CYP2D6 Polymorphisms in Patients with Porphyrias

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The cytochrome P-450 (CYP) isoenzymes, a superfamily of heme proteins which are the terminal oxidases of the mixed function oxidases system, metabolize more than 70% of all clinically approved drugs. The highly polymorphic CYP2D6 isoform metabolizes more than 25% of most common drugs, and the phenotypes of the 70-plus allelic variants range from compromised to excessive enzymatic activity. Porphyrias are a group of inherited or acquired metabolic disorders of heme biosynthesis, due to a specific decrease in the activity of one of the enzymes of the heme pathway. Clinical signs and symptoms of porphyrias are frequently associated with exposure to precipitating agents, including clinically approved drugs. CYP enzymes, including CYP2D6, participate in the metabolism of some porphyrinogenic drugs, leading to the deregulation of heme biosynthesis. Considering that some of the drugs not recommended for use in porphyric patients are metabolized by CYP2D6, the presence of CYP2D6 polymorphisms in porphyric patients would influence the triggering of the disease when these individuals receive a precipitating agent that is metabolized by CYP2D6. To investigate CYP2D6 polymorphisms in porphyric patients, healthy Argentinean volunteers, porphyric patients, and a group of individuals with high levels of iron were studied. Results indicated that the CYP2D6*3 and CYP2D6*4 alleles, in particular, would be linked to the onset of disease. Predictive genotyping for CYP2D6 in porphyric patients holds promise as a method to improve the clinical efficacy of drug therapy and to personalize drug administration for these patients.

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INTRODUCTION

The cytochrome P-450 (CYP) isoenzymes are a superfamily of heme proteins which are the terminal oxidases of the mixed-function oxidase system (1). The 1 to 3 families of CYP are responsible for 70% to 80% of all phase I–dependent metabolism of clinically used drugs (2). CYP2D6 isoform metabolizes more than 25% of most common drugs, including antiarrhythmics, antidepressants, beta blockers, neuroleptics, and opioids (3). CYP2D6 gene is extremely polymorphic, and more than 70 allelic variants have been described (4,5); as a result, metabolism and excretion rates of drugs vary between individuals, from extremely slow to ultra-fast. Different phenotypes can be distinguished: poor metabolizers (PM) lack the functional enzyme; intermediate metabolizers (IM) carry 2 different alleles, leading to partial activity; efficient metabolizers (EM) have 2 normal alleles; efficient intermediate metabolizers (EIM) are heterozygous for 1 deficient allele; and ultra-rapid metabolizers (UM) have multiple gene copies (6). The clinical consequences of genetic polymorphisms in drug metabolism depend on whether the activity of the drug lies with the substrate or its metabolite, as well as the extent to which the affected pathway contributes to the overall elimination of the drug (1). For example, the mutant CYP2D6*3 (CYP2D6A) allele with the A2637 deletion in exon 5 and the mutant CYP2D6*4 (CYP2D6B) allele with a G1934A splice site defect are among the most common mutations. These mutations result in decreased or lack of CYP2D6 isoenzyme activity, leading to PM phenotype (7-9). PM individuals have an increased risk for adverse side effects or therapeutic failure following drug treatment.

Porphyrias are a group of inherited or acquired metabolic disorders of heme biosynthesis in which specific patterns of overproduction of heme precursors are associated with characteristic clinical features. Each type of porphyria is the result of a specific decrease in the activity of one of the enzymes of the heme pathway (10-12). Porphyrias are classified as acute, cutaneous, or mixed according to clinical features. Acute attacks characteristic of acute intermittent porphyria (AIP) and variegate porphyria (VP), both hereditary, are often triggered by exposure to exogenous precipitating factors (13) including a wide range of commonly prescribed drugs. There are two main forms of porphyria cutanea tarda (PCT): type I (sporadic or acquired) and type II (familial or hereditary) in which the enzyme activity of uroporphyrinogen decarboxylase (URO-D) is reduced to approximately 50% of normal in all tissues. In type I PCT, subnormal URO-D activity is restricted to the liver. There is also a form of familial PCT called type III, in which a family history of PCT is observed, but subnormal URO-D activity is restricted to the liver. No mutations have been found in the URO-D gene in types I and III. PCT trig-

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Table 1. Data of patients with porphyria.

	PCT	AIP	VP
n	15	20	15
Mean age (range), y	36 (8-66)	30 (22-40)	43 (26-65)
Sex, M/F	7/8	6/14	4/11
Porphyria type	Gender		
I	1M/2F	—	—
II	4M/4F	—	—
III	0M/1F	—	—
No family history	2M/1F	—	—
Latent	0M/1F	5M/9F	2M/5F
Symptomatic	7M/7F	1M/5F	2M/6F

gering is frequently associated with exposure to precipitating agents, such as polyhalogenated aromatic hydrocarbons, alcohol abuse, estrogen ingestion, iron overload, and infections (14).

CYP enzymes participate in the metabolism of some porphyrinogenic drugs, leading to the deregulation of heme biosynthesis, which would influence the pathogenesis of porphyrias. Considering that some of the drugs not recommended for use in porphyric patients are metabolized by CYP2D6, inheritance of polymorphic CYP2D6 metabolizing genes would be an important determinant of individual variation in acute symptoms. Moreover, the presence of CYP2D6 polymorphisms in porphyric patients with genetic or biochemical alterations in any of the enzymes of the heme pathway would influence the triggering of the disease when these individuals received a precipitating agent that was metabolized by CYP2D6. The aim of this work was to investigate CYP2D6 polymorphisms in porphyric patients. To this end, CYP2D6*3 and CYP2D6*4 alleles were studied in healthy volunteers, porphyric patients, and a group of individuals with high levels of iron.

MATERIALS AND METHODS

All primers used for PCR analysis were synthesized by Fagos Laboratory (Buenos Aires, Argentina). All other chemicals and reagents were of molecular grade from Merck, Sigma, Promega, Ambion, Bio Labs and Amersham; Taq DNA polymerase was from Invitrogen. Digestion enzymes were from Bio Labs.

Subjects

A total of 120 subjects—51 healthy volunteers, 50 porphyric patients, and 19 individuals with high iron levels—were included in the study, all of Caucasian origin. The porphyric patient group (Table 1), previously studied in the Centro de Investigaciones sobre Porfirinas y Porfirias, consisted of 20 individuals diagnosed with AIP, 15 with PCT, and 15 with VP. All patients were diagnosed biochemically and genetically except 3 PCT individuals who were diagnosed only biochemically. All the individuals with high iron levels were normal for mutations in HFE gene responsible for hereditary hemochromatosis type I. All subjects gave their informed consent to participate in this study.

PCR-RFLP

Genomic DNA was extracted from EDTA-collected whole blood samples using the GFX Genomic Blood DNA Purification Kit (Amersham). Target DNA (0.5-1 µg) was amplified by PCR as described by Daly et al. (15) with slight modifications. CYP2D6*3 and CYP2D6*4 alleles were detected by amplification of the region of interest using primers G1 (bp 1827-1846; 5′-TGCCGCCTTCGCCAA CCACT-3′) and B1 (bp 2638-2657; 5′-GGCTGGGTCCCAGGTCATAC-3′). The reaction was carried out in a total volume of 50 µl containing 14 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 5% dimethyl sulfoxide with 0.2 mM dTNPs, 0.52 µM primers, and 2.5 units Taq DNA polymerase. For amplification, 30 cycles were carried out of 1 min at 95 °C, 1 min at 62 °C, and 3 min at 70 °C in a PTC 100 Research Mini Cycle thermocycler. The product (25 µL) was digested with 12.5 units of *Bsa*AI and 15 units of *Bst*NI restriction enzymes, at 50 °C for 3 h. To control the digestion by *Bsa*AI, GADPH gene, amplified by separate PCR reaction, was added to the CYP2D6 digestion. The GAPDH PCR was carried out using the primers R4 (5′-AGAAACAGGAGGTCCCTACT-3′) and R5 (5′-GTCGGGTCAACGCTA GGCTG-3′) and similar conditions to those for CYP2D6 amplification. The digest was analyzed on a 8% polyacrylamide gel using 1% TBE as the buffer, and the gel was stained with ethidium bromide.

Statistical analysis

Data were analyzed using χ^2 test. $P < 0.05$ was considered significant.

RESULTS

Figure 1 shows representative band patterns found for wild-type and CYP2D6*3 and CYP2D6*4 mutant variants of CYP2D6 gene in all the subjects analyzed. Between 3 and 6 bands for mutant and wild-type variants were observed. One of these bands was distinctive for CYP2D6*3 (160 bp) and the other was distinctive for CYP2D6*4 (388 bp). *wt*/*wt* pattern showed 4 bands (280, 168, 139, and 109 bp). In the case of *wt*/*3 or *wt*/*4, bands of 160 bp and 388 bp appeared, respectively. *3/*3 pattern presented the band of 160 bp but not the corresponding band of 168 bp; in *4/*4 pattern, the band of 388 bp was present while the bands of 109 bp and 280 bp were absent. *3/*4 individuals presented both bands of 160 bp and 388 bp. The bands of 500 bp corresponded to GAPDH gene.

Genotype distribution of CYP2D6 alleles among healthy subjects and porphyric patients is shown in Table 2. In the control group, 5.8% (3 of 51) were heterozygous for CYP2D6*3 and 33.3% (17 of 51) for CYP2D6*4 allele; 1 of 51 (1.9%) was homozygous for CYP2D6*3

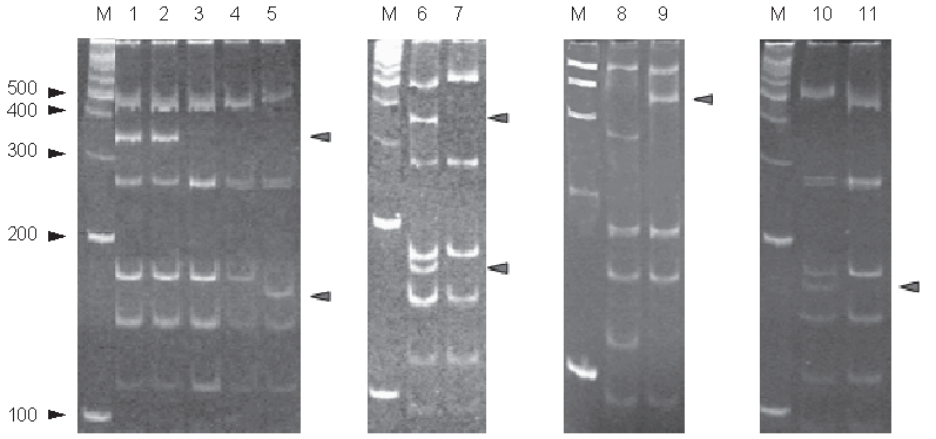

Figure 1. Representative band patterns of CYP2D6*3 and CYP2D6*4 alleles. Band pattern observed for the wild-type (*wt*/*wt*), heterozygous (*3/*wt*, *4/*wt*), compound heterozygous (*3/*4), and homozygous (*3/*3, *4/*4) CYP2D6 alleles. M indicates marker of 100 bp. Lanes 1, 2: *4/*wt*; 3, 4, 7, 8, 11: *wt*/*wt*; 5: *3/*3; 6: *3/*4; 9: *4/*4; 10: *3/*wt*. Other experimental conditions are described in Materials and Methods.

and CYP2D6*4. In the PCT group, 26.6% (4 of 15) were heterozygous for CYP2D6*4 and 1 of 15 (6.6%) was homozygous for this allele. In the AIP group, 10% (2 of 20) were heterozygous for CYP2D6*4. In the VP group, 26.6% (4 of 15) were heterozygous for CYP2D6*4, and 1 of 15 (6.6%) was double heterozygous (both CYP2D6*3 and CYP2D6*4). In individuals with high levels of iron, 15.7% (3 of 19) were heterozygous for CYP2D6*4; the same percentage were homozygous, significantly different from the control group ($P < 0.05$). None of the patients with porphyria or high iron was found to be homozygous for CYP2D6*3.

The frequency of CYP2D6 alleles observed is shown in Table 3. In the control group, 4.9% of alleles (5 of 102) were CYP2D6*3 and 18.6% (19 of 102) were CYP2D6*4. The PCT group showed a frequency of 20% (6 of 30) CYP2D6*4. In the AIP group only 5% (2 of 40) were CYP2D6*4 ($P < 0.05$); in the VP group, the frequency of this allele was 16.6% (5 of 30). The PV group showed a frequency of 3.3% (1 of 30) CYP2D6*3. In individuals with high levels of iron, CYP2D6*4 frequency was 23.6% (9 of 38). Results obtained for the PCT, PV, and high iron groups were no different from the control group.

The predicted phenotype distribution is shown in Table 4. The phenotype was predicted according to the genotypes as follows: EM carried 2 CYP2D6 wild-type alleles, EIM carried 1 deficiency and 1 wild-type allele, and PM were homozygous for 2 deficiency alleles. In the control group, 56.9% were identified as EM, 39.1% as EIM, and 3.9% as PM. In the AIP group, 90% were classified as EM and 10% as EIM. In the PCT and VP group, 66.8% were classified as EM, 26.6% as EIM, and 6.6% as PM. In the group with high levels of iron, 68.6% were identified as EM and 15.7% as EIM and PM.

DISCUSSION

CYP2D6 is one of the most studied polymorphic genes, and its clinical relevance and allelic frequency have been extensively investigated in different ethnic groups (16,17). To date, no data in the Argentinean population or in porphyric individuals worldwide have been reported. Our results showed a frequency of 18.6% CYP2D6*4 allele in the healthy group. The frequency of this allele was similar to that reported for other Caucasian populations (8,18,19). The CYP2D6*3 frequency found in our study (4.9%) was slightly higher than others (9,20,21).

The analysis of predicted phenotype distribution showed a difference in the frequency of CYP2D6*3 and CYP2D6*4 alleles between the control group and porphyric patients. When the results obtained in all porphyric patients studied were analyzed, the frequency of EM phenotype was higher ($P < 0.05$) than in healthy controls. When each type of porphyria was analyzed separately, only the EM phenotype in the AIP group was significantly higher than the healthy group. These results indicate that polymorphisms in porphyric patients might also have a significant influence on the individual susceptibility to foreign substances and the triggering of this disease. EM would be more exposed than EIM and PM to genotoxic porphyrinogenic xenobiotic metabolites. Several authors have presented similar results in different studies performed with lung cancer and urinary bladder cancer patients (22-24). Agündez et al. (25) observed a significant correlation between EM and the risk of liver cancer development.

In this study, the CYP2D6 polymorphism was also investigated in nonporphyric individuals presenting with some

Table 2. Genotype distribution.

	Healthy		PCT		AIP		VP			High Iron	
Alleles	*3	*4	*3	*4	*3	*4	*3	*4	*3/*4	*3	*4
Mut/*wt*, *n* (%)	3/51 (5.8)	17/51 (33.3)	0	4/15 (26.6)	0	2/20 (10.0)	0	4/15 (26.6)	0	0	3/19 (15.7)
Mut/Mut, *n* (%)	1/51 (1.9)	1/51 (1.9)	0	1/15 (6.6)	0	0	0	0	1/15 (6.6)	0	3/19 (15.7)[a]

[a] $P < 0.05$, calculated using χ^2 test.

Table 3. Frequency of CYP2D6 alleles.

	Alleles, *n*	CYP2D6*3, *n* (%)	CYP2D6*4, *n* (%)
Control	102	5 (4.9)	19 (18.6)
PCT	30	0	6 (20.0)
AIP	40	0	2 (5.0)[a]
VP	30	1 (3.3)	5 (16.6)
High iron	38	0	9 (23.6)

[a]$P < 0.05$, calculated using χ^2 test.

Table 4. Phenotype distribution.

	EM	EIM	PM
	Frequency (%)	Frequency (%)	Frequency (%)
Control	29/51 (56.9)	20/51 (39.1)	2/51 (3.92)
PCT	10/15 (66.8)	4/15 (26.6)	1/15 (6.60)
AIP	18/20 (90.0)[a]	2/20 (10.0)[b]	0/20 (0)
VP	10/15 (66.8)	4/15 (26.6)	1/15 (6.60)
High iron	13/19 (68.6)	3/19 (15.7)	3/19 (15.7)

[a]$P < 0.01$, [b]$P < 0.05$, calculated using χ^2 test.

hepatic alterations and high levels of iron. A higher percentage of this polymorphism was found in these subjects than in the control group. These individuals would be particularly exposed for an extended time period to the possible toxic effects of unmetabolized chemicals. These findings are in agreement with other studies that revealed an association between PM and breast cancer or leukemia (26-28).

Gardlo et al. (29) analyzed the CYP1A1 and CYP1A2 polymorphism prevalence in patients with PCT and suggested that the *m*4 polymorphism in Caucasian PCT type II patients might contribute to a higher susceptibility to porphyrinogenic compounds. In a similar study, Christiansen et al. (30) reported that the A/A genotype of CYP1A2 occurred significantly more often in PCT compared with the healthy control group in a Danish population.

In this study, we observed that, in the AIP group, no presence of polymorphisms was found among the 6 symptomatic patients, although 2 men with latent AIP were heterozygous for CYP2D6*4. In patients with VP, only 1 of the 7 individuals with latent VP carried the polymorphism CYP2D6*4 in the heterozygotic state. In the symptomatic VP cohort, 3 of 8 individuals possessed a heterozygous *4 allele, only 1 of whom had undergone an acute attack, while the other 2 presented only cutaneous manifestations. One woman in this group carried both polymorphisms (*3/*4). In the PCT group, among 8 individuals with hereditary PCT, 3 were heterozygous for CYP2D6*4: 1 was latent PCT; among the 3 patients with acquired PCT, 1 was heterozygous for CYP2D6*4 and another carried the same allele in the homozygous form.

Results presented here, although preliminary, demonstrated for the first time that 13% of porphyric subjects analyzed carried the CYP2D6*4 allele and only 1% carried the CYP2D6*3 allele. However, further studies in greater cohorts should be analyzed to provide a clearer picture of the extent and correlation of mutant genotypes in porphyric patients compared with healthy controls. Also, other genotyping studies should be performed for detecting the presence of other mutant alleles in these individuals, such as CYP2D6D which, like CYP2D6*3 and CYP2D6*4, is among the most common mutations leading to PM phenotype in the Caucasian population.

The information obtained with this research would have an impact on the practice of medicine in the near future. Many authors (6,21,31) have already determined that genotyping P450 would be a useful tool to predict the pharmacogenicity of drugs. Currently, CYP2D6 polymorphisms cause interindividual variability in drug response, influencing the treatment of several diseases (4,6). In the case of porphyrias, some drugs have shown conflicting evidence about their porphyrinogenicity in some individuals. Predictive genotyping for CYP2D6 in porphyric patients holds promise as a method to improve the clinical efficacy of drug therapy and to personalize drug administration for these patients.

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