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Molecular Genetic Analysis of the Cytochrome P450-Debrisoquine Hydroxylase Locus and Association with Cancer Susceptibility

by C. A. Dale Smith,[1] Julie E. Moss,[1] Alan C. Gough,[2] Nigel K. Spurr,[2] and C. Roland Wolf[1,3]

The cytochrome P450-dependent monooxygenases play a central role in the metabolism of chemical carcinogens. The action of these enzymes can lead to either carcinogen detoxication or activation. Differences in P450 expression in animal models give rise to large differences in susceptibility to chemical carcinogens, so genetic polymorphisms in P450 expression may be expected to be an important factor in individual human susceptibility to cancer. Of particular interest is the genetic polymorphism at the cytochrome P450-debrisoquine/sparteine hydroxylase locus (*CYP2D6*). Although this is a minor liver P450, its polymorphic expression is associated with the abnormal metabolism of at least 30 therapeutic drugs, including β-blockers and tricyclic antidepressants. Conflicting reports have been made on the association of this polymorphism with cancer susceptibility. This disagreement may be attributable to limitations of the phenotyping assay used to identify affected individuals (poor metabolizers, PMs). In order to clarify these anomalies, we have developed a simple DNA-based assay with which we can identify the majority of PMs. The assay is centered around the primary gene defect responsible for the polymorphism, a G to A transition at the junction of intron 3/exon 4 which results in a frame-shift in the resultant mRNA. The frequency of this mutation is 70–80% in PMs. We have studied the frequency of mutated alleles in a control population and in a wide range of cancer patients. No association between this polymorphism and lung cancer susceptibility was observed; however, in other populations of cancer patients some very interesting shifts were found in the proportion of PMs and heterozygotes from that in the normal population.

Introduction

Understanding the genetic factors that may affect the susceptibility of an individual to disease or the side effects of drugs is of enormous value for predicting risk groups and for the treatment of specific disorders. It has been suggested that many cancers are initiated by either environmental carcinogens or carcinogens generated by our industrial environment (*1*). The cytochrome P450 monooxygenases play a central role in the metabolism of such foreign compounds, and on this basis and their polymorphic expression, they may be an important factor in assessing susceptibility to disease (*2*).

The P450-debrisoquine hydroxylase polymorphism in humans is one of the best-studied enzyme deficiencies, and it has been variously linked with different types of cancer, including those of the lung (*3–5*), bladder (*6*) and breast (*7*). This polymorphism is characterized by the inability of certain individuals to metabolize specific drugs (e.g., debrisoquine, sparteine, and bufuralol); and affected individuals, termed poor metabolizers (PMs), are known to be deficient in the minor liver enzyme P450-CYP2D6 (*8*), which is responsible for the metabolism of these compounds. The PM phenotype is inherited in an autosomal recessive manner in 5–10% of the Caucasian population and is now associated with the inefficient metabolism of over 30 drugs with a wide range of clinical indications (*9,10*).

Most studies to date on the association of the *CYP2D6* polymorphism with cancer have relied on phenotyping assays which require administration of a test drug to subjects followed by urine collection for several hours to determine the concentration of unchanged drug, 4-hydroxy metabolite, and hence metabolic ratio (*11*). There are certain limitations to this assay, such as drug–drug interactions, hormonal effects, effects of the disease state, and the inability to identify heterozygotes. The develop-

[1]ICRF Molecular Pharmacology Group, University of Edinburgh, Edinburgh, United Kingdom.
[2]ICRF Human Genetic Resources Laboratory, Clare Hall, Potter's Bar, London, United Kingdom.
Address reprint requests to C. A. D. Smith, ICRF Molecular Pharmacology Group, University of Edinburgh, Edinburgh, United Kingdom.

ment of a reliable DNA-based genetic assay to predict the PM phenotype is thus essential for more comprehensive study of its association with disease susceptibility and the prediction of individuals at risk from drug side effects.

The *CYP2D* locus in humans has been identified as an array of at least three highly homologous gene sequences on the long arm of chromosome 22. In addition to the active *CYP2D6* gene, an inactive pseudogene, *CYP2D8P*, and an inactive homolog, *CYP2D7* are present in a tandem array (*12*). There is mounting evidence for the existence of a fourth gene, *CYP2D9*, but it has not been fully characterized to date (*13*). In initial experiments on genomic DNA, restriction fragment length polymorphisms were identified which could be associated with the PM phenotype; however these were found to be informative for less than 25% of PM individuals (*14*). Isolation of DNA clones associated with the normal and mutant alleles of the *CYP2D6* gene and subsequent sequence comparisons allowed identification of three mutations which explain the lack of enzyme in 90% of PM individuals (*15–17*). We have developed a polymerase chain reaction (PCR)-based genetic assay around one of these mutations, which allows the identification of approximately 70–80% of PMs (*16*). In the work presented here, we screened over 2500 subjects using this assay to determine whether any association exists between the debrisoquine polymorphism and cancer susceptibility.

Subjects and Methods

Sample Collection

Blood samples were collected from Caucasian individuals in EDTA or heparin vials from various locations within the United Kingdom. The samples were either frozen immediately or received at ambient temperature and then stored at −20°C until required. Tissue samples were frozen as soon as possible after excision and stored at −70°C prior to DNA isolation.

Target DNA Isolation

In some cases, genomic DNA was isolated from tissue samples, whole blood, or lymphocyte preparations using an Applied Biosystems 340A nucleic acid extractor according to the manufacturer's instructions. Most blood samples were processed rapidly to provide crude cell extracts suitable for PCR analysis as follows: Whole blood (0.1 mL) was thoroughly mixed (vortexed) with 0.75 mL of 10mM Tris/HCl (pH 8), 1mM EDTA (TE) in a microfuge tube prior to centrifugation at 12,000*g* for 20 sec. The supernatant was aspirated, and the pellet was further extracted twice with 0.5 mL TE, as described above, to complete the lysis of red blood cells and removal of plasma and other contaminants that may affect the PCR reaction. The final crude cell pellet (white blood cells) was lysed by the addition of 0.1 mL of buffer A: 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM Tris/HCl (pH 8), 0.45% Nonidet P-40, 0.45% Tween-20; containing 200 μg/mL proteinase K. After incubation at 55°C for 20 min, 0.1 mL of sterile water was added, and the lysate was heated to 90–100°C for 10 min to inactivate the proteinase. This rapid procedure provides sufficient lysate for 20-40 PCR analyses (*13*).

Polymerase Chain Reaction Analysis

Target DNA (1 μg genomic DNA or 5–10 μL crude blood lysate) was amplified by polymerase chain reaction [PCR (*18*)] using 300 ng of each *CYP2D6*-specific amplification primer (see legend to Fig. 1). The PCR was carried out using 2 U of either Amplitaq DNA polymerase (Cetus Corp.) or Hot-Tub DNA polymerase (Amersham PLC) according to the manufacturers' instructions, except that dimethyl sulfoxide was added to 10% (v/v) final concentration when using Amplitaq. In a Techne model PHC-2 thermocycler, an initial strand separation step was carried out at 94°C (5 min) prior to 30–35 cycles of strand separation, 94°C (30 sec), primer annealing, 60°C (60 sec), and polymerization, 72°C (60 sec). A final polymerization step at 72°C was carried out to complete elongation of all amplified strands. The high annealing temperature was required in order that the primers used annealed only to *CYP2D6* sequences and not to the homologous *CYP2D7* of *CYP2D8P* genes (*16*).

To distinguish between *CYP2D6* alleles, 30–40 μL of the amplified mixture were digested using 10 U *Bst* N1 (New England Biolabs) according to the manufacturer's instructions, and fragments were separated by electrophoresis through an 8% polyacrylamide/TBE (90 mM Tris-borate [pH 8.3], 2 mM EDTA) gel (*16*). Bands were visualized by ultraviolet irradiation of gels stained with ethidium bromide.

Statistical Analysis of Results

Differences in the distribution of assigned genotypes between the random normal and cancer populations were evaluated for significance using the two-tailed chi-square test (*19*). Contingency tables were constructed to compare observed genotypes and phenotype with expected values of homozygote and heterozygotes of the mutant and normal *CYP2D6* alleles.

Results and Discussion

Three common mutations have been identified that can explain the absence of a functional P450-CYP2D6 (debrisoquine hydroxylase) enzyme in the livers of PMs: *a*) major deletions of *CYP2D6* coding sequences, accounting for approximately 10% of mutant alleles; *b*) an A deletion at nucleotide 2637 in exon 5 (*14*), which results in a frame-shift and premature termination in mRNA, seen in approximately 2–5% of mutant alleles; and *c*) a G to A transition at the intron 3/exon 4 boundary of the *CYP2D6* gene, which leads to incorrect splicing of the resultant mRNA, resulting in a frame-shift and premature termination. The G to A transition has been identified as the primary gene defect at the *CYP2D6* locus and is estimated to account for 80-90% of mutant alleles in PMs (*16*). This site is the foundation of the DNA-based genetic assay used

in this study. The intron 3/exon 4 junction has the sequence CCCCC*AG*/**GACGCC** (bold letters indicating exon 4) in the normal allele but CCCCCA*AG*/**ACGCC** in mutant alleles. The mutation shifts the 3′-splice acceptor site (italics) one base pair downstream, resulting in the loss of the first nucleotide (G in the normal allele) of exon 4 and a premature termination at position 544 of the mRNA (*14,17*). The mutation also leads to the loss of a *Bst* N1 restriction site (CCAGG to CC.' 4G).

Figure 1 shows a diagrammat : representation of the assay for the PM genotype. PC.. amplification of DNA and subsequent digestion of the products, allows assignment of an individual's *CYP2D6* genotype with respect to the intron 3/exon mutation within the gene. The diagnostic pattern of bands produced after gel electrophoresis categorizes an individual as follows: EM, carrying two wild-type (normal) *CYP2D6* alleles at the site of the G to A mutation (almost exclusively extensive metabolizers), or one normal allele and a deletion; HEM, heterozygous at this site, i.e. carrying one wild-type and one mutant allele; PM, poor metabolizers, carrying two mutant alleles or one mutant G to A allele and a gene deletion. We estimate that

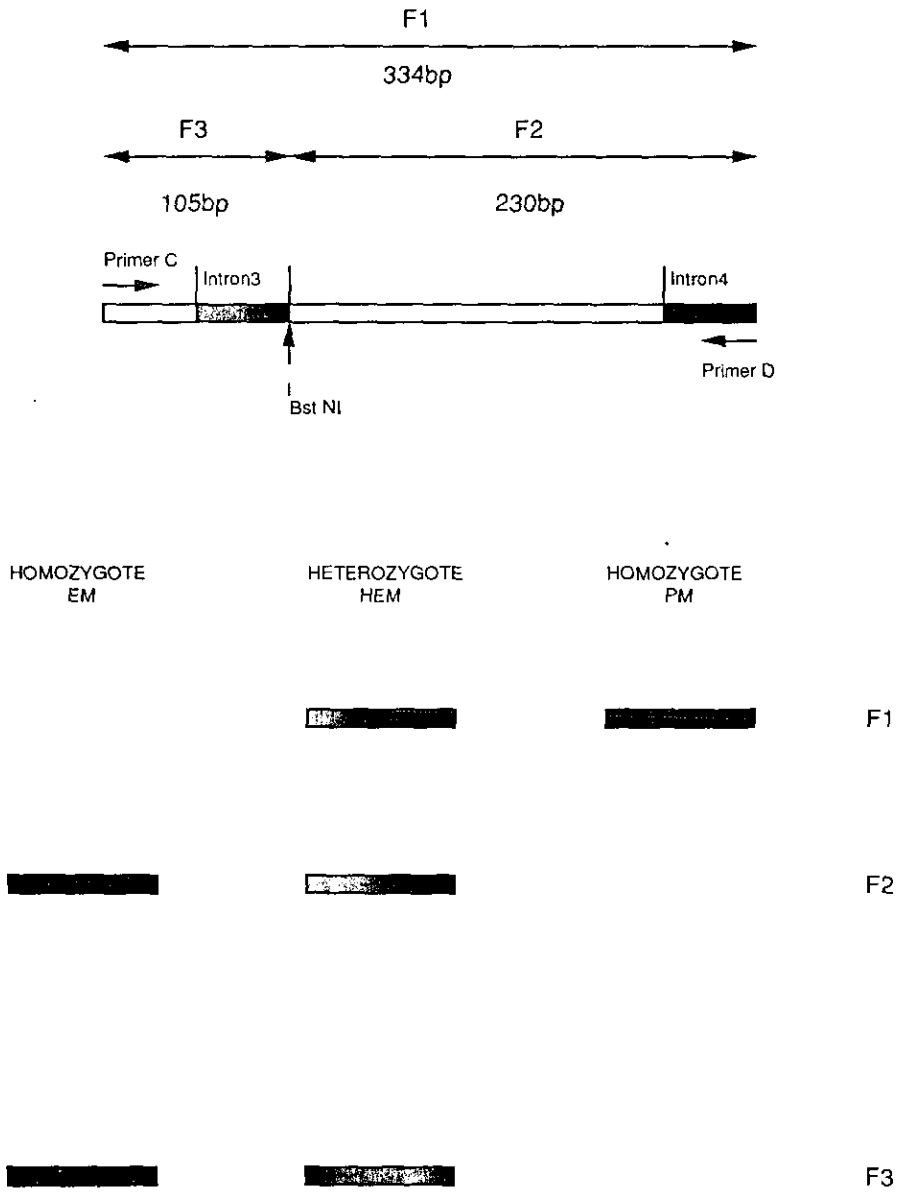

FIGURE 1. Polymerase chain reaction (PCR) assay for the G to A transition mutation at the intron 3/exon 4 junction of the *CYP2D6* gene. Diagrammatic representation of the method used and predicted banding patterns of individuals carrying the normal *CYP2D6* allele (EM), mutant *CYP2D6* allele (PM), or both (HEM). PCR amplification of DNA using primer pair C (exon 3: 5′-GCCTTCGCCAACCACTCCG-3′) and D (intron 4: 5′-AAATCCTGCTCTTCCGAGGC-3′) results in a fragment of 334 bp (F1) spanning the diagnostic polymorphic *Bst* N1 restriction enzyme site (CCNGG) at the intron 3/exon 4 boundary. This fragment is digested by *Bst*N1 into two bands of 230 bp (F2) and 105 bp (F3) in samples obtained from individuals carrying the normal *CYP2D6* allele, while the F1 fragment produced from the mutant allele is resistant to digestion. Primer D contains sequence mismatches with the *CYP2D8P* and *CYP2D7* genes.

Table 1. Distribution of *CYP2D6* alleles in cancer patients.

Subjects/patients	No. of samples	Genotype % EM	% HEM	% PM	%MAF[a]	χ^{2}[b]	$p<$
Random controls	720	66.1	29.6	4.3	19.1	–	–
Emphysema	151	61.6	35.1	3.3	20.9	2.35	0.50
All cancers	1759	63.4	31.6	5.0	20.8	6.38*	0.05
Lung cancer[c]	361	64.8	31.6	3.6	19.4	0.96	0.90
Breast cancer	437	66.8	29.3	3.9	18.5	0.22	0.90
Colon cancer	115	63.8	29.6	6.9	21.7	2.88	0.50
Leukemia[d]	312	65.7	26.9	7.4	20.8	7.65*	0.02
Teratoma[e]	169	65.7	30.8	3.5	18.9	0.32	0.90
Melanoma	127	54.3	39.4	6.3	26.0	7.91*	0.02
Bladder cancer	184	53.8	41.8	4.4	25.3	13.50*	0.005
Prostate cancer	54	59.2	31.5	9.3	25.0	3.62	0.50

Abbreviations: EM, extensive metabolizer; HEM, heterozygous at site of G to A mutation; PM, poor metabolizer.
[a]Mutant allele frequency: proportion of *CYP2D6* alleles that carry the intron 3/exon 4 mutation.
[b]Calculated with two degrees of freedom.
[c]Includes 145 squamous cell carcinomas, 74 adenocarcinomas, 52 small-cell lung cancers, and 90 other cancers (large-cell, mesothelioma, apical, bronchogenic carcinomas) and unknowns.
[d]Includes 141 cases of acute myeloid, 117 cases of chronic myeloid, 37 cases of myelodysplastic syndrome, and 17 cases of other leukemic disorders.
[e]Includes data obtained from teratocarcinoma and seminoma patients.
*Statistically significant.

we can identify 80% of all PM individuals in this manner in any given population (*16*). A small proportion of individuals homozygous or heterozygous for the G to A mutation will be PMs because they carry the base-pair deletion in exon 5; however, this will not affect the comparison of the frequency of mutant alleles in normal and cancer patient populations, described below.

Table 1 and Figure 2 summarize the results of the screening of over 2500 individuals for the major mutation in CYP2D6. The distribution of apparent EMs, HEMs, and PMs in a random population of Caucasians (n = 720) was 66.1, 29.6, and 4.3%, respectively. Within this group, results of genotyping in control populations in Sheffield (206) and Edinburgh (366) were combined with regionally random samples obtained mainly from the ICRF, Clare Hall Laboratories. The apparent proportions of PMs in these separate control groups were 4.9, 3.8, and 4.7%, respectively. The frequency of the G to A mutant allele in the population, calculated from the proportion of heterozygotes and assuming two mutant alleles in PMs, was 19.1% of all *CYP2D6* alleles. On this basis, it would be predicted that 4% of the population are homozygous for this allele. The proportion of genotyped PMs in this control population is slightly less than the expected number (5–10%), often observed using the phenotyping assay. If this assay can predict 80% of PMs, however, then the observed value represents a PM proportion of 5.5–6% in the Caucasian population, in good agreement with previous reports (*9,10*).

If we consider the distribution of *CYP2D6* genotypes in all the cancer patients studied (1759 individuals), statistically significant shifts were observed in the proportions of EMs, HEMs and PMs compared with the control population (Table 1). This deviation can be attributed, however,

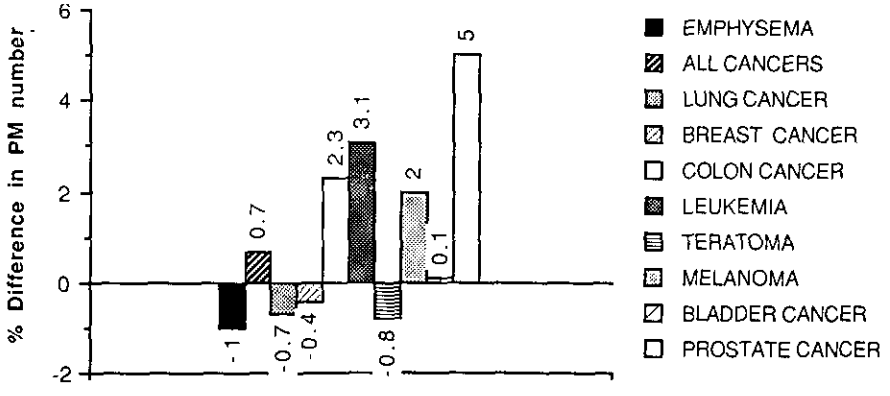

FIGURE 2. Differences in the distribution of poor metabolizers (PM) among cancer patients. Diagram shows the difference from control of the proportion of PMs determined in each cancer cohort. The percentage PM distributions shown in Table 1 were compared by subtracting the percentage PM in the control population. The differences are shown as +/− differences of the population over that seen in the control (0).

to an increase in mutant allele frequency as the difference between PMs and EMs is not significant (all mutant alleles, $\chi^2 = 6.38$, $p < 0.05$, homozygous G to A, $\chi^2 = 2.13$, $p < 0.5$), and the overall proportion of PMs is only slightly higher than that observed in the control population (Fig. 2). This association, although statistically significant, does not appear to have any biological relevance. Interestingly, the frequency of HEMs among the melanoma and bladder cancer patients was also significantly higher than in the control population (Table 1); and a relatively high (50% increased) PM frequency (6.3%) was observed among melanoma patients but not among bladder cancer patients, in whom the proportion of PMs was almost identical to that in the control group (Fig. 2). In melanoma and in bladder cancers, the mutant allele frequency of approximately 25% was significantly greater than that in controls. Why heterozygotes (in addition to PMs) have an altered susceptibility to these diseases requires further study.

In the series of lung cancer patients, there was a very slight reduction in the proportion of PMs (Fig. 2) but the observed change in *CYP2D6* genotype distribution was not statistically significant (Table 1). Interestingly, a virtually identical reduction in the proportion of PMs was observed in the emphysema cohort (Table 1 and Fig. 2). Emphysema patients have been used as a direct control group in previous studies associating debrisoquine hydroxylation phenotype with lung cancer (*3,20*). In addition, no difference from control was evident in mutant allele frequency in lung cancer patients, but slight increases in the mutant allele frequency and the proportion of heterozygotes was observed in emphysema patients. The lung cancer data are in contrast with those from studies in which greatly reduced proportions of PMs were observed in lung cancer cohorts and a link was suggested with the EM phenotype (*3,4,20*). In previous studies, patients were phenotyped directly for debrisoquine hydroxylase activity using marker drugs; therefore, fundamental differences in the two types of assay may account for the apparent anomaly. It must be emphasized that phenotyping assays may be directly affected by the disease state of the individual and complications may arise from the pharmaceutical and other procedures used in the treatment of cancer patients. Such considerations are irrelevant when using DNA-based genetic assays, even if, as is the case here, only 80% of PMs can be predicted. When the data on lung cancer are subdivided into the tumor types, squamous-cell carcinomas and other smoking-related cancers (EM, 64.5%; HEM, 32%; PM, 3.5%; $n = 287$) and adenocarcinoma (EM, 66.2%; HEM, 29.7%; PM, 4.1%; $n = 94$), no significant reduction in the proportion of PMs is observed (squamous-cell; $\chi^2 = 0.53$, $p < 0.9$), and the distribution of genotypes in cases of adenocarcinoma of the lung is virtually identical to that in the control population. In view of the central role of P450s in the activation of carcinogens, an association between the expression of these enzymes, including CYP2D6, and lung cancer cannot be completely ruled out. They may be implicated at some stage in tumor progression, although it appears unlikely that this is determined predominantly at the genetic level.

Although slight reductions in the proportion of PMs were observed in the teratoma/seminoma and breast cancer cohorts (Fig. 2) there was no significant variation from normal of the distribution of genotypes and mutant allele frequency in these two cancer groups (Table 1). In the cohorts of colon cancer and prostate cancer patients, there is a relatively large increase in the proportion of PMs, to 6.9% and 9.3%, respectively, although these did not reach statistical significance. These increases may represent some level of risk associated with the PM genotype, as the apparent mutant allele frequency is substantially increased in cases of both colon and prostate cancer. Many more patients should be studied, however, especially those with prostate cancer, to provide a clearer picture of the extent of correlation between these two cancer types and the PM genotype.

The difference in distribution of genotypes in leukemic patients relative to controls was highly significant, with an increase in the proportion of PMs (Fig. 2) and a slight decrease in the proportion of heterozygotes (Table 1). This cohort included 54 individuals with preleukemic myelodysplastic syndrome and other lymphatic disorders (see footnotes to Table 1). When these are discounted and only "true" leukemic disorders (acute myeloid, chronic myeloid, acute lymphocytic, chronic lymphocytic) are analysed, the level of statistical significance is increased [$n = 266$: EM, 66.9%; HEM, 25.2%; PM, 7.9%: $\chi^2 = 9.62$, $p < 0.01$ for genotypes: $\chi = 8.45$, $p < 0.005$, for G to A homozygotes). This suggests a strong association between the PM genotype and leukemia. It is known that many chemicals and some anti-cancer agents can induce leukemia, but it is not clear whether they are metabolized by CYP2D6. Alternatively, it is possible that the data obtained in this cancer study could be better explained by linkage between *CYP2D6* and a gene involved in the pathogenesis of this disease. It is already known that the c-*sis* (platelet-derived growth factor B) proto-oncogene and thyroid-stimulating hormone receptor gene are located on chromosome 22 (*21*), and we have recently mapped the *CYP2D6* locus to 22q13.1, very closely linked with these two candidate oncogenes (unpublished observation). As the DNA from the leukemia cases was obtained from blast cells, the increase in the proportion of PMs and the slight decrease in that of heterozygotes could be due to allelic loss in this region of chromosome 22. Translocations involving chromosome 22 are known to be involved in the pathogenesis of leukemia, although they predominantly involve translocations around the c-*abl* locus. The possibility of allele loss is therefore worthy of further study.

In summary, the data presented in this report suggest that there is no reduction in the proportion of PMs in many of the cancer types that have been previously associated with the *CYP2D6* polymorphism. It is thus unlikely, at least at the genetic level, that the debrisoquine polymorphism is a susceptibilty factor in these diseases; however, several of the cancer types studied showed increases in the proportion of PMs and significant increases in the fre-

quency of mutant alleles as a percentage of all *CYP2D6* alleles. In addition, significant changes in the frequency of heterozygotes were observed in certain cancer groups.

Several important questions regarding the *CYP2D6* polymorphism remain to be resolved. These include: *a*) the relationship between different mutations within the gene. Are they independent? *b*) isolation and characterization of further genes in this gene cluster, e.g., *CYP2D9*. Do they represent active genes? *c*) are some of the "pseudogenes" found in Caucasians active in other racial groups? *d*) is the *CYP2D6* gene in linkage disequilibrium with an oncogene? *e*) can substrates for CYP2D6 be found which are known carcinogens? *f*) more work is required on the association of *CYP2D6* with other environmentally linked disorders, particularly neurological diseases, *g*) does CYP2D6 have an important physiological function? *h*) how is *CYP2D6* regulated? Consideration of these issues and continued studies with DNA-based analysis of the locus should give a clearer insight into the role of P450s in cancer susceptibility.

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