Identification of Subtypes of CYP2D Gene Rearrangements among Carriers of CYP2D6 Gene Deletion and Duplication

MARÍA C. LEDESMA and JOSÉ A.G. AGÚNDEZ

Background: Cytochrome P450 2D6 (CYP2D6) is one of the best-known polymorphic drug-metabolizing enzymes. Rapidly evolving genotyping techniques permit the identification of single-nucleotide polymorphisms (SNPs) and thereby a prediction of individual metabolic capacities for CYP2D6 substrates. A considerable part of interindividual variability in CYP2D6 enzyme activity, however, is not related to SNPs but to gene deletions and duplications. Currently used genotyping methods assume that these gene rearrangements are homogeneous.

Methods: We analyzed the interindividual variability in CYP2D6 gene arrangements in genomic DNA from 740 Caucasian individuals by allele-specific PCR to identify common SNPs of the CYP2D6 gene that correspond to the variant alleles CYP2D6*3, *4, and *9. We investigated the presence and variability of CYP2D6*5 (gene deletion), CYP2D6x2 (gene duplication), and CYP2D6xn (gene amplification) by EcoRI and XbaI restriction fragment length polymorphism analyses and by long PCR plus KpnI and BamHI digestion. The presence of new mutations at the CYP2D locus was analyzed by sequencing.

Results: CYP2D6 gene rearrangements were present in >12% of individuals. Variability in the rearrangements regarding both gene deletion and gene duplication existed, and one of the unusual arrangements led to incorrect phenotype prediction. The frequency for carriers of unusual gene rearrangements was <0.3% (95% confidence interval, 0%–0.6%) in the population studied.

Conclusions: Heterogeneity in CYP2D6 gene rearrangement exists, but the allele frequency indicates that the risk for an erroneous phenotype prediction related to such variability is extremely low and that this risk can be neglected in routine analyses.

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Cytochrome P450 2D6 (CYP2D6) has high relevance in drug metabolism. CYP2D6 is involved in the metabolism of 20%–25% of clinically used drugs (1) and exhibits a clinically relevant gene polymorphism that modifies the pharmacokinetics of nearly 10% of drugs (2). Recently developed genotyping techniques permit the detection of individuals carrying CYP2D6 mutations, and odds are high that in the next few years these genotyping techniques will be routinely used in clinical practice.

The CYP2D6 gene is part of a cluster of 3 genes arranged in tandem on chromosome 22q13.1. The CYP2D gene cluster is composed of 2 pseudogenes, CYP2D8P and CYP2D7P, as well as the CYP2D6 gene (3). The only functional gene present in the human CYP2D gene locus is inactive in nearly 5% of Caucasian individuals because of detrimental mutations. More than 45 major polymorphic CYP2D6 alleles have been described (4). The frequencies for these alleles vary depending on the ethnicity of the individual (5). Among major variant alleles in Caucasians, those caused by single-nucleotide polymorphisms (SNPs), namely CYP2D6*4 and CYP2D6*10, account for 15%–20% of alleles. In addition, variant alleles resulting from CYP2D rearrangements, characterized by CYP2D6 gene deletion (CYP2D6*5) or duplication/multiduplication (CYP2D6*xn), are present with allele frequencies of 2%–7% and 1%–10%, respectively, in Caucasian individuals (2).

1 Genetics Unit, Hospital Perpetuo Socorro, Badajoz, Spain.
2 Department of Pharmacology, Medical School, University of Extremadura, Badajoz, Spain.
*Address correspondence to this author at: Department of Pharmacology, Medical School, University of Extremadura, Avda. de Elvas s/n, 06071 Badajoz, Spain. Fax 34-24-27-96-76; e-mail jagunde@unex.es.
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Previous reports indicated that the origin of CYP2D6*5 is an unequal crossover between the 2 sister chromatids. The expected outcome of crossover is interchange of DNA information between sister chromatids, but the outcome of this unequal crossover is that 1 chromatid takes the CYP2D6 gene from the other and also keeps its own CYP2D6 gene. This unequal crossing over causes duplication of the CYP2D6 gene (CYP2D6*5x2) in 1 chromatid but its deletion in the other (2). When such unequal crossover happens, the individual affected must have the duplicated gene in 1 chromosome and the deleted gene in the complementary chromosome, but in future generations the duplicated and deleted genes can be inherited separately. The presence of multiduplicated genes is attributable to additional unequal crossover events affecting chromatids that already contain duplicated genes. Gene duplication causes increased enzyme activity in vivo (6) and is related to lung cancer risk (7). Although it is generally accepted that gene rearrangements at the CYP2D locus are limited to CYP2D6 gene deletion and duplications, which are based on homogenous crossover mechanisms (i.e., no interindividual differences exist on the crossover events), no studies have addressed the issue of whether interindividual variability in the crossover of CYP2D6 gene occurs. In this event, such interindividual variability may decrease the efficiency of some commonly used genotyping techniques by leading to misclassifications of certain individuals.

A certain degree of SNP heterogeneity among duplicated and multiduplicated CYP2D6 genes has been shown. The most common variant alleles among duplicated genes are CYP2D6*2 and CYP2D6*41 (2). No information is available, however, concerning other types of genetic heterogeneity present in carriers of CYP2D6 gene rearrangements, particularly those involving large gene fragments. To date, the relatively low frequency of individuals carrying gene deletions or duplications has precluded the analysis of whether interindividual variability exists in the processes involved in such gene rearrangements. To investigate this question, we studies gene arrangement at the CYP2D locus in a large population (n = 740 Caucasian individuals) by combining the information obtained by sequencing, allele-specific PCR, and XbaI and EcoRI restriction fragment length polymorphism (RFLP) analyses. The aims of the study were to analyze the molecular basis for uncommon CYP2D6 gene arrangements and the association of these arrangements with the oxidative phenotype, and to evaluate the frequency and impact of a putative heterogeneity of CYP2D6 gene deletions or duplications.

Materials and Methods

DNA samples obtained from 737 unrelated and 3 related individuals were included after informed consent. Most of these individuals (n = 640) participated in studies of the association of CYP2D6 genotype with spontaneous diseases (8–12). Only healthy volunteers or patients with pathologies unrelated to the CYP2D6 polymorphism were included. In addition, samples from 3 healthy individuals, the brothers of an individual who had a rare genotype, were also analyzed. These 3 related individuals were not included in the calculation of allele frequencies. The Ethics Committee of the University Hospital Infanta Cristina, Badajoz, Spain approved the protocol for this study.

We collected 10-mL blood samples in sterile glass tubes containing EDTA as anticoagulant (Vacutainer; Becton Dickinson Systems) and stored them as whole blood at −80 °C until DNA isolation. Genomic DNA was purified from peripheral leukocytes and kept in sterile plastic vials at 4 °C until analysis. We performed the CYP2D6 genotyping analysis by mutation-specific PCR, long PCR, and restriction mapping with the enzymes EcoRI and XbaI as described elsewhere (8,13–16). For the sequencing analyses of exons 1–9 and the 3′flanking region of CYP2D8P, we conducted a series of 12 PCR amplification reactions spanning areas of ~0.5 kb between the position −25 (CYP2D8P) and −276 (the 3′ intergenic region of CYP2D8P). The primers used for amplification and sequencing analyses are shown in Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue6/.

The reaction conditions were identical for all amplification reactions, except for the primers used. The amplification mixture contained 250 ng of genomic DNA of probands, 10 mM Tris-HCl, 2.5 mM MgCl2, 50 mM KCl, 125 μM each deoxynucleotide (dATP, dGTP, dCTP, and dTTP), 10 pmol of each primer, and 0.5 U of Taq polymerase in a final volume of 25 μL. The initial denaturation step of 2 min at 95 °C was followed by 35 cycles of 1 min at 95 °C, 45 s at 63 °C, and 1 min at 72 °C, with a final extension of 7 min at 72 °C at the end of the cycles. We sequenced the amplified fragments in an ABI Prism 310 Genetic Analyzer by the dye terminator cycle sequencing method according to the manufacturer’s recommendations (Applied Biosystems). We analyzed sequences of exons 8 and 9, as well as the 3′flanking region of CYP2D7P, after a PCR amplification reaction with the same amplification mixture detailed above and primers at positions 3843 and 57. The denaturation step of 1 min at 94 °C was followed by 35 cycles of 1 min at 94 °C, 45 s at 60 °C, and 5 min at 72 °C, with final extension for 7 min at 72 °C. We sequenced the amplified fragments as detailed below.

The 95% confidence limits were calculated according to Bulbitt (17). Comparative analyses were carried out with the χ² test unless the conditions for its applicability were not adequate. In such cases, we used the Fisher exact test.

Results

Of the 737 unrelated individuals analyzed, 38 (5.1%) were carriers of CYP2D6*5, as indicated by the presence of the 13.1-kb fragment by EcoRI RFLP analysis. The 15.1-
combined with the CYP2D6*1 duplications at the complementary allele is shown in Table 1. The CYP2D6*5 association of CYP2D6 with the endonucleases differences. In all cases, digestion of the PCR products in this study. We analyzed the variability in the sizes of CYP2D6*5 complementary gene in the subgroup of carriers of allelic variant by long-PCR analysis (16). The findings obtained by EcoRI and XbaI RFLP analysis and by long PCR were fully correlated in all carriers of CYP2D6*5 identified in this study. We analyzed the variability in the sizes of the long-PCR products and observed no interindividual differences. In all cases, digestion of the PCR products with the endonucleases KpnI and BamHI produced fragments identical to those described previously (16). The association of CYP2D6*5 with common CYP2D6 mutations at the complementary allele is shown in Table 1. The findings indicate that CYP2D6*5 in most cases is combined with the CYP2D6*1 allelic variant in the other chromosome. The frequency of CYP2D6 variants at the complementary gene in the subgroup of carriers of CYP2D6*5 was similar to the CYP2D6 allele frequency among unrelated Spaniard noncarriers of CYP2D6*5 (6, 8). We therefore observed no association between CYP2D6*5 and mutations in the complementary gene.

The study of CYP2D6*x2, as shown by the presence of the 12.1-kb band in the EcoRI RFLP analysis, revealed that 53 individuals (7.2%) were carriers of CYP2D6 gene duplications. In the complementary XbaI RFLP analysis, all carriers of gene duplications had the 42-kb band characteristic of CYP2D6 gene duplication (15), indicating a full association between the mutations that induce the presence of the 12.1-kb band in the EcoRI RFLP analysis and the 42-kb band in the XbaI RFLP analysis. The association of alleles containing duplicated genes with mutations on the complementary allele is shown in Table 2. The mutation distribution in the complementary gene was roughly the same as that observed in unrelated healthy individuals, indicating that there is no association of alleles containing CYP2D6 gene duplications with a determined mutation in the complementary chromosome.

One individual with a rare gene arrangement had only the EcoRI 13.1- and 8.8-kb products (Fig. 1, lane 1). The 15.1-kb band that usually corresponds to CYP2D7P was not present. This description is the first to our knowledge of such an EcoRI RFLP pattern. The XbaI RFLP analysis indicated that this individual had a single 13-kb band, combined with the invariant 4.4- and 4.8-kb bands. A likely explanation for this RFLP pattern is that the individual could have a combined loss of CYP2D6 and CYP2D7P. To obtain additional information, we analyzed the patterns in the 3 brothers of the individual. All were heterozygous for CYP2D6*5 and a complementary allele that lacked common mutations by PCR analysis (i.e., the CYP2D6*5/CYP2D6*1 genotype). The heterozygous individuals had, in addition to the 13.1- and 8.8-kb bands, the 15.1- and 9.4-kb bands corresponding to CYP2D7P and CYP2D6, respectively (Fig. 1, lanes 2 and 3). Sequencing analysis of exons 8 and 9 of CYP2D7P in the 4 brothers confirmed the presence of 2 CYP2D7P pseudogenes in the homozygous individual and in the heterozygous carriers of CYP2D6*5. The 4 brothers were heterozygous CYP2D7AP/CYP2D7P as indicated by the presence of heterozygosities at positions 3899A/G (exon 8), 4239 C/T, and 4289 C/T (exon 9). Our findings thus indicate that the absence of the 15.1-kb band is not the result of a loss of CYP2D7P or a change in the EcoRI recognition sequence located downstream of CYP2D7P (16, 18). The absence of the 15.1-kb band seems instead to result from a decrease in the CYP2D7P fragment size to 13.1 kb, probably because of crossover events involved in the deletion of the CYP2D6 gene. The proposed gene arrangement for this individual is shown in Fig. 1 (lane 1).

Another rare EcoRI RFLP pattern identified in this study is that of an individual lacking both the 15.1- and 9.4-kb bands. The EcoRI digestion products were 13.1, 12.1, and 8.8 kb (Fig. 1, lane 4). Long-PCR analysis confirmed the presence of CYP2D6*5, which is consistent with the presence of the 13.1-kb band. PCR analysis confirmed the presence of a CYP2D6*2 allelic variant. XbaI RFLP analysis of DNA from this individual indicated the absence of common band patterns: no bands of 44, 29, 16, 11.5, or 9 kb were present. On the other hand, 2 isolated bands of 13 and 12 kb were identified. The proposed gene arrangement for this individual is shown in Fig. 1 (lane 4). The sequencing analysis of the intergenic region between CYP2D8P and CYP2D7P revealed that the individual was heterozygous at several positions. Analysis of the region spanning exons 1–9 of CYP2D8P also revealed several mutations indicating the presence of an unknown CYP2D8P pseudogene variant. The new mutations identified in this individual are summarized in Table 3.
Discussion

Rapidly evolving high-throughput genotyping techniques will permit, in the next few years, the routine use of genotype information in clinical practice, which will require accuracy in phenotype prediction. Genotyping methods have been developed for the majority of individuals, but in some cases uncommon genotypes occur. To date, little attention has been paid to these uncommon genotypes. The actual frequencies of such genotypes and the actual impact in phenotype prediction, however,
individual among the 38 carriers of a genotype homozygous for pseudogene. The Intron 9 4960 T TC rearrangements at the routine genetic analyses. should be evaluated to increase the clinical efficiency of Exon 8 4852 A G Silent (G) Exon 2 1892 C C or T H-Y Exon 1 86 A A or C Y-F revealed a frequency for of 737 unrelated individuals. Analysis of the 1474 alleles further expansion of such populations distributed the 7.2%. In the population studied, the presence of population. The gene duplication carrier frequency is thus from northeast African populations favored the character- inactivating mutations).

poor metabolizers (i.e., homozygous carriers of enzyme- gene duplication carriers is more common than that of gene rearrangements and that CYP2D6 gene amplification were identified in the study population. The gene duplication carrier frequency is thus 7.2%. In the population studied, the presence of CYP2D6 gene duplication carriers is more common than that of poor metabolizers (i.e., homozygous carriers of enzyme-inactivating mutations).

It has been proposed that dietary selection pressure from northeast African populations favored the characteristics of carriers of CYP2D6 gene rearrangements and that further expansion of such populations distributed the variant alleles in other populations (2). In this study we show that these variant alleles are not completely homogeneous, thus reinforcing the hypothesis that external pressure factors may be responsible for the occurrence of such variant alleles by different mechanisms. Two rare gene arrangements were observed in 1474 alleles. One resulted from homozygous deletion of CYP2D6 combined with partial deletion of a fragment corresponding to the intergenic region located between CYP2D6 and CYP2D7P (Fig. 1, lane 1). The other rare case corresponded to an individual who carried several mutations in the CYP2D8P pseudogene. The EcoRI and Xbal RFLP patterns suggested a genotype homozygous for CYP2D6 gene deletion. That individual, however, carried a CYP2D6*2 gene (Fig. 1, lane 4). This description is the first of the presence of a 12-kb band in Xbal RFLP analyses that corresponds to the CYP2D6 gene. RFLP analyses would have erroneously classified the individual as a poor metabolizer. From a practical point of view, however, the impact of the variability observed is limited, and our findings therefore indicate that the different methods suitable for the detection of CYP2D6 gene arrangements, including previous methods (15, 16), as well as those described recently (19, 20), are expected to have identical capacities for phenotype prediction and thus for routine clinical use.

**Table 3. Mutations located in exons 1–9 of CYP2D8P and within the intergenic region between CYP2D8P and CYP2D7P.**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Position</th>
<th>Wild-type sequence</th>
<th>Mutation</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>69</td>
<td>A</td>
<td>A or G</td>
<td>Q/R</td>
</tr>
<tr>
<td>Exon 1</td>
<td>86</td>
<td>A</td>
<td>A or C</td>
<td>Y-F</td>
</tr>
<tr>
<td>Exon 2</td>
<td>1892</td>
<td>C</td>
<td>C or T</td>
<td>H-Y</td>
</tr>
<tr>
<td>Exon 8</td>
<td>4852</td>
<td>A</td>
<td>G</td>
<td>Silent (G)</td>
</tr>
<tr>
<td>Exon 1</td>
<td>4913</td>
<td>C</td>
<td>T</td>
<td>R-W</td>
</tr>
<tr>
<td>Intron 9</td>
<td>4960</td>
<td>T</td>
<td>TC</td>
<td></td>
</tr>
<tr>
<td>Intergenic region</td>
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<td>T</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−4512</td>
<td>C</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−3608–9</td>
<td>GC</td>
<td>CCGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−3413</td>
<td>T</td>
<td>G</td>
<td></td>
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<td></td>
<td>−3412</td>
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<td></td>
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<td>A</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>−3355</td>
<td>T</td>
<td>G</td>
<td></td>
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</tbody>
</table>

should be evaluated to increase the clinical efficiency of routine genetic analyses.

In this study we analyzed the frequency of gene rearrangements at the CYP2D locus in a large population of 737 unrelated individuals. Analysis of the 1474 alleles revealed a frequency for CYP2D6*5 of 2.6%. Only 1 individual among the 38 carriers of CYP2D6*5 was homozygous. With a single exception, the gene arrangement was identical in all individuals. The occurrence of mutations on the complementary alleles was similar to that described in healthy noncarriers of CYP2D6*5. Another gene rearrangement, which is involved in the duplication of CYP2D6, occurred with an allele frequency of 3.6%. No individuals homozygous for gene duplication or for CYP2D6 gene amplification were identified in the study population. The gene duplication carrier frequency is thus 7.2%. In the population studied, the presence of CYP2D6 gene duplication carriers is more common than that of poor metabolizers (i.e., homozygous carriers of enzyme-inactivating mutations).

It has been proposed that dietary selection pressure from northeast African populations favored the characteristics of carriers of CYP2D6 gene rearrangements and that further expansion of such populations distributed the variant alleles in other populations (2). In this study we show that these variant alleles are not completely homogeneous, thus reinforcing the hypothesis that external pressure factors may be responsible for the occurrence of such variant alleles by different mechanisms. Two rare gene arrangements were observed in 1474 alleles. One resulted from homozygous deletion of CYP2D6 combined with partial deletion of a fragment corresponding to the intergenic region located between CYP2D6 and CYP2D7P (Fig. 1, lane 1). The other rare case corresponded to an individual who carried several mutations in the CYP2D8P pseudogene. The EcoRI and Xbal RFLP patterns suggested a genotype homozygous for CYP2D6 gene deletion. That individual, however, carried a CYP2D6*2 gene (Fig. 1, lane 4). This description is the first of the presence of a 12-kb band in Xbal RFLP analyses that corresponds to the CYP2D6 gene. RFLP analyses would have erroneously classified the individual as a poor metabolizer. From a practical point of view, however, the impact of the variability observed is limited, and our findings therefore indicate that the different methods suitable for the detection of CYP2D6 gene arrangements, including previous methods (15, 16), as well as those described recently (19, 20), are expected to have identical capacities for phenotype prediction and thus for routine clinical use.

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