

Human debrisoquine hydroxylase gene polymorphisms in cancer patients and controls

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The extensive metabolizer phenotype of debrisoquine has been associated with increased risk of lung cancer, and it has been proposed that a molecular test for this phenotype is feasible. DNA restriction fragment length polymorphisms of the human debrisoquine 4-hydroxylase gene locus (*CYP2D6*), and the metabolic phenotype for debrisoquine have been studied in a group of healthy volunteers, a group of lung cancer patients and two control groups (chronic obstructive pulmonary disease patients and patients with cancers at sites other than the lung). Confirmation of four distinct *Xba*I allelic fragments (44, 29, 16/9 and 11.5 kb), previously identified among caucasians, was obtained. The 29 kb alleles were the most frequently observed in both poor and extensive metabolizers of debrisoquine. Alleles of 44 kb were found with approximately equal frequency among both poor and extensive metabolizers. The data are consistent with the hypothesis that the 11.5 and 44 kb fragments are associated with mutant alleles of the *CYP2D6* gene, but the power of phenotype prediction by these alleles was less than that previously reported for a European (Swiss-German) population. Similarly, the data also show that 8% of 29 kb homozygotes are poor metabolizers (indicating that at least 28% of 29 kb fragments are also associated with mutant alleles) and are not therefore informative for predicting the debrisoquine phenotype. The 16/9 allele may represent either wild-type or mutant alleles. Restriction fragments of 44 kb were found more frequently among cancer patients and chronic obstructive pulmonary disease patients (30%) than among the healthy volunteer group (7%). Genotypes observed were not related to lung tumor histology. Furthermore, at least three *Eco*RI alleles were found to be in linkage disequilibrium with the 'mutant' 44 kb allele. These data suggest that the 44 kb allele can comprise three distinct haplotypes, in contrast to studies of a European population. These studies indicate that no single mutant *CYP2D6* allele as determined by *Eco*RI appears to be associated with lung cancer, despite the findings that these patients are invariably of the extensive metabolizer phenotype.

Introduction

Human debrisoquine 4-hydroxylase (P450IID6) activity is known to be polymorphic among the caucasian population where

*Abbreviations: EM, extensive metabolizer; PM, poor metabolizer; DNA-RFLP, DNA restriction fragment length polymorphism.

individual capacities to metabolize substrates such as debrisoquine, dextromethorphan, propranolol and bufuralol are bimodally distributed (1-3). Epidemiological studies have provided support for the original observations of Ayesch *et al.* (4) that lung cancer risk is increased in extensive metabolizers (EMs*) of debrisoquine (5-7), while two other studies have reported equivocal (8) or no (9), support for this association. However, in the latter study by Speirs *et al.* (9), conclusions are drawn from comparison of lung cancer cases with historical controls. Roots *et al.* (8) found marginal statistical significance of the relationship between debrisoquine metabolism and lung cancer. Therefore, this polymorphism is currently the focus of studies attempting to identify individuals with increased susceptibility to lung cancer, based on the premise either that interindividual variations in metabolism of procarcinogenic xenobiotics may be a significant determinant in cancer susceptibility or that the debrisoquine 4-hydroxylase gene is linked to an oncogene or tumor suppressor gene (10). However, no procarcinogens have yet been identified as substrates for this enzyme (11). A goal of this study is to validate genetic markers that can then replace the difficult process of phenotyping in studies of cancer susceptibility.

The debrisoquine 4-hydroxylase cDNA was cloned and sequenced (12-14) and used as a probe to detect DNA restriction fragment length polymorphisms (DNA-RFLPs) (15). In this previous study, a relationship was found between metabolic phenotype and DNA-RFLP patterns in peripheral blood lymphocyte samples among a European (Swiss-German) population (15). In particular, the polymorphism revealed by the restriction enzyme *Xba*I was found to be the most promising for predicting the phenotype of an individual. Four different allelic *Xba*I fragments were detected with this enzyme: a 29 kb fragment thought to represent primarily wild-type alleles, 44 and 11.5 kb alleles thought to represent mutant alleles, and a pair of fragments of 16/9 kb found only in combination with the 29 kb band. Using these criteria, the *Xba*I DNA-RFLP was reported to identify 70% (17/24) of poor metabolizers (PMs) that had the mutant 44 or 11.5 kb alleles (15). The remaining PMs, 29 kb allele homozygotes, were explained by the presence of one or more mutant 29 kb alleles. In the present study, the relationship between debrisoquine metabolic phenotype and *Xba*I DNA-RFLPs among lung cancer patients and controls has been examined. In addition, the previously reported *Eco*RI polymorphism has also been studied, since it was reported to be in linkage equilibrium with the *Xba*I predictive alleles.

Materials and methods

Study subjects

Thirty-eight healthy volunteers (20 male; 18 female), 35 of whom were white, were recruited from the Laboratory of Human Carcinogenesis and the Environmental Epidemiology Branch of the National Cancer Institute (Bethesda, MD) between March and September 1988. Forty-five lung cancer patients (43 male, 2 female; 22 whites, 23 blacks) and 42 control subjects (39 male, 3 female; 23 whites, 19 blacks) consisting of 28 chronic obstructive pulmonary disease patients and 14 patients with tumors at sites other than the lung or bladder (16) were analyzed. These subjects were recruited as part of the ongoing case-control study at the University of Maryland and Baltimore Veterans Administration Hospitals

between July 1985 and December 1988. DNA samples were analyzed in all subjects with adequate DNA available. Non-essential medication use was withheld on the day when phenotyping was performed. Individuals who received drugs known to influence the metabolic ratio (i.e. quinidine) were excluded from the analysis.

Determination of debrisoquine metabolic phenotype

Debrisoquine (10 mg) was administered orally and urine was collected over an 8 h period. Levels of debrisoquine and its principal metabolite, 4-hydroxydebrisoquine, were measured in an aliquot of urine according to the method of Mahgoub *et al.* (1). The metabolic ratios (percentage of dose eliminated as unmetabolized debrisoquine divided by the percentage of the dose eliminated as 4-hydroxydebrisoquine) were calculated as previously described (6). Cutoff points for the determination of the metabolic phenotype from the metabolic ratio were determined according to a previously published method (17). Briefly, a computerized optimization method (3,18) was used to generate three log-normal distributions of the metabolic ratios of controls of each race and the healthy volunteers, under an autosomal codominant model of inheritance. Extensive, intermediate and poor metabolizers correspond to homozygous dominant, heterozygous and homozygous recessive metabolizers respectively. Since the metabolic ratios defining the EMs and intermediate metabolizers overlap, these two groups were combined in this report as EMs for a comparison with the PM group (4,18). The natural log metabolic ratios above which an individual was considered to be a PM were 4.2 for the healthy volunteers, and 2.5 and 3.3 respectively for the white and black controls. Study subjects were then placed into phenotypic categories according to their metabolic ratios.

DNA restriction fragment length polymorphism analyses

Samples of DNA were digested with either *Xba*I or *Eco*RI (Pharmacia, Piscataway, NJ) and subjected to agarose gel electrophoresis (0.4–0.7%). The gels were treated with NaOH:NaCl (400 mM:0.6 M) and neutralized with NaCl:Tris (1.5 M:500 mM, pH 8.0) prior to transfer of the DNA to nylon membranes (Bio Trace-Gelman, Ann Arbor, MI or Gene Screen Plus, New England Research Products, Boston, MA). The DNA samples were then hybridized with a 1.6 kb cDNA clone of human *CYP2D6* (12) under stringent conditions (1% dextran sulfate, 1 M NaCl, 1% SDS at 65°C). The membranes were washed with sodium chloride:sodium citrate:SDS (300 mM:30 mM:1%) twice for 30 min at 65°C. X-ray films (Eastman Kodak Company, Rochester, NY) were then exposed to the membranes for the detection of restriction fragments and their sizes were determined by comparison with high mol. wt size markers (New England Biolabs, Beverly, MA).

Results

Samples of DNA isolated from peripheral blood lymphocytes of 38 healthy volunteers, 45 lung cancer patients, 28 chronic obstructive pulmonary disease patients and 14 patients with tumors at various sites other than lung or bladder were digested to completion with *Xba*I and subjected to Southern hybridization with a cDNA clone (1.6 kb) of the human *CYP2D6* gene. These experiments detected four different alleles among the 125 study subjects (Figure 1). The most common allele was a 29 kb restriction fragment. Fragments of 16 kb and 9 kb together (16/9) represented the least common allele and intermediate frequency alleles were found at 44 or 11.5 kb. The following genotypes were detected: heterozygotes 29:11.5, 44:29 and 44:16/9 (lanes B, C and D respectively), and the 29 kb homozygote (lane A). No 44, 11.5 or 16/9 kb homozygotes were observed in these studies.

The relationships between the genotype (RFLP analysis) and phenotype (metabolic ratio) for each group are given in Table I. Ninety per cent of PMs and 99% of EMs were found to have at least one 29 kb allele, therefore, this allele was the most frequently observed irrespective of metabolizer phenotype. There was no significant difference in the allelic distribution between each study group. The 44 kb allele was found in almost equal proportions between PMs and EMs (36 and 34% respectively). The 44:29 and 29:11.5 genotypes were found mainly in EMs (93 and 86% respectively), in marked contrast with those determined among Europeans (Swiss–Germans) by Skoda *et al.* (15) in which these genotypes were associated almost exclusively with the PM phenotype.

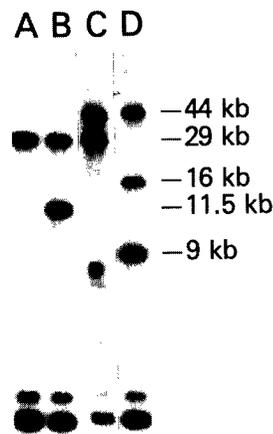


Fig. 1. Representative Southern analysis for human genomic DNA digested with *Xba*I. Following agarose gel electrophoresis, DNA samples were immobilized on a nylon filter and hybridized to the *CYP2D6* cDNA clone (10,15). The genotypes shown are for a 29 kb homozygote (A), and heterozygotes 29:11.5 kb (B), 44:29 kb (C) and 44:16/9 kb (D).

Table I. Comparative analysis of debrisoquine metabolic phenotypes and the *Xba*I DNA-RFLP at the *CYP2D6* gene locus in lung cancer patients and controls

Genotype ^a	Lung cancer ^b		Control group ^c		Healthy volunteers	
	EM	PM	EM	PM	EM	PM
29/29	18	0	18	3	26	2
44/29	20	0	15	2	3	1
29/11.5	7	0	2	2	3	0
29/16/9	0	0	0	0	1	0
44/16/9	0	0	0	0	1	1

^aGenotype is the determination of *Xba*I DNA restriction fragment size (kb).

^bCategorization of metabolic phenotypes were determined by the maximum likelihood method (3,18). EMs are defined as having a metabolic ratio of less than the cut-off value between the two metabolic groups, and PMs are defined by a metabolic ratio of greater than that value. The cut-off value was calculated by the method described in the text; calculations were made separately for each race and the cut-off values were determined to be $\epsilon_{3,3}$ in blacks, $\epsilon_{2,5}$ in whites and $\epsilon_{4,2}$ in healthy volunteers.

^cControl subjects in this group consisted of age, race and smoking matched patients with chronic obstructive pulmonary disease or cancer at sites other than the lung or bladder.

Our studies did not reveal the previously reported 44:11.5, 44:44 or 11.5:11.5 genotypes (Figure 1), therefore it is not possible to confirm or deny the hypothesis that the 44 and 11.5 kb alleles are mutant forms of the gene. However, two subjects with the 44:16/9 genotype were found; one was an EM, and one was a PM. Therefore, these data would support the possibility that the allele represented by the 16 and 9 kb fragments could be either wild-type or mutant, assuming that the 44 kb fragment is a mutant form.

No PMs were found among the group of 45 lung cancer patients. This is consistent with the previously reported association between lung cancer risk and debrisoquine metabolic phenotype (4–7). Furthermore, no differences in allelic distributions were found between race, histologic type of lung cancer, or non-lung cancer patients when compared to chronic obstructive pulmonary disease controls (Table II). Thus, there was no association between the *Xba*I polymorphism and disease category or phenotype. The data also revealed that 29:29 kb homozygotes

Table II. *Xba*I polymorphic genotype and histology of lung cancer

Genotype ^a	Lung cancer ^b			
	Adenocarcinoma ^c	Squamous cell carcinoma	Small cell carcinoma	Large cell carcinoma
29/29	4	11	2	1
44/29	8	10	2	0
29/11.5	2	3	1	1

^aDetermination of the *Xba*I DNA-RFLP by Southern analysis, sizes of restriction fragments are given in kilobases.

^bNo poor metabolizers were found among this sub-set of 45 lung cancer patients.

^cNo relationship between genotype 29/29 versus 29/X and adenocarcinoma versus non-adenocarcinoma was found ($\chi^2 = 0.52$, $df = 1$, $P = 0.47$).



Fig. 2. Representative Southern analysis for human genomic DNA digested with *Eco*RI. Following agarose gel electrophoresis, DNA samples were immobilized on a nylon filter and hybridized to the *CYP2D6* cDNA clone (10,15). Where it was previously shown that individuals with a 44 kb *Xba*I allele had an associated 15 kb allele (1), the data shown here revealed either no associated band (A), a 16 kb band (B), a 15 kb band (C), a 13 kb band (D) or a 16:13 heterozygote (E).

Table III. Comparison of the *Eco*RI DNA-RFLP with the *Xba*I DNA-RFLP^a

<i>Xba</i> I restriction fragments	<i>Eco</i> RI restriction fragments				Total
	13	15	16	None ^b	
44	4	0	6	13	23
11.5	0	4	0	0	4

^aRestriction fragment sizes (kb) determined by Southern analysis for *Eco*RI or *Xba*I digests of high mol. wt DNA.

^bNo variant fragment detected.

were not more likely to be EMs than heterozygotes (Table I).

Figure 2 shows representative Southern analyses for *Eco*RI-digested DNA samples. The restriction fragments of 18, 10 and 8 kb are invariant, while the 13, 15 and 16 kb fragments are polymorphic, as previously described (15). The 15 kb fragment was always found in samples where *Xba*I digestion revealed an 11.5 kb restriction fragment (Table III), indicating that these two fragments are in linkage disequilibrium. In 23 samples where *Xba*I had revealed a 44 kb fragment, *Eco*RI revealed a 16 kb fragment in six samples, a 13 kb fragment in four samples and no variant band in 13 samples (Table III). Furthermore, when the 44:16/9 genotypes were revealed by *Xba*I, the associated *Eco*RI fragments were 13 kb for the EM and 16 kb for the PM.

Discussion

Previous evaluation of the *Xba*I DNA-RFLP of the human debrisoquine hydroxylase locus (14) was in part predictive of the debrisoquine metabolic phenotype. These data showed that the presence of a 44 or 11.5 kb restriction fragment was associated with the PM phenotype, indicating that these restriction fragments were associated with or contained mutant *CYP2D6* alleles. The relationship between *Xba*I DNA-RFLPs and

metabolic phenotype has now been evaluated in a United States population of healthy volunteers, chronic obstructive pulmonary disease patients and cancer patients, which included patients with lung, breast, skin, esophageal and colon cancer. The overall frequency of the distribution of the various alleles (Table I) that were detected was in agreement with previous data (14). The common 29 kb allele was observed with almost equal frequency in poor and extensive metabolizers; therefore, the probability that a 29 kb allele represents a mutant form is ~20% in the 29:29 kb homozygous population.

In the populations studied here, 55% of PMs were found to have the 44 or 11.5 kb alleles, and 45% of EMs have at least one of these alleles. The observation that 44 kb alleles are present among 34% of EMs in the current study is significantly different from that of 3.4% reported previously in a European population ($\chi^2 = 5.72$, $P < 0.001$) (15), but consistent with data on a Chinese population (19). This trend would appear to argue against the hypothesis that the 44 kb allele is always associated with a mutant form of the gene, but since 44 kb homozygotes were not found among the populations studied here this hypothesis could not be adequately tested. However, Table I shows that 3 of 41 individuals who were found to be 44:29 kb heterozygotes were also found to be PMs. This is approximately the expected proportion given that 15–25% of 29 kb alleles are mutant alleles.

One reason for the discrepancies between these data sets might be that the European population studied earlier consisted of an unusually large number of PMs (45%), in comparison with the expected frequency (<10%) among most Western populations (3,19). Alternatively, these observations are consistent with the contention that mutant alleles act in a recessive manner and the different populations contain variable proportions of presumed 29 kb mutant alleles. This hypothesis (variable prevalence of recessive 29 kb alleles among subpopulations) would also explain the observation that a higher frequency of 44 kb alleles was found among cancer patients (all EMs) and chronic obstructive pulmonary disease patients that were part of a case-control study than were observed among the healthy volunteer group. However, it was recently reported (19) that among Chinese subjects the 44 kb allele was present in 50% of EMs. Further evidence that the 44 kb allele is not sufficient to predict poor metabolizer status is provided in our data by the detection of the 44:16/9 genotype in both a poor and an extensive metabolizer.

In general, the *Xba*I DNA-RFLP and phenotyping data presented here are consistent with previous reports (15,19), however, the *Eco*RI DNA-RFLP data showed more and different polymorphic bands in comparison to earlier reports. In a previous study (15) an *Eco*RI digest was found to be interchangeable with *Xba*I for identification of the 'mutant' 44 kb *Xba*I allele. However, that finding has not been corroborated in the current study. Three apparently polymorphic restriction fragments may be detected with *Eco*RI at the *CYP2D6* gene locus: 13, 15 and 16 kb (Figure 2). Whereas the 15 kb *Eco*RI allele was always found to be associated with the 11.5 kb *Xba*I allele, the 13 and 16 kb *Eco*RI alleles were found in association with the 44 kb *Xba*I alleles in only 10 of 23 samples, indicating that the 44 kb allele is heterogeneous. This supports the statement that not all 44 kb 'mutant' alleles are responsible for the poor metabolizer phenotype.

The data presented here are inconsistent with the assertion that the *Xba*I and *Eco*RI DNA-RFLPs can be used interchangeably to identify mutant alleles of human *CYP2D6*. These data and those of earlier reports (19) are more consistent with the hypothesis that *Xba*I can be used to identify a significant proportion of mutant

forms of the *CYP2D6* gene. Differences in the overall distribution of DNA restriction fragments using this enzyme were not found to vary with ethnicity in this study, even though geographical differences have been observed for the metabolic phenotype (19–22). These findings demonstrate the complexity of P450 metabolism and suggest the possibility that mutations previously presumed to be responsible for the debrisoquine phenotype only partially explain interindividual variation. A diagnostic restriction enzyme might be variable among populations or between families; furthermore, the regulation of debrisoquine gene expression remains to be studied. Studies at the RNA level may indicate more clearly the appropriate probes and restriction enzymes to use to predict individual phenotypes. Recent molecular studies of debrisoquine by Kimura *et al.* (14) have described the transcription start site of *CYP2D6*. These types of studies may help to elucidate whether there are other controlling factors, e.g. an independent gene, influencing debrisoquine hydroxylase expression. A direct approach, the amplification of debrisoquine cDNA, has recently been reported by Redlich *et al.* (23). This strategy is promising for establishing a molecular test for phenotypic expression of debrisoquine and may have epidemiological application in the future.

Previous studies have clearly demonstrated an association between metabolic phenotype and lung cancer (4–7, 18), although studies with negative or ambiguous results have been reported (8, 9). This association between EM, phenotype and lung cancer could be due to either a direct action of the P450 in activating a procarcinogen, or perhaps *CYP2D6* allele is in linkage disequilibrium with another gene that predisposes for lung cancer. It appears from the data presented here that the *CYP2D6* locus is heterogeneous in cancer patients, indicating that a number of different restriction fragments are wild-type alleles and further suggest the existence of heterozygotes who are EMs.

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