Molecular heterogeneity of the XbaI defined 44kb allele of the CYP2D locus within the Caucasian population

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1 Cytochrome P450 debrisoquine (CYP2D6) activity is polymorphic and under genetic control. Most Caucasians are extensive metabolizers, but 5%-10% are poor metabolizers.
2 Restriction fragment length polymorphism analysis of the CYP2D6 locus identifies a 29kb XbaI fragment, either normal (D6-wt) or mutated, and three mutated XbaI alleles (44kb, 11.5kb and 16 + 9kb). The 44kb allele was initially considered as a poor metabolizer allele owing to a D6-B mutation, but cases of 44kb allele not carrying the D6-B, and therefore potentially functional, have been found. The degree of molecular heterogeneity of this allele was investigated by phenotype and genotype analysis of families.
3 Thirty-one French Caucasian families, representing 117 individuals, possessing at least one 44kb allele in each family were selected. Phenotypes were determined using dextromethorphan, and the XbaI, NcoI and BamH1 RFLPs of 42 independent chromosomes were analyzed.
4 80% of the XbaI 44kb alleles carried the CYP2D6-B mutation and had an additional NcoI fragment (12.5kb or 4.8kb). The remaining 20% did not carry the CYP2D6-B or A mutations and had no extra NcoI fragment.
5 Information on three families demonstrated that 44kb alleles not carrying the CYP2D6-B mutation were associated with the extensive metabolizer phenotype.
6 We conclude that a substantial percentage of XbaI 44kb alleles is associated with a functional CYP2D gene, and therefore, that the XbaI 44kb allele is not consistently a poor metaboliser allele.

Keywords debrisoquine polymorphism metabolism genotype phenotype cytochrome P4502D6

Introduction

The cytochrome P-450 gene superfamily encodes for enzymes which catalyze oxidative biotransformations of hydrophobic endo and xenobiotics. Among the thirteen families described in man, four are involved in the detoxication of exogenous compounds, including drugs. Several cytochrome P450 loci are polymorphic in humans and contribute to genetically determined interindividual variation in drug response (Brosen, 1990; Nebert & Gonzalez, 1987).

The debrisoquine-type polymorphism has been widely investigated (Brosen & Gram, 1990; Eichelbaum & Gross, 1990; Jacqz-Aigrain et al., 1988; Maghoub et al., 1977) using either debrisoquine as the probe drug or other drugs, such as dextromethorphan, sparteine and encainide. The reaction involves an enzymatic oxidation in the liver catalysed by a specific cytochrome P450 originally termed debrisoquine 4-hydroxylase (CYP2D). Two metabolic phenotypes, extensive (EM) and poor (PM) metabolizers, were defined among Caucasian populations, PM have deficient oxidation, and account for 5 to 10% of the population (Alvan et al., 1990; Gonzalez & Meyer, 1991). Family studies and genetic analyses reveal that the PM phenotype is monogenically inherited as an autosomal recessive trait. Therefore,
co-segregation of two PM alleles at the CYP2D locus results in the PM phenotype (Meyer et al., 1990; Price-Evans et al., 1980; Sloan et al., 1983). Accordingly, three theoretical genotypes: homozygous for two EM alleles (em/em), heterozygous for one EM and PM allele (em/pm) and homozygous for two PM alleles (pm/pm) should have three corresponding phenotypes: extensive, intermediate and poor metabolizers. However, graphical and statistical analyses of individual metabolic ratios obtained in large populations with various probe drugs have revealed no clear cut intermediate group.

Once the molecular DNA probes for CYP2D6 became available, several DNA nucleotide polymorphisms were described, especially those affecting the restriction sites (restriction fragment length polymorphism: RFLP). Three distinct patterns of XbaI RFLP (44kb, 11.5kb and 16 + 9kb) are associated with mutant alleles, while a 29kb XbaI fragment could represent either a normal (D6-wt) or a mutant allele (Dahl et al., 1992; Evans & Relling, 1990; Skoda et al., 1988).

Molecular cloning identified the CYP2D locus as a cluster of three tandemly arranged genes. One (CYP2D6) codes for a functional protein, whereas the other two (CYP2D8 and CYP2D7), based on nucleotide sequence data, appear not to code for a cytochrome related protein (Kimura et al., 1989). The XbaI-defined 11.5kb allele corresponds to a deletion of the CYP2D6 gene (Gaedigk et al., 1991), and the 44kb allele to a cluster with an additional gene (CYP2D7*). In this rearranged cluster the CYP2D6 gene carries a mutation, designated CYP2D6-B, at the splice site junction of intron 3 and exon 4 (Hanioka et al., 1990; Kagimoto et al., 1990). A substantial percentage of XbaI 29kb mutated alleles also carry an identical mutation in the CYP2D6 gene. Clearly both the 11.5kb allele with the deletion of the functional gene and the 44kb allele with the CYP2D6-B mutation (44kbD6-B) cannot encode a functional protein, and hence must be poor metabolizer alleles. However, some cases in which the 44kb allele does not carry the D6-B mutation have been reported (Broly et al., 1991; Daly et al., 1990).

A 44kb allele without the D6-B mutation could therefore either be functional, as recently reported by Dahl et al. (1992) in one case, or carry other mutation(s). In order to clarify this issue we have undertaken family studies at the phenotype and genotype levels, including molecular characterization for known mutations. The purpose of this work was to understand the degree of molecular heterogeneity of this allele through family studies and its relationship to the phenotype diversity.

### Methods

Thirty-one French Caucasian families, representing 117 individuals, possessing at least one 44kb allele were selected from a large study population.

DNA was extracted by the standard phenol/chloroform method. Five μg of each DNA was digested to completion with three restriction endonucleases: XbaI, NcoI and BamH1 and the RFLPs were determined for each digest (Skoda et al., 1988) by Southern hybridization with a randomly primed CYPD6-cDNA probe (kindly provided by U.A. Meyer). Screening for D6-B and D6-A mutations was performed by the procedures described earlier (Gough et al., 1990; Wolf et al., 1990). Two polymorphic BamH1 sites are located at an homologous position in the intervening sequence 1 of D7 and D6 genes, and the absence (-) or the presence (+) of these sites generate 6.0kb (-) or 4.9kb (+) fragments for D7 and 4.1kb (-) or 2.2kb (+) fragments for D6 (Kagimoto et al., 1990; Skoda et al., 1988). Haplotypes defining the polymorphism of BamH1 at D7 and D6 on a chromosome were assigned as either ++, ---, + - or -- from the fragment sizes obtained in family studies.

Individual phenotypes were determined with dextromethorphan hydrobromide (30 mg in adults, 15 mg in children). The urinary dextromethorphan and dextrorphan concentrations were measured by high performance liquid chromatography (Motassim et al., 1987) and the parent drug to metabolite ratio for each family member was calculated. Statistical analysis was performed using Student's t-test (P < 0.05 as minimum significance threshold).

### Results

Parent/offspring segregation was used to align the chromosomal polymorphic restriction patterns of the three enzymes (NcoI, BamH1, XbaI) defining the haplotype. A total of 42 independent chromosomes (one or two from each parent) having the XbaI-defined 44kb allele was found. Thirty-two of them (80%) carried the CYP2D6-B mutation. They were further characterised by the presence of an extra NcoI fragment (as compared with the normal pattern): either 12.5kb in 30 cases (typical) or 4.8kb in 2 cases (atypical). The remaining ten chromosomes (20%) of XbaI 44kb did not carry the CYP2D6-B mutation, the CYP2D6-A mutation nor the additional NcoI fragment.

The molecular heterogeneity of this XbaI 44kb allele, was further examined by additional RFLP analyses with the BamH1 restriction enzyme. Distinct polymorphic patterns were observed for the chromosomes with and those without the D6-B mutation. All 44kbD6-B alleles (with the D6-B mutation) were associated with the BamH1 ++ haplotype, including the two atypical haplotypes. The others (no D6-B mutation) were heterogenous, and were either BamH1 --- (n = 6), ++ (n = 3) or -- (n = 1) (Table 1).

#### Table 1 NcoI and BamH1 polymorphisms associated with 44kb alleles

<table>
<thead>
<tr>
<th>Group</th>
<th>NcoI</th>
<th>BamH1</th>
<th>Number of XbaI 44kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>44kbD6-B typical</td>
<td>NP</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>44kbD6-B atypical</td>
<td>NP</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>44kb-em</td>
<td>NP</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>NP</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

NP: Normal profile
SF: Supplementary fragment.
Three families were informative for determining the phenotype associated with the XbaI 44kb allele having no point mutations (D6-B and D6-A) or extra NcoI fragment (Figure 1). All of the members of the families were extensive metabolizers, as shown by their metabolic ratios. In family A, individual I2, an extensive metabolizer, is heterozygous for XbaI 44kb and 29kbD6-B, whereas I1 who is homozygous for XbaI 44kb is an extensive metabolizer. Thus, unambiguously the 44kb allele is associated with an extensive metabolizer allele, designated 44kb-em, in this family. Family B contains another example of the 44kb-em allele, although its combination with a XbaI 11.5kb allele (lack of functional CYP2D6 gene) results in an extensive metabolizer phenotype (individual I2). A third family (Family C) provides further evidence for the existence of another 44kb-em allele. Here, the D6-B mutation was assigned to the maternal XbaI 44kb fragment as this allele was associated with a NcoI 12.5kb fragment.

Families D and E, have genotypes comparable with family A and B, but the XbaI 44kb allele carries the D6-B mutation and is combined with known PM alleles. The 44kb allele in these two families is undoubtedly a PM allele, as indicated by the value of their log metabolic ratio.

We selected 47 subjects having comparable genotypes to determine whether the two previously defined XbaI 44kb haplotypes (XbaI 44kbD6-B and XbaI 44kbD6-em) correspond to two distinct phenotypic groups. Group I XbaI 44kbD6-B/29kbD6-wt contained 35 cases and group II XbaI 44kbD6-em/29kbD6-wt contained 12 cases. The mean urinary dextromethorphan excretion over 8 h in group I was significantly lower than that in group II (P < 0.005) (Table 2). However, the mean dextromethorphan excretion and metabolic ratios of the two groups were not statistically different owing to the low concentrations of unchanged drug.

**Discussion**

The advantage of DNA-based identification of poor and extensive metabolizer alleles over clinical phenotyping procedures is that it is free from interference from coadministered drugs or confounding diseases. It is also, in principle, a less invasive procedure as no probe drug administration is required to determine the phenotype, and is thus particularly suitable for pediatric and pregnant
patients. Genotyping should be capable of identifying EM heterozygotes without a family study; this is not, at present, feasible at the phenotype level. These potential advantages will have a practical impact on clinical pharmacology only when a clearcut correlation has been established between genotype and phenotype. Genotypes defined by RFLPs are genetic markers, and their association with a mutation-conferring enzyme deficiency may or may not be very strong within an ethnic group. The correlation is likely to be even poorer in admixed populations, such as Europeans or North Americans.

The heterogeneity of the XbaI-defined 44kb allele of the CYP2D locus with respect to phenotype has been noted, but not explored in detail (Broly et al., 1991; Daly et al., 1990). The present results demonstrate the molecular heterogeneity of the XbaI-defined 44kb allele. The first level of heterogeneity is related to mutation in the functional gene. Although 75% of the 44kb alleles carry the D6-B mutation and represent the PM allele, 25% do not. This defines two groups of 44kb alleles: XbaI 44kbD6-B (group I) and XbaI 44kb-em (group II). The second level of heterogeneity is the associated polymorphisms for other restriction enzymes, such as NcoI and BamH1. The group I allele differs from that of group II in having an extra fragment in NcoI digests and a more intense regular D7-related BamH1 fragment. The extra NcoI fragment (12.5kb or 4.8kb) in the XbaI 44kbD6-B haplotype strongly suggests the presence of an additional CYP2D6-related gene, probably D7*, confirming published data (Hanioka et al., 1991). Similarly, XbaI 44kb-em alleles do not appear to possess an additional gene, as neither an extra NcoI fragment nor a more intense BamH1 fragment were observed. In this regard, our data do not support the hypothesis of Broly et al. (1991) of an additional gene in XbaI-44kb regardless of whether or not the D6-B mutation was present. Their assumption that XbaI 44 kb-E (44kb-em in our study) is the 'Chinese' allele (Johanson et al., 1991), is probably not true as the latter has an additional 12.5kb NcoI fragment, whereas XbaI 44kb-em (and hence 44E) does not. Our previous results demonstrate that these two alleles are distinct (Mura et al., 1991). Alternatively, the 44-E allele and our XbaI44-em may be distinct, and hence not comparable as there is no NcoI data for the former. The group II alleles appear to be more heterogeneous with respect to the associated BamH1 polymorphism than the group I alleles, which all have the + + BamH1 pattern. The homogeneous + + BamH1 polymorphic pattern of group I alleles is of interest. Over 90% of the chromosomes bearing the D6-B mutation on a XbaI 29kb fragment also had a + + BamH1 pattern (unpublished data). This tight linkage between BamH1 polymorphism and D6-B mutation suggests that XbaI-44D6-B might have been generated by a recombination event involving a mutant and a 29kbD6-wt allele.

Our genotype-phenotype data plus family information, indicate that ten of the forty-two 44kb alleles studied are extensive metabolizer alleles (44kbD6-em), so that individuals with a 44kb XbaI restriction fragment are not obligate extensive metabolizer heterozygotes. These 44-kb-em alleles also lacked a 12.5kb or a 4.8kb NcoI fragment. Subjects with a 44kb allele may therefore be characterised by the combined information obtained by XbaI and NcoI RFLPs and PCR screening for D6-B. This is relevant and important, especially in epidemiological genotypic screening of individuals to predict the metabolic phenotype. They also explain some of the reported discrepancies in genotype-phenotype correlation (Broly et al., 1991; Daly et al., 1990; Turgeon et al., 1991). However, it is intriguing to find that the presence of an additional gene coincides with the occurrence of the D6-B mutation in the XbaI 44kbD6-B allele. Further data from different ethnic groups is necessary to understand this enigma.

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References


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