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## Frequency of the variant allele *CYP2D6(C)* among North American Caucasian lung cancer patients and controls<sup>1</sup>

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### Abstract

Previous reports of the association between the debrisoquine polymorphism and lung cancer risk are conflicting. Following the report of an association between lung cancer risk and the variant allele *CYP2D6(C)*, we examined the presence of this allele in 98 incident Caucasian lung cancer patients and 110 age, race, and sex matched hospital controls from a case-control study conducted at the National Naval Medical Center in Bethesda, MD. Debrisoquine metabolic phenotype was determined by debrisoquine administration and analysis of debrisoquine and 4-hydroxydebrisoquine in the subsequent 8 h urine collected.

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Genomic DNA was genotyped by a specific polymerase chain reaction amplification and subsequent restriction enzyme digestion, and Southern analysis. Twenty subjects were heterozygous for the *CYP2D6(C)* allele but none were homozygous for this allele. There was no significant difference in frequency of *CYP2D6(C)* between lung cancer patients and controls (5.61% and 4.09%, respectively), and there was no significant heterogeneity among cases by histologic type of lung cancer ( $P = 0.08$ ). However, 7 of 11 cases (64%) with the *CYP2D6(C)* allele had small cell lung cancer, and none had squamous cell carcinoma. Carrying the *CYP2D6(C)* allele did not impair debrisoquine metabolism to the same degree as the known inactivating mutations, *CYP2D6(A)* and *CYP2D6(B)*, or deletion of *CYP2D6*. Thus, the *CYP2D6(C)* allele does not encode a completely inactivating mutation, and the suggestion of a role for this variant allele in the risk for specific histologic types of lung cancer justifies further investigation. © 1997 Elsevier Science Ireland Ltd.

**Keywords:** Debrisoquine; Lung cancer; Genetic susceptibility

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## 1. Introduction

The differential susceptibility of tobacco smokers to lung cancer has led to a search for other contributing etiologic factors. Genetic predisposition characterized by polymorphic oxidation of debrisoquine has been an area of active investigation [13]. Previous studies using debrisoquine genotype have yielded conflicting results regarding the association with lung cancer risk [1,10,16]. Our study enrolled frequency matched hospital-based controls and had an 80% power to detect an odds ratio of 2 for excess lung cancer risk among extensive metabolizers [14]. Approximately 8–9% of Caucasians lack the ability to metabolize debrisoquine and are considered poor metabolizers [13]. Specific mutations in both alleles of the *CYP2D6* locus on chromosome 22 result in an absent or dysfunctional P450 isozyme [5]. Several mutations in the *CYP2D6* gene have been identified [11]. These mutations are polymorphic and not all are functional mutations which result in deficient metabolism. One of the variant alleles, *CYP2D6(C)*, is characterized by a 3-base pair deletion in exon 5 of the *CYP2D6* gene, resulting in deletion of Lys<sub>281</sub> [15], but does not appear to result in deficient metabolism of debrisoquine [2]. Recently Agúndez et al. reported increased frequency of the *CYP2D6(C)* allele among Spanish lung cancer patients compared to normal volunteers [1]. We examined the frequency of the *CYP2D6(C)* allele and its influence on the debrisoquine metabolic phenotype in individuals participating in an incident case-control study of lung cancer.

## 2. Subjects and methods

A case-control study of incident lung cancer was conducted at the National Naval Medical Center, Bethesda, Maryland, USA from August 1988 through

February 1992 as previously reported [14]. The protocol was approved by all participating institutional review boards, and all study subjects gave signed informed consent. Patients undergoing evaluation for possible lung cancer were identified and recruited from all departments that treat lung cancer patients and metabolic phenotyping was conducted before any therapy for lung cancer. Control subjects were recruited from outpatients with scheduled appointments in the Urology and Orthopedic Surgery Clinics at the National Naval Medical Center in Bethesda, and matched to cases by 5-year age group, sex and race.

Debrisoquine (Declinax<sup>®</sup>) was obtained from Hoffman-LaRoche Limited under an Investigational New Drug approval. Metabolic phenotyping was conducted overnight as previously described [14]. Urine samples were analyzed by fluorescence detection high-performance liquid chromatography for debrisoquine and 4-hydroxydebrisoquine in order to calculate the natural log of the debrisoquine metabolic ratio (LMR) [3,4]. Genotypes were determined by PCR-based tests for mutations described as the *CYP2D6(A)* allele [9], *CYP2D6(B)* allele [6], and by Southern blot analysis for the *CYP2D6(D)* deletion allele [4]. Mutations considered to be inactivating were the deletion of *CYP2D6* or the presence of the *CYP2D6(A)* or the *CYP2D6(B)* allele [6].

A primer-directed mismatch PCR-based test for the *CYP2D6(C)* allele was developed to facilitate screening large numbers of samples [8,12]. *CYP2D6*-specific forward primer 58 (GCGGAGCGAGAGACCGAGGA), located in intron 4, and *CYP2D6*-specific reverse primer 59 (CCGGCCCTGACACTCCTTCT), located in intron 6, were used in a 40- $\mu$ l PCR with 250 ng of DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.2 mM each dNTP, 2 mM MgCl<sub>2</sub>, 0.2  $\mu$ M each primer, and 1 unit of Taq polymerase (Perkin-Elmer/Cetus) as diagrammed in Fig. 1. The PCR parameters included a hot start at 94° for 2 min, followed by denaturation at 94° for 30 s, primer annealing at 60° for 45 s, extension at 72° for 45 s, with an extension time of 3 min for the last cycle. The reaction was stopped after 30 cycles in a Perkin-Elmer/Cetus 9600 thermal cycler. The PCR product containing both exons 5 and 6 was diluted 500-fold, and 4  $\mu$ l was used for each of the subsequent nested PCRs. Both nested PCRs were performed under the following conditions: 40  $\mu$ l PCR with 2 mM MgCl<sub>2</sub>, 10 mM Tris-HCl pH 8.3, 0.2 mM each dNTP, 0.2  $\mu$ M each primer, and 1 unit of Taq polymerase (Perkin-Elmer/Cetus). The PCR cycle parameters were similar to those of the primary amplification except that the annealing temperature was 55°. The 'wt-cuts' test was performed by PCR amplification using forward primer 60 (CCTTCCTGGCAGAGATGAAG) and reverse primer 35 (CCTCATTCCTCCTGGGACGC). Digestion of the PCR product with *Mbo*II followed by gel electrophoresis in an 8% polyacrylamide gel resolves two fragments of 55 bp and 29 bp from the normal allele and one 81 bp fragment from the *CYP2D6(C)* allele (Fig. 1). The 'mut-cuts' test was performed by PCR amplification with forward primer 61 (GACTGAGGCCTTCCTGGCAG) and reverse primer 35. Primer 61 introduces a mismatch which creates a *Mn*II recognition site in the PCR product from the *CYP2D6(C)* allele but not in the PCR product from the normal allele. Digestion of the PCR product with *Mn*II generates three fragments of 55 bp, 17 bp, and 18 bp from the *CYP2D6(C)* allele and two

fragments of 76 bp and 17 bp from the normal allele (Fig. 1; 17 and 18 bp fragments not shown).

Statistical analyses were conducted using the chi-square test comparing the difference between controls and each histologic type. Statistical significance was defined as a two-sided  $P < 0.05$ . Kruskal-Wallis one way analysis of variance on ranks and the Mann-Whitney rank sum test were used to compare the median LMR between genotypes.

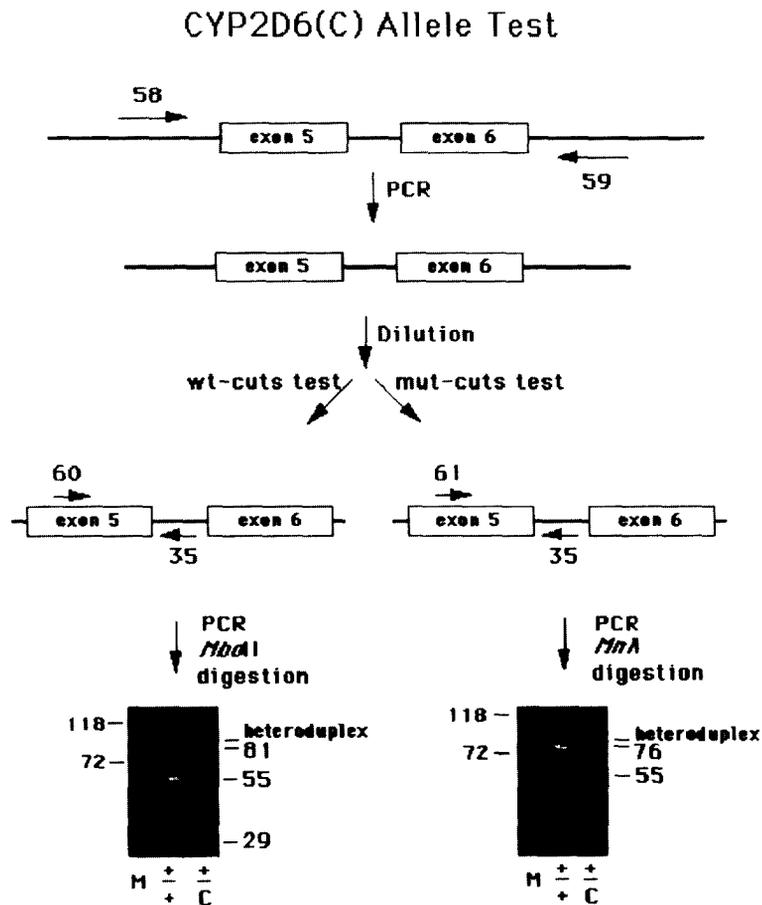


Fig. 1. Primer-directed mismatch polymerase chain reaction (PCR)-based test for the *CYP2D6(C)* allele. *CYP2D6(C)*-specific PCR is performed using primers that flank exons 5 and 6. After dilution, two independent PCRs are performed with nested primers. The products of each nested PCR are digested with restriction endonucleases and electrophoresed on 8% acrylamide gels. An additional band is only seen in PCRs of *CYP2D6(C)* heterozygotes. This band is likely due to heteroduplex molecules. +, Indicates *CYP2D6* wild-type allele; c, indicates *CYP2D6(C)* allele. M, Indicates the molecular weight marker  $\phi$ X174 *Hae*III digest.

Table 1

Frequency of the *CYP2D6(C)* allele in lung cancer cases by histologic type compared to matched controls

	No. C alleles	Total alleles	C allele frequency (%)
Controls	9	220	4.09
Cases (all)	11	196	5.61
SCLC	7	60	11.67
All NSCLC	4	136	2.94
Adenocarcinoma	3	72	4.17
Squamous cell	0	28	0
Other NSCLC	1	36	2.78

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer.

### 3. Results

Ninety-eight of 109 (90%) Caucasian patients with histologically confirmed lung cancer and 110/135 (81%) age, race, and sex matched control subjects had both debrisoquine phenotype and genotype determined. All subjects had phenotype determined; subjects without genotyping results refused phlebotomy. Eleven of 98 lung cancer cases and 9 of 110 controls were heterozygous for the *CYP2D6(C)* allele. Of these, 15 had no additional mutations identified, but five also had other known mutations identified. The *CYP2D6(C)* allele frequencies of 5.61% and 4.09% for cases and controls respectively were not significantly different ( $\chi^2 = 0.24$ ,  $P = 0.62$ ).

Table 1 shows the frequency of *CYP2D6(C)* by histologic type. Although the frequency of the *CYP2D6(C)* allele varied by histologic type of lung cancer, the proportions of observations in controls and the different histologic types were not significantly different ( $\chi^2 = 8.22$ ,  $P = 0.08$ ). When allele frequency of each histologic type was compared to the controls, the allele frequency of 11.67% among patients with small cell lung cancer (SCLC) was higher, but was not statistically significant. Of note, none of the 15 patients with squamous cell carcinoma carried the *CYP2D6(C)* allele. Among patients with NSCLC, there was a significant difference in the stage distribution with all four NSCLC patients with the *CYP2D6(C)* allele having Pathologic Stage I (3) or II (1) disease, in contrast to 38% of all NSCLC patients having Stage I or II disease ( $P = 0.02$ ). There was no association between smoking and frequency of the *CYP2D6(C)* allele overall or by histologic type.

Among the 20 subjects heterozygous for the *CYP2D6(C)* allele, the *CYP2D6* wild-type/*CYP2D6(C)* (wt/c) heterozygotes had significantly different LMR than the *CYP2D6*-wild-type (wt/wt) homozygotes ( $P < 0.0001$ ). The three subjects heterozygous for both the *CYP2D6(C)* allele and the *CYP2D6(B)* allele (c/mut) had LMR ranging from 0.887 to 2.27. However, there was no significant difference between wt/c heterozygotes and either wt/mut heterozygotes or c/mut heterozygotes, or between c/mut heterozygotes and subjects homozygous for inactivating mutations. However, the power to detect a difference in these data is limited by

the number of observations in each group. The mean ( $\pm$  standard error) of the LMR is plotted by genotype suggesting a trend for increasing LMR from wt/wt homozygotes to mut/mut homozygotes, as shown in Fig. 2. These findings are consistent with the *CYP2D6(C)* allele encoding an enzyme with activity intermediate between the active enzyme encoded by the wild-type allele and an inactive enzyme resulting from one of the inactivating mutations. This intermediate enzyme activity appears sufficient to metabolize debrisoquine in vivo, although less efficiently than the wild-type allele.

#### 4. Discussion

We examined 208 Caucasian subjects from a case-control study of lung cancer to determine whether there was an association of the *CYP2D6(C)* allele with lung

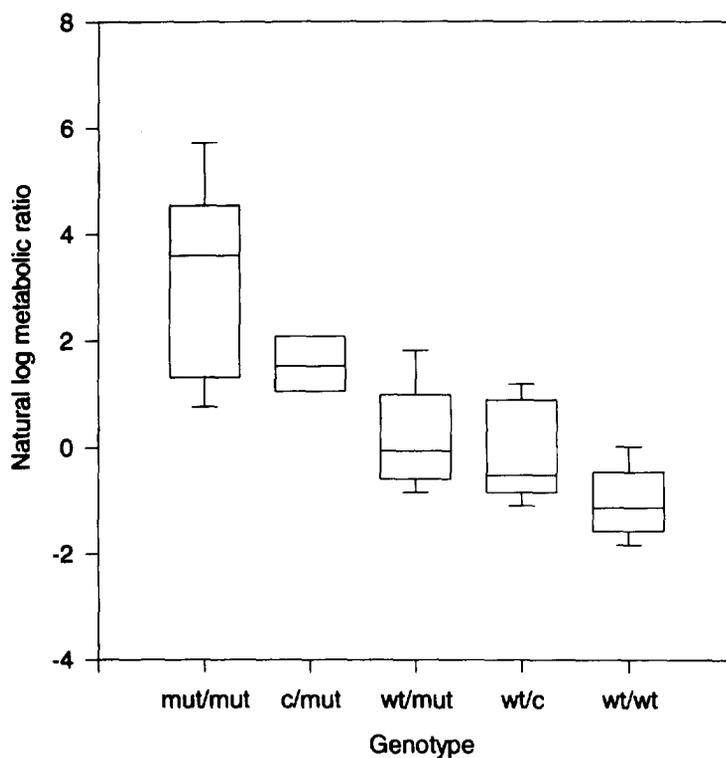


Fig. 2. Plot of the distribution of natural log debrisoquine metabolic ratio by genotype with the median indicated by the horizontal line within the box, the 25th and 75th percentiles indicated by the boundaries of the box, and the 10th and 90th percentiles indicated by the error bars. Abbreviations: wt/wt, homozygous *CYP2D6* wild-type; wt/c, heterozygous *CYP2D6* wild type/*CYP2D6(C)*; wt/mut, heterozygous *CYP2D6* wild-type/*CYP2D6(A)*, (B), or (D); c/mut, heterozygous *CYP2D6(C)*/*CYP2D6(A)*, (B), or (D); mut/mut, homozygous *CYP2D6(A)*, (B), or (D).

cancer as reported by Agúndez et al. [1]. Contrary to their findings, we find a similar frequency of the *CYP2D6(C)* variant allele among lung cancer cases and age, race and sex matched controls. However, when examined by histologic type of lung cancer, patients with SCLC had a higher frequency of the *CYP2D6(C)* allele compared to the controls, although this was not statistically significant. While the determinants for differentiation into the different histologic types of lung cancer remain unknown, it is likely that the association observed with the *CYP2D6(C)* allele and SCLC is due to chance. The proportion of our cases with SCLC was similar to that reported by Agúndez et al. for microcytic lung cancer, and they observed no overrepresentation of the *CYP2D6(C)* allele among these patients compared to other histologic types. Differences in ethnicity may account for variations in the frequency of variant alleles, and 8% of our North American Caucasian controls carried a *CYP2D6(C)* allele compared to 3% of Spaniards [1] and 4% of Europeans [7]. These differences in study populations may explain some of the discrepancy in results. Further investigation of the role of the *CYP2D6(C)* allele is needed to clarify its role in risk for lung cancer, particularly in different histologic types.

Subjects heterozygous for the *CYP2D6(C)* allele in combination with either the wild-type allele or a variant allele known to result in impaired metabolism had LMRs consistent with extensive and intermediate metabolism reported among heterozygotes [2]. These findings are consistent with the previous report that the *CYP2D6(C)* allele seems to encode a functional *CYP2D6* enzyme [2]. Our results do suggest that this enzyme is less efficient than that encoded by the wild-type allele. The impact of this allele on metabolizing capacity will need further characterization to determine whether it is clinically significant.

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