures for single, typical duplicated and deleted arrangements. A, single *CYP2D6* arrangement. *CYP2D8* (not shown) and *CYP2D7* pseudogenes are located 5′ to the *CYP2D6* gene. Similar 0.6-kb repeats follow the *CYP2D7* and *CYP2D6* sequences as do rep 7 and rep 6, respectively, which differ by just a few nucleotides in the 5′ and 3′ regions of 5 rep. Note that the 1.6-kb spacer is located 3′ to the *CYP2D7* pseudogene, which is absent downstream of the *CYP2D6* gene. B, typical *CYP2D6* duplication arrangement. The first *CYP2D6* gene is followed by rep dup, a hybrid containing a 5′ rep 6 sequence and a 3′ rep 7 sequence. Multiplications of the sequence shown between the brackets are known to exist. C, *CYP2D6* deletion arrangement (*CYP2D6*5*), in which *CYP2D7* is followed by a rep del that is a hybrid containing a 5′ rep 7 sequence and a 3′ rep 6 sequence. PCR fragments used in this investigation are depicted as lettered lines under the structures (Table 1). Probe locations for TaqMan copy number assays are designated as * and + for the 5′ flanking *CYP2D6* assay and the *CYP2D6* intron 6 assay, respectively.

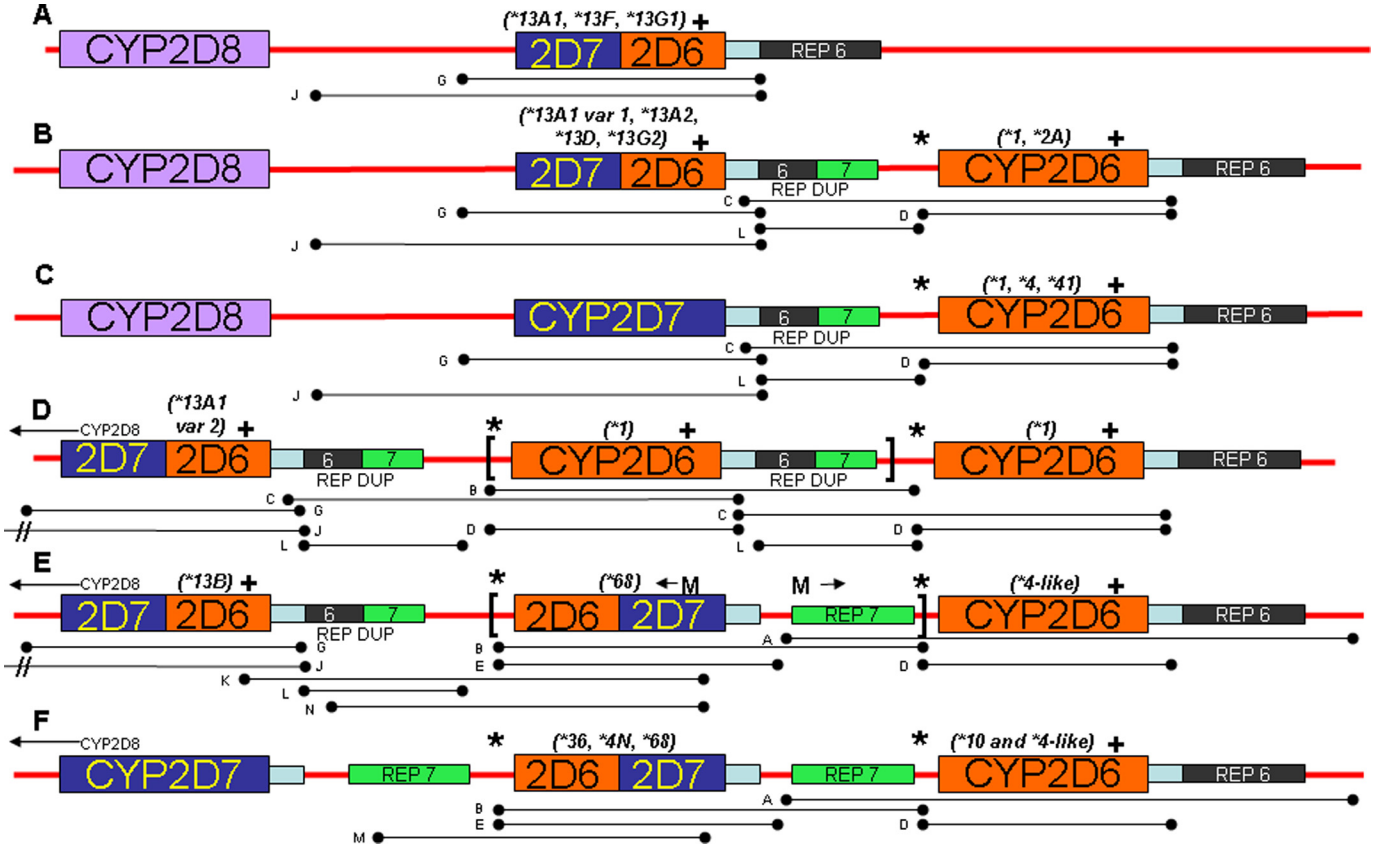

FIG. 2. *CYP2D* locus structures for single and hybrid tandems of *CYP2D7-2D6* and *CYP2D6-2D7*. A, single *CYP2D7-2D6* arrangement (e.g., *CYP2D6*13A1*, *CYP2D6*13F*, and *CYP2D6*13G1*). B, *CYP2D7-2D6* + *CYP2D6* arrangement (e.g., *CYP2D6*13A1*, *CYP2D6*13A2*, *CYP2D6*13D*, and *CYP2D6*13G2* with a tandem *CYP2D6*1, CYP2D6*2A*). Notice that the hybrid gene is followed by rep dup. C, *CYP2D7* gene in a duplication arrangement that is followed by rep dup upstream of the tandem *CYP2D6* gene (e.g., *CYP2D6*1*, *CYP2D6*4*, and *CYP2D6*41*). D, novel *CYP2D7-2D6* + *CYP2D6*×*N* hybrid tandem multiplication arrangement. In this case, the *CYP2D7-2D6* hybrid is a *CYP2D6*13A1 variant 2* and the *CYP2D6* alleles are *CYP2D6*1*. The absolute number of *CYP2D6*1* alleles in tandem could not be determined. E, novel *CYP2D7-2D6* hybrid (*CYP2D6*13B*) followed by rep dup, *CYP2D6-2D7* hybrids (*CYP2D6* 68×2*) and a *CYP2D6* gene (*CYP2D6*4*-like). The ←M and M→ designations show the relative locations of fragment M primers in this arrangement, which generates a product because the *CYP2D6-2D7* hybrid is found in tandem in this arrangement. F, *CYP2D6-2D7* + *CYP2D6* arrangement (e.g., *CYP2D6*36*, *CYP2D6*4N*, *CYP2D6*68* with a tandem, *CYP2D6*10*, and *CYP2D6*4*-like). Note that the *CYP2D6-2D7* gene is followed by rep 7 rather than rep dup. Multiduplications have multiples of the sequence shown between the brackets and were observed in D and E. PCR fragments used in this analysis are depicted as lettered lines under the structures (Table 1). Probe locations for TaqMan copy number assays are designated as * and + for the 5′ flanking *CYP2D6* assay and the *CYP2D6* intron 6 assay, respectively. Note that *CYP2D8* is thought to be present in all of the structures but is only shown in structures A to C for simplicity.

2D6 hybrid is *CYP2D6*13A1*, in which the switch from *CYP2D7* to *CYP2D6* sequence occurs in intron 1 (Fig. 2A).

Hybrid genes can also occur in tandem arrangements. We recently described the *CYP2D6*68* + **4*-like and *CYP2D6*4N* + **4*-like hybrid tandem arrangements (Kramer et al., 2009), and others described *CYP2D6*36* + **10* (Chida et al., 2002; Gaedigk et al., 2006). These three structures are of the *CYP2D6-2D7* + *CYP2D6* hybrid tandem variety (Fig. 2F). Another hybrid tandem arrangement is the

CYP2D7-2D6 + *CYP2D6* variety (Fig. 2B), an example of which is *CYP2D6*13A2* (EU530609) + **2A* (Kramer et al., 2009; Gaedigk et al., 2010a).

The aim of this study was to determine the frequency of hybrid genes present in a large number of samples originally genotyped using the Luminex Tag-It Mutation Detection Kit for Cytochrome P450 2D6 (*CYP2D6* ASPE kit) and to identify the impact of these variants on phenotype prediction. The *CYP2D6* ASPE kit does not detect hybrid genes because the kit generates A and B amplicons for the 5′ and 3′ portions of the *CYP2D6* gene, respectively. Amplicon A mix also contains additional primers that allow for the amplification of rep dup (Fig. 1B) and its subsequent detection by an ASPE primer. In the case of *CYP2D7-2D6* + *CYP2D6*, a duplication signal is detected even though a hybrid gene is present in the 5′ position (Fig. 2, B and C). *CYP2D6-2D7* and *CYP2D7-2D6* genes will cause failure of A and/or B amplicon generation during the PCR step, depending upon the location of the switch from *CYP2D6* to *CYP2D7* sequence. Because there is a tendency for the *CYP2D6* portions in the hybrid tandem gene to be identical to the *CYP2D6* portion in the normal gene (Kramer et al., 2009), the kit will not detect partial failures in the ASPE step. In the case of a single *CYP2D7-2D6* gene, total failure of PCR of the hybrid gene can occur, which can lead to a homozygous call on the basis of amplification of the nonhybrid allele. In this research, the samples studied included: 1) *CYP2D6*4*-containing samples, 2) *CYP2D6*10*-containing samples, 3) samples with homozygous genotypes, and 4) samples with duplication signal.

Materials and Methods

Samples and Initial Clinical Genotyping. A total of 1390 deidentified samples submitted to the Nucleotide Polymorphism Laboratory of the Department of Laboratory Medicine and Pathology at the Mayo Clinic for clinical *CYP2D6* genotyping using the Luminex Tag-It Mutation Detection Kit for Cytochrome P450 2D6 version 1 or 2 (hereafter named *CYP2D6* ASPE kit version 1 or version 2, respectively; Luminex Corporation, Austin, TX) were studied with Mayo Clinic institutional review board approval. The samples were submitted from practices both internal and external to the Mayo Clinic system, but all were submitted to predict tolerability and response to either tamoxifen or psychotropic drugs. Samples were submitted from all over the world, and because they were deidentified, the ethnic and racial mixture of these samples could not be determined. To meet the criteria for study, a sample needed to have 1) at least one *CYP2D6*4* allele ($n = 508$), 2) at least one *CYP2D6*10* allele ($n = 209$), 3) a homozygous genotype ($n = 332$), or 4) a duplication signal ($n = 341$).

The *CYP2D6* ASPE kit v2 detects the following *CYP2D6* recombinants and alleles: gene duplication, gene deletion (**5*), −1584C>G (**2A*), 100C>T (**4* and **10*), 124G>A (**12*), 138insT (**15*), 883G>C (**11*), 1023C>T(**17*), 1661G>C (**2*, **4*, **17*, and others), 1707T>del (**6*), 1758G>T/A (**8*, **14*), 1846G>A(**4*), 2549A>del (**3*), 2613–2615 del AGA (**9*), 2850C>T (**2*, **17*, **41*, and others), 2935A>C (**7*), 2988G>A (**41*), and 4180G>C (**2*, **4*, **17*, and others). The *CYP2D6* ASPE kit v1 lacked the 138insT, 1661G>C, 1758G>A, 2988G>A, and 4180G>C polymorphisms. All samples originally genotyped with the v1 kits that contained a *CYP2D6*2* allele with duplication were retested using the v2 kit to identify *CYP2D6*41* alleles. The 138insT and 1758G>A have a very low frequency, because we have found 4 and 2 alleles, respectively, in our experience of more than 11,000 samples; thus, we did not retest our samples for these. The 1661G>C and 4180G>C polymorphisms are part of several alleles and were added to the *CYP2D6* ASPE kit v2 to help confirm allelic calls; thus, retesting for these was not done. In 240 of 285 heterozygous duplication samples, the duplicated allele was predicted on the basis of the mutant allelic ratios as established for the clinical assay.

DNA Extraction. DNA was extracted from blood using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) with the method described in the product literature.

PCR and Amplicon Verification. *CYP2D6-2D7* and *CYP2D7-2D6* genes were detected by PCR using primers specifically designed to allow them to be amplified as described in Kramer et al. (2009) (Table 1; Figs. 1 and 2). The PCR primers used in this study were selected using Oligo Primer Analysis Software (version 6.71; Molecular Biology Insights Inc., Cascade, CO). PCR amplicon locations are shown in Figs. 1 and 2 as black bars under each structure. Specific primers were designed to prevent unintentional nonspecific annealing between the *CYP2D6* gene, *CYP2D7* pseudogene, and *CYP2D8* pseudogene. All samples were screened for *CYP2D6-2D7* genes using amplicon E and *CYP2D7-2D6* genes using amplicon G, regardless of the original genotype.

The PCR Master Mix was composed of 12-μl reactions containing 0.12 μl of LA Taq HS (5 U/μl; TaKaRa Bio Inc., Otsu, Shiga, Japan), 1.2 μl of 10× LA PCR buffer II (25 mM Mg^{2+}), 2 μl of dNTP mixture (2.5 mM each), 6.48

TABLE 1

PCR primers and amplicon characteristics

The primers shown were used to generate PCR amplicons for testing by allele-specific primer extension assay or DNA sequencing or for PCR fragment analysis. Refer to Figs. 1 and 2 for approximate annealing sites of these primers on the various genomic structures.

PCR Product	Sequence	Arrangement Amplified	Size
			base pairs
A	GTCCCACACCAGGCACCTGTACT GAATTAGTGGTGGTGGGTGTTTG	Nonduplicated gene and deletion arrangement	15,629, 3471
B	TCACCCCCAGCGGACTTATCA CCACAGCCCTCAATAAGTGAA	5′ gene of duplication arrangement	12,052
C	CACCACCCTCAGCCTCGTC TAGGTAGCCCTGGCCTATAGCTCCCTGACGCC	3′ gene of duplication arrangement	12,103
D	TTGCCACATTATCGCCCGTGAAA TAGGTAGCCCTGGCCTATAGCTCCCTGACGCC	Any normal arrangement of *CYP2D6*	8433
E	TCACCCCCAGCGGACTTATCA TACGGTGGGCTCCCTGCGAG	*CYP2D6-2D7* gene	6714
G	CCTGTGTGGGCTTGGGGAGCTTG TGTGGTGAGGTGACGAGGCTGA	*CYP2D7-2D6* gene	5742
J	ACCTGGACGCCTGACTTTA TAGGTAGCCCTGGCCTATAGCTCCCTGACGCC	PCR fragment from *CYP2D8*-unique 3′ region to *CYP2D6* 3′ region	9525
K	GCCACCATGGTGTCTTTGCTTTC AAAGCTGACGACACGAGAGTGGCT	PCR fragment from exon 9 in *CYP2D6* to exon 9 in *CYP2D7*	12,152
L	TCAGCCTCGTCACCTCACCACAGG CCACAGCCCTCAATAAGTGAA	Any rep dup region	5410
M	CCTCAGGGATGCTGCTGTCTG AAAGCTGACGACACGAGAGTGGCT	PCR fragment from a unique region in rep 7 to a *CYP2D7*-specific exon 9 sequences and only amplifies if there are two rep 7 regions	11,432
N	CCTCAGGGACGCTGCTGTACA AAAGCTGACGACACGAGAGTGGCT	PCR fragment from a unique region in rep 6 to a *CYP2D7*-specific exon 9 sequence	11,432

μl of water, and 1 μl of betaine monohydrate (16.25 M; Fluka Biochemical, Steinheim, Germany), 0.5 μl of each primer, and 0.2 μl of genomic DNA (250 ng/μl).

Amplicons A to E, G, and J (Table 1) were generated as described by Kramer et al. (2009). Thermocycler parameters for amplicon K were as follows: 94°C for 1 min followed by 96°C for 10 s, 64°C for 30 s, 68°C for 11 min for 30 cycles, and a final extension at 72°C for 10 min and a 4°C hold. Thermocycler parameters for fragment L were as follows: 94°C for 1 min followed by 35 cycles of 96°C for 10 s and 68°C for 4 min, a final extension at 72°C for 10 min, and a 4°C hold. Thermocycler parameters for fragment M and N were as follows: 94°C for 1 min followed by 30 cycles of 96°C for 10 s and 68°C for 9 min, a final extension at 72°C for 10 min, and a 4°C hold.

PCR fragment sizes were analyzed using an Agilent Technologies DNA 12000 Kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocol. Expected fragment sizes are shown in Table 1.

Genotyping by Allele-Specific Primer Extension Assay. Genotyping of amplicons A to E, G, and J to M (Table 1) was performed using the *CYP2D6* ASPE kit v2 as described in the product literature but without the original amplification step using the A and B primer sets. All samples were analyzed on the Luminex 100 IS device using IS 2.3 software (Luminex).

DNA Sequencing. DNA sequencing was performed using an Applied Biosystems 3730xl DNA Analyzer and the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Foster City, CA). All *CYP2D7-2D6* genes (amplicon G) and their associated rep dup regions (amplicon L) were sequenced bidirectionally using multiple overlapping amplicons. In addition, a unique structure containing *CYP2D6*13B + *68×2 + *4*-like was discovered (described below) and was sequenced from exon 9 of *CYP2D6*13B* through exon 9 of *CYP2D6*68* using amplicons E, G, and K. The source of DNA for all sequencing was genomic DNA isolated from blood, and amplicons were generated per PCR section noted previously.

Real-Time PCR *CYP2D6* Copy Number Assays. The *CYP2D6* copy number in samples containing both a *CYP2D7-2D6* gene and a duplication signal was determined using two real-time (rt) PCR assays, which targeted different regions of the gene (Figs. 1 and 2). This assay was done only on the duplication samples because accurate copy number determination was needed to predict phenotype. Phenotype prediction for *CYP2D6*4*- and *CYP2D6*10*-containing samples is not affected by copy number as will be discussed below; thus, copy number was not determined for these samples. One assay, Hs04502391_cn (intron 6) was commercially available (Applied Biosystems). The other assay targeting the 5′ flanking region as described by Hosono et al. (2009) was synthesized and purchased from Applied Biosystems. Both assays were performed in triplicate with an internal control RNaseP TaqMan copy number reference and TaqMan Genotyping PCR Master Mix on a StepOnePlus rtPCR instrument as directed by the manufacturer (Applied Biosystems). Relative quantification was performed using CopyCaller software (Applied Biosystems) following the comparative $\Delta\Delta C_T$ method. The confidence estimate (the probability that the calculated copy number is the correct assignment compared with other copy numbers that have nonzero probability of occurring) for a given sample was automatically generated. CopyCaller software uses the copy number and observed ΔC_T values of a sample to calculate the confidence estimate.

Bioinformatics Tools. DNA sequence data were compiled using Sequencher software (version 4.9; Gene Codes Corp., Ann Arbor, MI) and Mutation Surveyor (version 3.13; SoftGenetics LLC, State College, PA). GenBank entry M33388 served as a reference sequence for *CYP2D6*, and GenBank entry NC_000022, the reference sequence for chromosome 22, served as reference sequences for *CYP2D7* and *CYP2D8*. The latter sequence was used because of sequencing variations in the original reference sequence for *CYP2D7* and *CYP2D8*, M33387. Comparisons were performed using the Specialized BLAST program, bl2seq (http://blast.ncbi.nlm.nih.gov/) and CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw/index/html) at default settings.

***CYP2D6* Allelic Nomenclature.** Sequences were compared with the *CYP2D6* alleles listed by the *CYP2D6* nomenclature committee (http://www.cypalleles.ki.se/cyp2d6.htm). The home page of the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee has reclassified *CYP2D7-2D6* hybrid genes that contain a *CYP2D7*-derived exon 1 (see http://www.cypalleles.ki.se/cyp2d6.htm for details), and this article uses this new classification for the first time. For this reason, all *CYP2D7-2D6* and *CYP2D7-2D6 + CYP2D6* alleles are designated *CYP2D6*13A1-*13H* according to this new nomenclature. The suffixes designate the switch location of the *CYP2D7* to *CYPD6* sequence. Examples of alleles discussed in this article include *CYP2D6*13A1* and *CYP2D6*13A2* (formerly *CYP2D6*13* and *CYP2D6*77*, respectively), which switch from *CYP2D7* to *CYP2D6* in intron 1. *CYP2D6*13B* (formerly CYP2D6*79) switches in exon 2, *CYP2D8*13D* (formerly *CYP2D6*78*) switches in intron 4, *CYP2D6*13F* (formerly *CYP2D6*16* and *CYP2D6*66*) switches in intron 7 to intron 8, and *CYP2D6*13H* (formerly *CYP2D6*76*) switches to *CYP2D6* in exon 9.

Phenotype Prediction. Phenotype prediction was based on the genotype and the activity listed on the *CYP2D6* Allele Nomenclature Web page (http://www.cypalleles.ki.se/cyp2d6.htm) and the literature cited below. Phenotype prediction was binned into four categories in a manner similar to that reviewed by Ingelman-Sundberg (2005) and Kirchheiner et al. (2004). It should be noted that other methodologies for phenotype prediction have been described, and this is a controversial area of pharmacogenomics (Steimer et al., 2004; Gaedigk et al., 2008). In particular, there is controversy about where to classify the *CYP2D6*2A* versus other *CYP2D6*2* alleles. There is evidence that the *CYP2D6*2* alleles (except *CYP2D6*2A*) have reduced function, although this is somewhat substrate-dependent (Raimundo et al., 2000, 2004; Bapiro et al., 2002; Yu et al., 2002; Abduljalil et al., 2010). However, the c.1584C>G polymorphism found in *CYP2D6*2A* increases protein production, possibly through increased induction, which compensates for the reduced function caused by the other polymorphisms found in the *CYP2D6*2* alleles, resulting in a function similar to and possibly greater than that of *CYP2D6*1* (Løvlie et al., 2001; Zanger et al., 2001).

The classification used here is as follows: an ultrarapid metabolizer (UM) had more than two normally functioning alleles (*CYP2D6*1* or *CYP2D6*2A*). An extensive metabolizer (EM) had two normally functioning alleles or one normally functioning allele and two reduced function alleles (e.g., *CYP2D6*2*, *CYP2D6*10*, *CYP2D6*17*, and *CYP2D6*41*) or two normally functioning alleles and a reduced function allele. An intermediate metabolizer (IM) had one normally functioning allele and either a reduced function allele or a null allele (e.g., *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6*, or single *CYP2D7-2D6* gene). Samples with two or three reduced function alleles were also considered intermediate metabolizers. A poor metabolizer had only null alleles or a null allele plus a reduced function allele.

Results

Summary of Hybrid Genes Found in *CYP2D6*4*, *CYP2D6*10*, Duplication, and Homozygous Samples. Overall, 8.5% of the 1390 samples studied had *CYP2D6*4N + *4*-like or *CYP2D6*68 + *4*-like, 3.2% had *CYP2D6*36 + *10*, 0.3% had a single *CYP2D7-2D6* gene, and 1.8% had a *CYP2D7-2D6 + CYP2D6*, *CYP2D7-CYP2D6 + CYP2D6×N*, or *CYP2D7-2D6 + CYP2D6-2D7×2 + CYP2D6* hybrid tandem arrangement (described below) (Table 2). However, this set of samples was enriched for *CYP2D6*4*, *CYP2D6*10*, homozygous samples, and samples with duplication signals. Therefore, the overall frequency is expected to be different in the general population, which should contain a random variety of alleles.

Of samples with either a heterozygous or homozygous *CYP2D6*4* allele ($n = 508$), 9 (1.8%) had *CYP2D6*4N + *4*-like and 109 (21.5%) had *CYP2D6*68 + *4*-like. Of samples with a heterozygous or homozygous *CYP2D6*10* allele ($n = 209$), 44 (21.1%) had *CYP2D6*36 + *10* (Fig. 2F). For homozygous *CYP2D6*4* and *CYP2D6*10* samples, we did not determine whether one or both chromosomes contained a hybrid tandem arrangement. To determine the risk that an individual *CYP2D6*4* or *CYP2D6*10* allele is actually in a hybrid tandem arrangement, we also calculated the percentage of heterozygous samples that had a hybrid tandem. Of *CYP2D6*4* heterozygotes ($n = 482$), 7 (1.5%) harbored *CYP2D6*4N + *4*-like and 83 (17.2%) harbored *CYP2D6*68 +*

TABLE 2

Frequency of hybrid genes per sample type

The frequency of hybrids genes in *CYP2D6*4* and *CYP2D6*10* samples is reported for both heterozygous or homozygous and heterozygous alone. We did not determine whether one or both chromosomes contained a hybrid tandem in homozygous samples. For this reason, to determine the risk that a *CYP2D6*4* or *CYP2D6*10* allele is actually in a hybrid tandem arrangement, we determined the percentage of heterozygous samples for each, which had a hybrid tandem. The "Any homozygous genotype" row included any sample with an initial homozygous genotype except those with duplications. Finally, the frequency of hybrid genes in samples originally genotyped as having duplications is shown. The percentage of hybrid alleles in the 1390 samples is also shown by category, although note that the sample set was not from a random population but was purposefully enriched for samples with *CYP2D6*4*, *CYP2D6*10*, duplication, and homozygous genotypes; thus, frequency of hybrid genes in a general population is likely to be different.

Category	*n*	%
Heterozygous or homozygous *CYP2D6*4*	508	
Containing *CYP2D6*4N* + **4*-like	9	1.8
Containing *CYP2D6*68* + **4*-like	109	21.5
Percentage of total samples (*n* = 1390) with *CYP2D6*4N* + **4*-like or *CYP2D6*68* + **4*-like (*n* = 118)		8.5
Heterozygous *CYP2D6*4*	482	
Containing *CYP2D6*4N* + **4*-like	7	1.5
Containing *CYP2D6*68* + **4*-like	83	17.2
Heterozygous or homozygous *CYP2D6*10*	209	
Containing *CYP2D6*36* + **10*	44	21.1
Percentage of total samples (*n* = 1390) with *CYP2D6*36* + **4*-like (*n* = 44)		3.2
Heterozygous *CYP2D6*10*	193	
Containing *CYP2D6*36* + **10*	28	14.5
Any homozygous genotype	332	
Containing *CYP2D7-2D6* hybrid genes	4	1.2
Percentage of total samples (*n* = 1390) with *CYP2D7-2D6* hybrid gene (*n* = 4)		0.3
Any duplication sample	341	
Containing *CYP2D7-2D6* + *CYP2D6* hybrid tandem present	25	7.3
Percentage of total samples (*n* = 1390) with *CYP2D7-2D6* + *CYP2D6* or novel structure (*n* = 25)		1.8

**4*-like. Of *CYP2D6*10* heterozygous samples (*n* = 193), 28 (14.5%) harbored *CYP2D6*36* + **10*.

Of samples homozygous for any allele (*n* = 332), 4 (1.2%) had a *CYP2D7-2D6* gene. Of samples with a duplication signal (*n* = 341), 25 (7.3%) contained a *CYP2D7-2D6* + *CYP2D6* hybrid tandem or similar arrangement as discussed below.

Characteristics of 5′ Genes in *CYP2D7-2D6* + *CYP2D6* Hybrid Tandem. Table 3 shows the actual 5′ genes present in the *CYP2D7-2D6* + *CYP2D6* hybrid tandem we observed. DNA sequence analysis was done for all of the 5′ genes found in this research. In seven samples, the DNA sequence analysis revealed that the 5′ gene was actually a *CYP2D7* that converted to a *CYP2D6* sequence in the 0.6-kb repeat located just downstream of the *CYP2D7* sequence and upstream of the rep dup sequence (Figs. 1A and 2C). Four *2D7* + *CYP2D6*1* (cases 1, 10, 20, and 22), two *2D7* + **41* (case 4 and 16), and one *2D7* + *CYP2D6*4* (case 8) were observed.

Twelve *CYP2D6*13A2* + **2A,* one *CYP2D6*13A1* (*variant 1*) + **1* (case 7), one *CYP2D6*13A1* (*variant 2*) + *CYP2D6*1×N* (case 15), two *CYP2D6*13D* + **2A* (cases 13 and 18), one *CYP2D6*13G2* + **1* (case 5), and one *CYP2D6*13B* + **68×2* + **4* (case 19) were also observed. GenBank entry numbers for *CYP2D6*13A1 variant 1* and *variant 2* are HQ670230 and HQ670231, respectively. *CYP2D6*13A1 variant 1* has intron 4 c.667–149G, *CYP2D6*13A1* has a T and *variant 1* has an intron 5 c.844–49G, and *CYP2D6*13A1* has a T. *CYP2D6*13A1 variant 2* has only the intron 4 c.667–149G. (Note that locations are derived from the *CYP2D6* reference sequence M33388.) An additional novel allele was identified, which was assigned *CYP2D6*13G2* by the nomenclature committee, GenBank entry number HQ670229). In this sample, the conversion from *CYP2D7* to *CYP2D6* occurred in intron 7 on the basis of sequence data and comparison to *CYP2D7* and *CYP2D6* sequence data, although it also contains one *CYP2D7*-like SNP in exon 8.

To confirm the presence of a rep dup in each of these samples, DNA sequence analysis was performed using amplicon L (Table 1; Figs. 1B and 2, B–D). In every case, the first 53 nucleotides of rep dup contained the five nucleotides specific to rep 6 and the last 39 nucleotides of rep dup contained the 4 nucleotides that define rep 7 (Steen et al., 1995; Soyama et al., 2006). Absence of the *CYP2D7* pseudogene 5′ to all *CYP2D7-2D6* hybrids was confirmed by the presence and size of fragment J (Fig. 2, B–E), which cannot be generated if *CYP2D7* is present because of the size of the amplicon.

Table 4 summarizes the frequency with which a given duplicated allele was found to have an associated *CYP2D7-2D6* gene. A *CYP2D7-2D6* hybrid tandem was present in 7 of 91 (7.7%) samples involving a *CYP2D6*1* allele. Likewise, a hybrid tandem was present in 14 of 98 (14.3%) of samples involving a *CYP2D6*2A*, 2 of 25 (8%) alleles involving a *CYP2D6*4* allele, and 2 of 4 (50%) samples involving a *CYP2D6*41* allele. Of note, there were 20 samples in which a *CYP2D6*2×N* (not *CYP2D6*2A×N*) was predicted to be duplicated, and none of them were found to have a hybrid tandem. Although these numbers are small, the risk of an undetected hybrid tandem being present in a sample appears to be greatest in those samples in which the *CYP2D6*1, CYP2D6*2A, CYP2D6*4,* and *CYP2D6*41* is predicted to be duplicated using the *CYP2D6* ASPE kits as described here.

Characteristics of Single *CYP2D7-2D6* Genes. DNA sequencing was performed on all homozygous samples that were found to contain a single *CYP2D7-2D6* gene (Fig. 2A). Of these, three were found to be *CYP2D6*13F* and one was found to have a novel allele named *CYP2D6*13G1* (JN618990).

Gene Copy Numbers of *CYP2D7-2D6* + *CYP2D6* Hybrid Tandem. The *CYP2D6* copy number was determined for all samples that gave a duplication signal and were determined to have a *CYP2D7-2D6* + *CYP2D6* arrangement (Table 3). rtPCR assays were performed, which used one primer pair plus a TaqMan probe to target the intron 6 region of C*YP2D6* and another primer pair plus TaqMan probe to target the 5′ flanking region of the *CYP2D6* promoter region (Figs. 1 and 2). *CYP2D7-2D6* genes were detected on the basis that the copy number varied between the two regions. In particular, *CYP2D7-2D6* genes have a *CYP2D7* promoter region so the 5′ flanking probe will not bind there in distinction to any *CYP2D6* genes present in a sample. *CYP2D7-2D6* genes that switch to *CYP2D6* sequence upstream of intron 6 have a binding site for the intron 6 probe. Therefore, early crossing *CYP2D7-2D6* genes (e.g., *CYP2D6*13A1*, *CYP2D6*13A1 variant 1* and *variant 2*, *CYP2D6*13A2*, *CYP2D6*13D*, and *CYP2D6*13B*), which convert to the *CYP2D6* sequence upstream of intron 6 were detected with the intron 6 probe but not with the 5′ *CYP2D6* probe. The *CYP2D7-2D6* gene, which was not detected by the intron 6 probe, was *CYP2D6*13G2* because of its conversion to *CYP2D6* in intron 7. Neither probe detected instances in which the 5′ *CYP2D7* pseudogene converted to the *CYP2D6* trailing sequence in the 0.6-kb repeat region just downstream of *CYP2D7* and upstream of the rep dup sequence.

CYP2D6-2D7 hybrids (cases 19 and 24) were detected with the *CYP2D6* 5′ probe because they have a *CYP2D6* promoter sequence (Fig. 2F). The intron 6 assay did not yield an amplification product from the *CYP2D6*68* hybrid because this allele switched to the *CYP2D7* sequence in intron 1. Copy numbers for each assay were as expected for all but two samples (Table 3). Five to six copies of *CYP2D6* were observed with both the intron 6 and the *CYP2D6* promoter probes for case 15, indicating that *CYP2D6*1* is multiplied, but the total number could not be determined. The product

TABLE 3

Characteristics of duplication samples found to contain CYP2D7-2D6 genes

In the original *CYP2D6* genotype column, parentheses around the star alleles designate samples in which the duplicated allele could not be determined in the clinical assay. Correspondingly, this led to some ambiguity in the original phenotype prediction for a subset of samples. Samples originally genotyped as a *CYP2D6*2* that were later found to be *CYP2D6*41* are designated with a #. This occurred because of limitations of the *CYP2D6* ASPE kit version 1. Subsequent testing was done with the *CYP2D6* ASPE kit version 2, which does detect *CYP2D6*41*. TaqMan copy number assay probe locations for these structures are found in Figs. 1 and 2.

Case No.	Original *CYP2D6* Genotype	Copy Number per TaqMan Results		Actual *CYP2D6* Genotype	Predicted Phenotype Comparing Original Genotype to Actual Genotype
		CYP2D6 Promoter Assay	*CYP2D6* Intron 6 Assay		
1	**1/*1×N*	2	2	**1/2D7 + *1*	UM to EM
2	**2A×N/*17*	2	3	**13A2 + *2A/*17*	EM to IM
3	**1/*2A×N*	2	3	**1/*13A2 + *2A*	UM to EM
4	*(*2A/*41)×N#*	2	2	*2D7 + *41/*2A*	EM or IM to IM
5	**1×N/*2A*	2	2	**13G2 + *1/*2A*	UM to EM
6	**2A/*2A×N*	2	3	**2A/*13A2 + *2A*	UM to EM
7	**1×N/*5*	1	2	**13A1 variant 1 + *1/*5*	EM to IM
8	**2A×N/*4*	2	2	**2A/2D7 + *4*	EM to IM
9	**2A/*2A×N*	2	3	**2A/*13A2 + *2A*	UM to EM
10	**1×N/*2A*	2	2	*2D7 + *1/*2A*	UM to EM
11	**2A/*41×N#*	2	3	**13A2 + *2A/*41*	EM or IM to IM
12	**2A×N/*4*	2	3	**13A2 + *2A/*4*	EM to IM
13	*(*2A/*41)×N#*	2	3	**13D + *2A/*41*	EM or IM to IM
14	*(*2A/*4)×N*	2	3	**13A2 + *2A/*4*	EM or IM to IM
15	**1/*1×N*	5–6[a]	5–6[b]	**1/*13A1 variant 2 + *1×N*	UM (no change)
16	**41/*41×N*	2	2	**41/2D7 + *41*	IM (no change)
17	**2A/*2A×N*	2	3	**2A/*13A2 + *2A*	UM to EM
18	*(*1/*2A)×N*	2	3	**1/*13D + *2A*	UM to EM
19	*(*1/*4)×N*	4[c]	3	**1/*13B + *68×2 + *4*	EM or IM to IM
20	*(*1/*41)×N#*	2	2	*2D7 + *1/*41*	EM or IM to IM
21	*(*1/*2A)×N*	2	3	**1/*13A2 + *2A*	UM to EM
22	*(*1/*6)×N*	2	2	*2D7 + *1/*6*	EM or IM to IM
23	**2A/*2A×N*	2	3	**2A/*13A2 + *2A*	UM to EM
24	*(*2A/*4)×N*	3	3	**13A2 + *2A/*68 + *4*	EM or IM to IM
25	*(*2A/*41)×N*	2	3	**13A2 + *2A/*41*	EM or IM to IM

Confidence estimates for the copy number assays were all >0.99 with the following exceptions: [a]0.56; [b]<0.50; [c]0.85.

TABLE 4

Frequency of CYP2D7-2D6 genes in tandem to specific CYP2D6 alleles

This table shows the frequency for which a predicted duplicated allele was found to have a *CYP2D7-2D6 + CYP2D6* arrangement. Star alleles predicted to be duplicated using the *CYP2D6* ASPE kits are shown in column 1.

Predicted Duplicated *CYP2D6* Allele	*n*	*CYP2D7-2D6* Gene Present	%
**1*	91	7	7.7
**2*	20	0	0.0
**2A*	98	14	14.3
**4*	25	2	8.0
**9*	1	0	0.0
**10*	1	0	0.0
**41*	4	2	50.0
Total	240	25	

literature states that decreased confidence estimates are observed with increased copy numbers. In addition, case 19 had three and four copies with the intron 6 and *CYP2D6* promoter assay, respectively, suggesting two copies of *CYP2D6*68*. Cases 15 and 19 are discussed in detail below.

Novel *CYP2D7-2D6 + CYP2D6×N* Hybrid Tandem. We could not reliably determine the number of *CYP2D6* genes by rtPCR in case 15 (Table 3). We expected that the intron 6 assay would show one more copy than the *CYP2D6* promoter assay, because the *CYP2D6*13A1* gene would not be detected by the TaqMan probe in the promoter region. The presumed reason for this finding is that reliability of rtPCR to detect copy numbers higher than 5 is poor (see product literature). The structure of this sample was supported by *CYP2D6* ASPE kit analysis of fragments A and B (Table 1; Fig. 2D), both of which were genotyped as *CYP2D6*1*. This is the first time that a *CYP2D7-2D6* hybrid gene has been reported as part of a multiplied *CYP2D6* arrangement.

Novel *CYP2D7-2D6 + CYP2D6-2D7×2 + CYP2D6* Hybrid Tandem. The results from PCR fragment analysis (Fig. 3), the *CYP2D6* ASPE kit, sequence analysis (amplicons E, G, and K), and copy number assays were compiled to generate the structure of case 19 (Table 3; Fig. 2E), a novel *CYP2D7-2D6 + CYP2D6-2D7×2 + CYP2D6* hybrid tandem. The presence of *CYP2D6*13B* followed by *CYP2D6*68* on the same chromosome was confirmed by the amplification of fragment K (Fig. 3, lane 7), which spans from exon 9 in *CYP2D6* to exon 9 in *CYP2D7*. This amplicon was sequenced using multiple amplicons and primers to ensure continuity between the *CYP2D6*13B* and *CYP2D6*68* alleles. In addition, four copies of the *CYP2D6* promoter region were observed with the rtPCR *CYP2D6* promoter copy number assay, consistent with two *CYP2D6*68* alleles plus *CYP2D6*4* on one chromosome and *CYP2D6*1* on the other chromosome, whereas three copies of *CYP2D6* intron 6 were found using the intron 6 copy number assay. This occurred because *CYP2D6*13B* contains *CYP2D6* intron 6 as does *CYP2D6*4* on the same chromosome and *CYP2D6*1* on the other chromosome, but *CYP2D6*68*, which converts to *CYP2D7* in intron 1, is not detected by the intron 6 copy number assay. The presence of tandem *CYP2D6*68* alleles was confirmed by PCR amplification using forward primers located in the rep 7 (fragment M) and rep 6 (fragment N) regions of the gene with a reverse primer located in exon 9 of *CYP2D7* (Fig. 3, lane 10). These amplifications would occur only if both rep dup and rep 7 each were followed by a *CYP2D6*68* (*CYP2D6-2D7*) arrangement (Fig. 2E). Original genotyping of case 19 yielded a *CYP2D6*1/*4* genotype. This genotype is consistent with the arrangement shown in Fig. 2E because the locations of the Luminex kit primers allows PCR amplification of both the normal *CYP2D6* (Fig. 1A) as well as the 3′ most *CYP2D6* structure in the novel arrangement (Fig. 2E). It should be noted that although

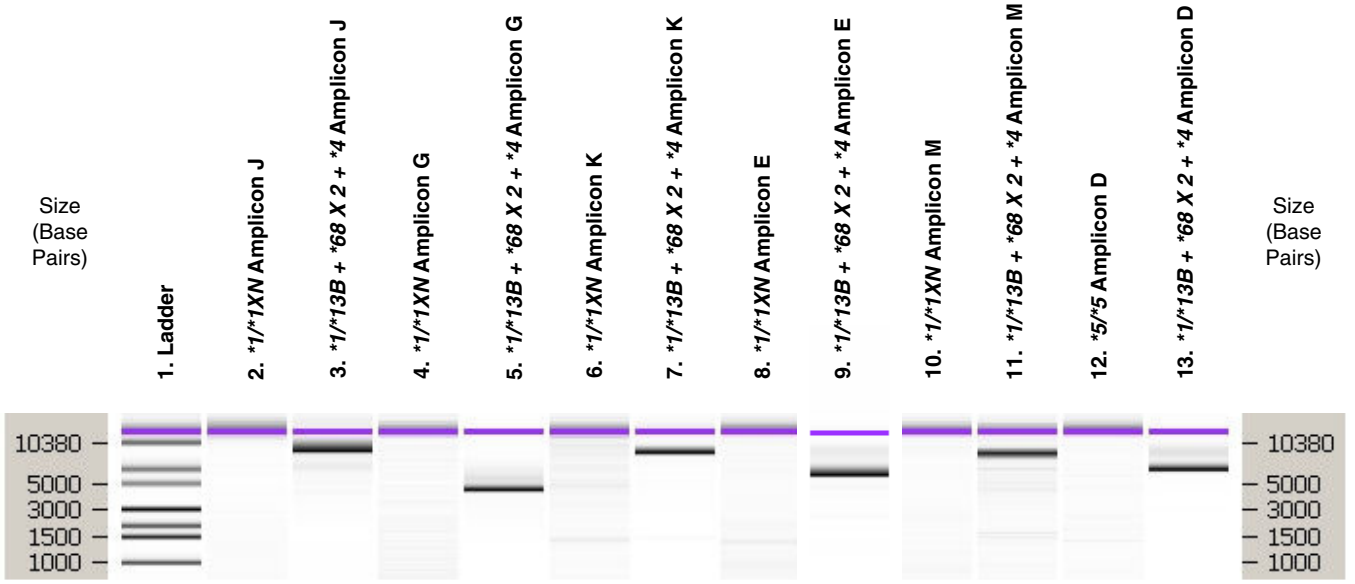

FIG. 3. Amplicon analysis of novel *CYP2D6*13B + *68×2 + *4*. PCR fragment analysis for case 19 (Fig. 2E). PCR primers and expected fragment sizes for products D, E, G, J, K, and M are listed in Table 1. Fragment analysis was performed using the Agilent Technologies DNA 12000 kit. The ladder in lane 1 shows the size in base pairs of the molecular weight marker. For each amplicon, a negative control is shown first followed by amplicons generated for case number 19. Amplicon J (lanes 2 and 3) primed from the trailing sequence of *CYP2D8* to the beginning of rep 6 or rep dup. When the *CYP2D7* pseudogene was present, no amplicon was generated because of the lengthy span of DNA. In this case (lane 3), a fragment was generated indicating that *CYP2D7* was absent. Amplicon G (lanes 4 and 5) uniquely amplified the *CYP2D7-2D6* gene (*CYP2D6*13B*) and was used in sequencing. Amplicon K (lanes 6 and 7) primed from the *CYP2D6* exon 9 sequence in *CYP2D6*13B* to the *CYP2D7* exon 9 sequence in *CYP2D6*68*. This amplicon was sequenced using multiple amplicons to ensure continuity between the *CYP2D6*13B* and *CYP2D6*68* alleles. Amplicon M (lanes 8 and 9) primes from a unique forward region in rep 7 to the exon 9 sequence in *CYP2D7* found in *CYP2D6*68*. Amplification would only occur with a complete rep 7 (not rep dup) upstream of *CYP2D6*68*. Amplicon E (lanes 10 and 11) uniquely amplified *CYP2D6-2D7* genes (*CYP2D6*68*) and was used to generate an amplicon that was genotyped using the *CYP2D6* ASPE v2 kit, thus verifying the *CYP2D6*68* allele. Amplicon D (lanes 12 and 13) amplified *CYP2D6* gene in any normal arrangement (e.g., Fig. 1A), any *CYP2D6* gene 3′ allele in a duplication arrangement (Fig. 1B), and any *CYP2D6* gene in a tandem (Fig. 2, B–F). This amplicon generated the genotype of heterozygous *CYP2D6*4* from the tandem gene in Fig. 2E and the *CYP2D6*1* allele on the other chromosome using the *CYP2D6* v2 kit.

*CYP2D6*13B* has been observed alone (Gaedigk et al., 2010b), this is the first time it has been reported in tandem with other genes.

Impact of Hybrid Genes on Predicted Phenotype. *CYP2D6-CYP2D7 + CYP2D6 hybrid tandems.* The *CYP2D6*4*, *CYP2D6*4N*, and *CYP2D6*4*-like alleles have the 1846A variation, which results in a splicing defect that encodes an enzyme with no activity. The *CYP2D6*68* converts to the *CYP2D7* sequence from intron 1 forward and is also expected to produce a null allele because of the presence of the *CYP2D6*10* polymorphism c.100C>T plus the presence of an exon 4 c.631dupG resulting in a frameshift that causes a stop codon 43 bases downstream on the insertion (p.Glu211GlyfsX43) although no in vivo or in vitro research has been reported for this allele. The *CYP2D6*10* allele is described as having reduced function and the *CYP2D6*36* allele is described as having poor function (Gaedigk et al., 2006). Thus, the presence of a *CYP2D6*36*, *4N*, and *68* in a tandem does not affect the predicted phenotype of the sample.

Single CYP2D7-2D6 samples. All *CYP2D7-2D6* genes carry a T-insertion in exon 1 that causes a frameshift and a premature termination (c.137_138insT, p.Leu47AlafsX207). These hybrids are nonfunctional (Gaedigk et al., 2010b). Four (100%) of four samples originally genotyped as having a homozygous genotype, which were found to have a *CYP2D7-2D6* gene on one chromosome (Fig. 2A), had a change in phenotype prediction. These samples were originally genotyped as *CYP2D6*1/*1* but were found to actually be heterozygous for *CYP2D6*13G1* ($n = 1$) and *CYP2D6*13F* ($n = 3$), resulting in a change in phenotype prediction from EM to IM.

CYP2D7-2D6 + CYP2D6 hybrid tandem. Table 3 shows the change in phenotype of the samples with *CYP2D7-2D6 + CYP2D6* hybrid tandems (Fig. 2, B and C) observed in this research. Detection of the hybrid gene resulted in a change of predicted phenotype in 23 (92%) of 25 samples containing a hybrid. In 9 of the samples (cases 4, 11, 13, 14, 19, 20, 22, 24, and 25), the duplicated allele could not be predicted at the time of original genotyping and the original phenotype was specified "EM or IM." After the correct genotype was determined, the predicted phenotype was confirmed to be IM. Thus, in these cases, more precise phenotype prediction could be determined using the methods described here. If these 5 cases are also included in the "no change" group, then 18 samples (56%) had a change in phenotype.

Discussion

The intent of this study was to determine the frequency of *CYP2D6-2D7* and *CYP2D7-2D6* genes in samples containing *CYP2D6*4* alleles, *CYP2D6*10* alleles, homozygous samples of any variety, and samples with duplication signals that were originally genotyped with a commercially available ASPE kit. Furthermore, we wanted to determine the impact of these undetected genes on predicted phenotype.

The main findings of this research are that hybrid genes 1) are relatively common in clinical samples containing *CYP2D6*4* alleles, *CYP2D6*10* alleles, and duplication signals, 2) are uncommon in homozygous samples, and 3) frequently affect phenotype prediction in samples originally genotyped as homozygous and, separately, those that were originally genotyped as having a duplication. However, hybrid tandems do not change the predicted phenotype in *CYP2D6*4*- and *CYP2D6*10*-containing samples.

Our results involving duplication samples support those described by Gaedigk et al. (2010a), in which three *CYP2D6*13A2 + *2* and one *CYP2D6*13D + *2* were found in 32 duplication-positive white samples (12.5%) and one *CYP2D6*13H + *1* was found in 59 duplication-positive African-American samples (1.6%) for an overall

frequency of 5.4% compared with our 7.3%. The difference between these two studies is probably due to a difference in the ethnic mix of patients, although ethnic and racial data are not available for our samples. We also saw more *CYP2D6*13A2 + *2A* ($n = 12$) versus *CYP2D6*13D + *2A* ($n = 2$) (Table 3). Of interest, no hybrid genes were observed with other *CYP2D6*2* variants. We also identified seven samples with duplication signals, which contained a *CYP2D7* gene with a *CYP2D6*-like region downstream of exon 9 similar to that described by Gaedigk et al. (2010b). In particular, cases 1, 10, 20, and 22 had *CYP2D7 + *1*, cases 4 and 16 had *CYP2D7 + *41*, case 8 had *CYP2D7 + *4*, and all cases had sequence-confirmed rep dup 3′ to the *CYP2D7* hybrid allele.

In our collection of 341 samples originally genotyped with duplications, a hybrid tandem gene was present in 25 (7.3%), and this had an impact on phenotype prediction for 92% of these samples. Furthermore, a single *CYP2D7-2D6* gene was only found in 4 of 332 samples originally genotyped as homozygous, but it changed predicted phenotype in 100% of cases. Although the need to test for the rare single *CYP2D7-2D6* gene in a clinical setting can be debated in the absence of actual evidence that a given patient has genotype-phenotype discordance, the authors argue that the frequency of hybrid tandems in duplication samples and their impact on phenotype prediction warrants their analysis.

The *CYP2D6* ASPE kit that was used to originally genotype our samples was not designed to detect hybrid genes; thus, it is not surprising that none were detected by this kit during original clinical genotyping. Hybrid genes will also not be detected in a sequence-based analysis unless primers are designed to specifically allow for the PCR amplification of these hybrids. Furthermore, microarray-based assays, such as the AmpliChip CYP450 Test (Roche Diagnostics, Basel, Switzerland), will not detect hybrid genes unless the initial PCR amplification step uses primers that amplify the hybrid gene and then hybrid gene-specific probes are designed into the array.

Another feature that can affect phenotype is gene multiplication. In this study, 2 of 25 (8%) samples tested had otherwise undetected multiplications. This allowed for more accurate phenotype prediction in case 15 (*CYP2D6*1/*13A1 variant 2 + *1×N*), and the rtPCR copy number assays allowed for the identification of two unique *CYP2D7-2D6* hybrid arrangements (case 15 and 19). These findings show that duplication samples are challenging to accurately genotype, and it appears that use of an array of analyses, such as we have done here, is necessary to be sure of completely accurate genotyping.

One limitation of this study is that no ethnic or racial data were available for these samples because, per the institutional review board requirement for this type of study, all samples needed to be deidentified. Likewise, no phenotypic data are available for these individual samples.

In conclusion, to be detected, *CYP2D7-2D6* genes must be specifically tested for using methods comparable to those outlined here. *CYP2D7-2D6* genes were found frequently in samples originally genotyped to have duplications using a *CYP2D6* ASPE kit. Their detection resulted in a change in phenotype prediction in a high percentage (92%) of cases in which a duplication call was made. Furthermore, phenotype changed in 100% of rarer cases in which a single *CYP2D7-2D6* gene was present in our samples. *CYP2D6-2D7* genes must also be specifically tested for to be detected, but they have no effect on phenotype prediction because they were found in tandem with *CYP2D6*4* and *CYP2D6*10* alleles, which are null or have reduced function, respectively, and the hybrid genes (*CYP2D6*36, CYP2D6*4N*, and *CYP2D6*68*) have poor phenotype activity.

Two new structures, *CYP2D6*13B + (*68×2) + *4* and *CYP2D6*13A1 variant 2 + (*1×N)*, were described as were two new alleles, *CYP2D6*13G1* and *CYP2D6*13G2*, and two variations of the *CYP2D6*13A1* allele.

The information presented here is crucial for detection of hybrid genes regardless of the platform used to genotype *CYP2D6*. Probe sets for microarray detection must be designed with knowledge of hybrid genes or probes will not bind in a fashion that will allow their detection. In addition, *CYP2D6* full gene sequencing and high throughput genotyping using next-generation DNA analyzers will need to use the DNA sequences and genomic structures presented here as scaffolds for interpretation of sequence results.

Authorship Contributions

Participated in research design: Black, Walker, and O'Kane.

Conducted experiments: Walker and Harmandayan.

Contributed new reagents or analytic tools: Black, Walker, and O'Kane.

Performed data analysis: Black, Walker, O'Kane, and Harmandayan.

Wrote or contributed to the writing of the manuscript: Black, Walker, O'Kane, and Harmandayan.

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