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*Xba*I 16- plus 9-Kilobase DNA Restriction Fragments Identify a Mutant Allele for Debrisoquin Hydroxylase: Report of a Family Study

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SUMMARY

Previous studies have established that two *Xba*I polymorphic restriction fragments [11.5 and 44 kilobases (kb)] hydridizing to a full length debrisoquin hydroxylase cDNA are associated with two different mutant alleles for the debrisoquin hydroxylase gene (IID6). An independent allele, defined by *Xba*I 16- and 9-kb fragments, has previously been identified only in extensive metabolizers, precluding determination of whether this restriction fragment length polymorphism (RFLP) pattern identifies a functional or nonfunctional mutant allele of IID6. We phenotyped and performed RFLP studies in the family of a poor metabolizer (PM) propositus with the *Xba*I 16+9 allele. Three family members were of the PM phenotype, two of whom had the 16+9 allele, indicating that this RFLP pattern identified a nonfunctional IID6 allele in this family. Moreover, additional RFLP analyses indicated that the PM mother had two different IID6 mutant alleles that produce the *Xba*I 29-kb fragment, indistinguishable from the RFLP pattern produced by a functional IID6 allele. These data indicate that the *Xba*I 16+9 RFLP pattern can identify a mutant IID6 allele and that at least two different nonfunctional mutant alleles can produce the *Xba*I 29-kb fragment found in all extensive metabolizers.

Genetic regulation of enzymes involved in drug metabolism is a major determinant of interindividual variability in the pharmacokinetics of many drugs (1–3). One of the most widely studied polymorphisms of drug metabolism is the debrisoquin-type oxidative pathway, which is now known to involve the metabolism of over 24 drugs (4–8). In American and European Caucasians, about 5–10% of the population is deficient in the ability to metabolize drugs by the debrisoquin-oxidative pathway (9–11). Clinical studies have demonstrated that these individuals (i.e., PMs) are at increased risk for adverse drug effects if these pathways are involved in drug detoxification (10, 12, 13) or are less likely to derive therapeutic benefit if these pathways are required for activation of the drug (14).

Population studies and family pedigree analyses have indicated that the enzyme involved in this metabolic pathway [debrisoquin hydroxylase, P450 IID6 (15)] is under monogenic control and the PM phenotype is inherited as an autosomal recessive trait (5, 10, 16). Zanger and co-workers (17) have demonstrated the complete or almost complete absence of the high affinity, highly stereoselective, P450 IID6 enzyme in human liver microsomes prepared from PM phenotypes. A full length cDNA for P450 IID6 has now been cloned and expressed, and variant messenger RNAs have been identified as products of mutant genes by cloning of cDNAs from several PM livers (18). Recently, Skoda and co-workers (19) identified 14 allelic forms of the IID6 gene, but to date only two have been linked to the PM phenotype. By probing leukocyte DNA obtained from unrelated PM and EM subjects with the full length P450 IID6 cDNA, two polymorphic *Xba*I restriction fragments were associated with the PM phenotype (19). The predominant mutant allele, identified by a 44-kb restriction fragment following digestion with the *Xba*I endonuclease, was found in 58% of PM phenotypes but only 3% of EM subjects. A second mutant allele, identified by an 11.5-kb fragment following *Xba*I digestion, was found in 33% of PMs and no EMs.

As concluded by Skoda and co-workers (19), additional mutant alleles must exist for the IID6 gene, because no mutant allele was identified in 25% of the PMs in their study. One additional allele found by Skoda *et al.* (19) was identified by the combination of 16- and 9-kb fragments, following digestion of leukocyte DNA with *Xba*I restriction endonuclease. This RFLP pattern was found in 2 of 29 EM subjects, in combination

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ABBREVIATIONS: PM, poor metabolizer; EM, extensive metabolizer; kb, kilobase; RFLP, restriction fragment length polymorphism.

with a 29-kb band, but in none of the 24 PMs studied. It was, therefore, not possible to determine whether the 16+9 RFLP pattern identified a functional allele or a nonfunctional mutant allele for the debrisoquin hydroxylase gene, because the two EM subjects with this allele could have either one or two functional alleles for the debrisoquin hydroxylase gene, given the autosomal recessive inheritance of the PM trait.

In this paper, we report the RFLP analysis of a family in which two individuals with the *Xba*I 16- and 9-kb fragment allele are PM phenotypes, representing the first report of this allele in a PM subject and demonstrating that the *Xba*I 16+9 RFLP pattern identified a nonfunctional mutant allele in this family.

Materials and Methods

Human subjects. The PM propositus (D.J.) is a white female, age 29 years, with no known medical illnesses or organ dysfunction. She was identified as a debrisoquin hydroxylase PM phenotype during participation in a volunteer study, using dextromethorphan as the test substrate, as described below. The family comprises the father (age, 62 years), mother (age, 58 years), and sister (B.S.) (age, 32 years) of the PM propositus. All were without known illness, major organ dysfunction, or therapy with medications that would interfere with the dextromethorphan phenotyping procedure (6).

Debrisoquin hydroxylase phenotype determination. After informed consent was obtained, all family members received an oral dose of dextromethorphan (30 mg, given as 10 ml of Pertussin 8-HR cough syrup). A predose urine sample and postdose 4-hr urine collection were obtained. This method and duration of urine collection has been shown to adequately characterize debrisoquin hydroxylase metabolizer phenotype (20). A 10-ml aliquot from the entire urine collection was stored at −70° until assayed for dextromethorphan and dextrorphan.

Assay of dextromethorphan and its major metabolite, dextrorphan, was performed by a modification of the high performance liquid chromatography-UV method of Park *et al.* (21), as previously described in detail (6).

The urinary ratio of molar dextromethorphan to dextrorphan concentrations was used to determine debrisoquin hydroxylase oxidative metabolism phenotype (8). EMs are defined as those with ratios <0.3, and PMs as those >0.3. In a study of 268 adults (8), there was a clear separation between the urinary metabolic ratios for EM and PM phenotypes, because no patients had ratios between 0.2 and 0.6 (antimode).

Nucleic acid procedures. DNA was isolated from leukocytes by phenol and chloroform extractions and isopropanol/sodium acetate precipitation, as previously described in detail (22). DNA yields from leukocytes in 30 ml of whole blood ranged from 500 to 1000 μg, with 260 nm/280 nm ratios ranging from 1.6 to 2.0 for all subjects.

DNA (5–10 μg) was digested to completion with a restriction endonuclease (*Xba*I or *Bam*HI), under conditions recommended by the source (New England Biolab). Southern blotting (23) was performed by alkaline transfer to Biotrace RP membranes (Gelman Scientific, Inc.). The full length IID6 cDNA hybridization probe (kindly provided by Dr. Frank Gonzalez, National Institutes of Health) was prepared by nick translation with [^{32}P]dCTP (6000 Ci/mmol, Amersham Corp.) to a specific activity of 1–3 × 10^8 dpm/μg of DNA.

Results

The family (father, mother, and two daugthers) contained one EM (father) and three PM phenotypes (mother and both daughters). The dextromethorphan to dextrorphan metabolic ratios for each family member are shown in Fig. 1, demonstrating a clear difference (>20-fold) between the EM and PM phenotypes. Because the father is an EM phenotype with PM phenotype offspring, he is an obligate heterozygote for the IID6 gene.

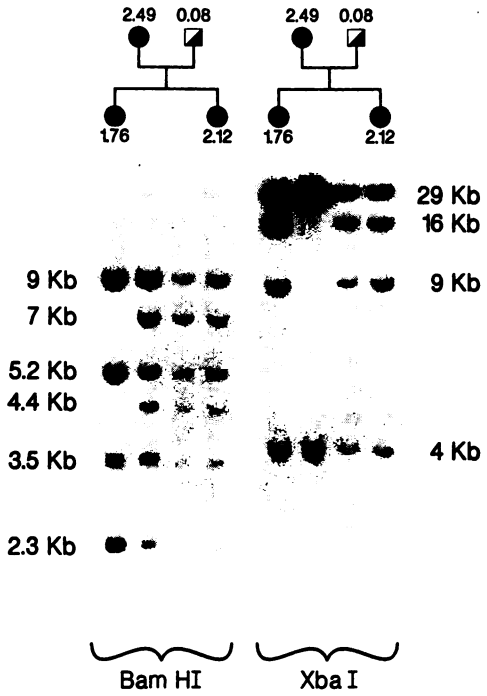

Fig. 1. Family pedigree and autoradiographs of Southern blots of leukocyte DNA obtained from the family of a PM propositus (*2.12*). ●, Debrisoquin hydroxylase PMs (mother and two daughters). The EM father is an obligate heterozygote (◪). The urinary metabolic ratios (dextromethorphan/dextrorphan) used to assign phenotype are given for each family member. DNA fragment lengths with the two restriction endonucleases (*Bam*HI, *Xba*I) were determined based on migration of DNA markers of known length.

When DNA was digested with *Xba*I, the RFLP patterns for three of the family members (father and two daughters) contained bands of 29, 16, and 9 kb that hybridized with the IID6 cDNA probe, plus the 4-kb band found in all subjects (19). The fourth family member (mother) had only the 29-kb band after *Xba*I digestion of DNA. As previously reported (19), the 29-kb fragment is associated with at least two different alleles for IID6 (at least one functional and one nonfunctional), whereas a separate allele is defined by the presence of 16- plus 9-kb fragments.

Results of the clinical phenotyping and *Xba*I RFLP analysis, taken together, indicate that the 29-kb allele of the father is a functional allele, because he is an obligate heterozygote EM and his 16+9 allele was inherited by the two PM phenotype daughters. Moreover, because both daughters inheriting the 16+9 allele are PM phenotypes, this RFLP pattern must be associated with a mutant (nonfunctional) allele for IID6 in this family.

The third PM individual in this family (mother) has only the 29-kb *Xba*I fragment. By definition, the 29-kb fragment has identified nonfunctional alleles in this PM individual, as was the case in 79% of unrelated PMs previously reported by Skoda *et al.* (19).

To further elucidate the molecular characteristics of the IID6 gene in this family, additional RFLP analyses were performed with other restriction enzymes that were previously reported to produce RFLPs with IID6 (19). As shown in Fig. 1, the two daughters had different *Bam*HI RFLP patterns, despite having

the same *Xba*I 29/16+9 RFLP pattern. This indicates that each of the two daugthers inherited a different mutant allele from their mother, because both inherited the *Xba*I 16+9 allele from their father. The father (heterozygote EM) had the same *Bam*HI RFLP pattern as one PM daughter (D.J.) and the PM mother (i.e., 9-, 7-, 5.2-, 4.4-, 3.5-, and 2.3-kb fragments), whereas the other PM daughter (B.S.) had a different *Bam*HI RFLP pattern (i.e., 9-, 5.2-, 3.5-, and 2.3-kb fragments). Thus, the mother must have two different nonfunctional IID6 alleles, which produce the same *Xba*I 29-kb fragment but different fragments with *Bam*HI.

Moreover, the fact that one daughter (B.S.) inheriting the father's *Xba*I 16+9 allele lacks the *Bam*HI 7- and 4.4-kb fragments, whereas the father and mother have these fragments, is consistent with the father's functional *Xba*I 29-kb allele and one of the mother's nonfunctional alleles yielding the 7- and 4.4-kb *Bam*HI fragments. This finding (i.e., *Bam*HI producing RFLP patterns not discriminating the PM phenotype) is consistent with previously reported studies (19), and yet the different *Bam*HI RFLP patterns in the two PM daughters indicate that there are at least two mutant IID6 alleles that produce a *Xba*I 29-kb fragment, as does the wild-type allele.

Discussion

This family study has identified debrisoquin hydroxylase PM phenotype subjects who have the *Xba*I 29-, 16- and 9-kb fragments on Southern blots, using the IID6 cDNA probe. Skoda and co-workers (19) originally observed this RFLP pattern and described an independent mutant allele for the IID6 gene by the presence of *Xba*I 16+9 kb fragments, which was found only in a heterozygous state with the 29-kb allele in 2 of 26 EM subjects. It was, therefore, not possible for Skoda and coworkers to determine whether the *Xba*I 16+9 allele was associated with a functional or nonfunctional allele, because it was not found in any PM subjects.

The present study demonstrates that the *Xba*I 16+9 RFLP pattern can identify a nonfunctional allele for the IID6 gene. It is possible that the 16+9 RFLP pattern always identifies a nonfunctional mutant allele for IID6, because the previously reported cases were likely heterozygote EMs with a functional allele identified by the *Xba*I 29-kb fragment. However, additional unrelated EMs and PMs with this allele must be studied to establish whether the 16- and 9-kb fragments consistently identify a mutant IID6 allele, as do the 11.5- and 44-kb fragments. To date, there are no published reports of individuals homozygous for the 16+9 allele, although this is likely due to the relative rarity of this genotype and the small number of individuals studied by RFLP analysis to date.

A second finding in the present study is that two nonfunctional mutant alleles (in the mother) produced the *Xba*I 29-kb fragment, yet produced two different RFLP patterns when digested by another restriction enzyme (i.e., different RFLP patterns in the two daughters, who inherited the same *Xba*I 16+9 allele from the father). This indicates that at least two different mutations occurred in the mother's two nonfunctional alleles for the IID6 gene, yet both nonfunctional alleles produced only the *Xba*I 29-kb fragment, indistinguishable from the wild-type (functional) allele in the father and all EM subjects studied to date (19). The finding that the mother's nonfunctional mutant alleles produced the *Xba*I 29-kb fragment is not unusual, because this fragment was identified in 79% of PMs reported by Skoda *et al.* (19), and was the only polymorphic fragment found in 25% of their PMs. Thus, the *Xba*I 29-kb fragment is often associated with a nonfunctional IID6 allele; our findings indicate that at least two different nonfunctional IID6 alleles can produce the *Xba*I 29-kb fragments. Thus, at least two, and possibly several IID6 mutant alleles yield a *Xba*I 29-kb fragment, consistent with the relatively high prevalence (79%) of the *Xba*I 29-kb allele in PM phenotype subjects (19).

The previously reported mutant alleles, identified by *Xba*I 11.5- or 44-kb fragments, are the most prevalent mutant alleles for IID6. The present study indicates that a third nonfunctional allele can be identified by the presence of *Xba*I 16- and 9-kb fragments on Southern blots, using the full length IID6 cDNA probe. This mutant allele appears to be relatively rare in Caucasians, occurring considerably less frequently than either the 11.5- or 44-kb alleles. A major limitation of current RFLP analysis is that the majority of PM individuals have a *Xba*I 29-kb fragment that is indistinguishable from the 29-kb fragments found in all EM individuals, limiting the use of RFLP analysis to determine phenotype. Results of the current study indicate that at least two different mutations in the IID6 gene can yield a nonfunctional allele that produces the *Xba*I 29-kb fragment, suggesting that gene probes more specific than the full length cDNA will be required to develop genetic analyses that unequivocally identify the debrisoquin hydroxylase PM phenotypes.

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