The Debrisoquine Metabolic Phenotype and DNA-based Assays: Implications for Misclassification for the Association of Lung Cancer and the Debrisoquine Metabolic Phenotype

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Debrisoquine is an antihypertensive drug that is metabolized by cytochrome P4502D6. Deficient metabolism is inherited as an autosomal recessive condition. We previously reported in a case-control study that extensive metabolizers of debrisoquine were at greater risk of lung cancer compared to poor and intermediate metabolizers. Cloning of the gene that encodes P4502D6 (CYP2D6) led to the identification of both wild-type and mutant forms of the gene. Subsequently, a DNA-restriction fragment length polymorphism (RFLP) was identified, and a Southern hybridization-based test was developed in an attempt to define the genotype. When the DNA-RFLP test was applied to stored DNA from our study subjects there was neither a significant association with the metabolic phenotype nor an association with lung cancer. Further work has demonstrated that the wild-type gene, which was characterized by a 29-kb allele, can also contain mutations that result in nonfunctional or absent proteins. When these mutations are present, individuals exhibit the poor or intermediate metabolizer phenotype in spite of the presence of the 29-kb putative wild-type allele. Sequence determination of the mutants led to the development of techniques to exploit the polymerase chain reaction, which, together with Southern analysis, have been reported to detect as many as 95% of poor metabolizers. This technique is being used to examine the association of the extensive metabolizer genotype with lung cancer in the subjects from the case-control study. Preliminary results indicate a weak association between the homozygous wild-type genotype and lung cancer; in contrast, the extensive metabolizer phenotype is strongly associated with lung cancer in this subset. Employing this polymerase chain reaction method only, misclassification in the genotype assignment continues to occur, and work is in progress to identify further mutations that may account for subjects who are phenotypically poor metabolizers but possess “wild-type” alleles. The phenotyping approach is currently more sensitive, while the genotyping method may be more specific with regard to detection of the deficient metabolizer state in the context of population studies. Increasing use of genotyping is anticipated in future studies.

Introduction

While exposure to tobacco smoke is widely accepted as the major etiologic factor in lung cancer, there are clear differences in individual susceptibility consistent with a heritable component to risk. The metabolism of the antihypertensive drug debrisoquine (DBR) is under autosomal genetic control (1-3), and inheritance of the trait conferring ability to “extensively” metabolize the drug has been suggested as a host susceptibility factor for lung cancer. Studies consistent with a genetic component to lung cancer susceptibility (4-7) and studies to test the hypothesis of an association between DBR metabolism and lung cancer (8-14) are reported.

In a case-control study, we tested the hypothesis that the ability to metabolize DBR is related to lung cancer risk. Overall, extensive metabolizers of DBR, as determined by the metabolic ratio after administration of DBR, were at significantly elevated risk of lung cancer compared to poor or intermediate metabolizers (odds ratio = 7.5...
was Amary patients and DBR, were determined in an aliquot of urine using the method of Idle et al. (20). DBR (Declinax, Roche) is an adrenergic blocker used as an antihypertensive drug in Canada and Europe. Following an overnight fast, 10 mg (tracer dose) of DBR were administered orally. After the initial voiding had been discarded, urine was collected over the next 8 hr. Nonessential medications were not given on the morning of DBR administration; fluids and a light breakfast were permitted 1 hr after the dose. No significant hypotensive or other adverse reaction was noted in the study (21).

The DBR metabolic phenotype was determined by calculating the metabolic ratio, i.e., percent dose excreted as unchanged DBR divided by percent dose excreted as 4-hydroxy-DBR. This ratio can be used to classify individuals into one of three categories: extensive, intermediate, and poor metabolizers of DBR. The method used for cutpoint determination involves a mixture model to fit three normal distributions to the frequency distribution of metabolic ratios observed in controls. Cutpoints for the determination were derived in blacks (extensive: intermediate = 4.2, intermediate:poor = 26.4) and whites (extensive:intermediate = 4.8, intermediate:poor = 11.7) from the intersections of the three theoretical distributions (22).

Statistical analyses were performed using the SAS statistical analysis package (23).

**Genotype.** The method of Gough et al. (18), employing PCR to characterize the 29-kb allele, identifies a base deletion at the junction of intron 3 and exon 4, which results in a splice-site defect. This mutation, designated the CYP2D6 “B”, is the most common one among poor metabolizers and accounts for 75% of the alleles in this group. Next most common is CYP2D6 “D,” a complete deletion of gene, associated with the 11.5 kb XbaI haplotype and accounting for slightly greater than 10% of alleles in poor metabolizers. CYP2D6 “A” (deletion in exon 5) and “C” (single base pair deletion in exon 5) are less common and at least 5% of mutations are as yet uncharacterized (24). When these point mutations are present, individuals exhibit the poor or intermediate metabolizer phenotype in spite of the presence of the putative 29-kb wild-type allele (17,25,26). The PCR is used to amplify a 298-bp fragment, using primers from an area that is not homologous with CYP2D7 and CYP2D8. The product is then enzymatically digested with BstN1 (New England Biolabs, MA), according to the manufacturers’ instructions. Samples were analyzed by electrophoresis on agarose (2.2%) (17).

**Results and Discussion**

In the original case–control study, 13 poor metabolizers were identified from among 181 subjects who had undergone phenotyping [89 lung cancer cases, 1 poor metabolizer; 92 pooled controls, 12 poor metabolizers (15)]. Genotyping using the XbaI restriction fragment size in an overlapping subset of controls and normal volunteers
(n = 80) revealed 11 poor metabolizers with the following distribution of haplotypes: five were 29/29 homozygous, three were 29/44, two were 29/11.5, and one was 44/16/9 [see Table 1 in Sugimura et al. (16)]. These data failed to demonstrate that the 11.5 kb and 44 kb allele fragments were of value in identifying the deficient metabolizer phenotype. Finally, in a recent preliminary application of a modification of Gough’s (18) method for detection of the intron 3/exon 4 mutation in eight poor metabolizers, 3/3 were homozygous mutant subjects, but four were wild-type homozygous and two were heterozygotes. Misclassification is apparently possible with either assay, but the PCR method is clearly an improvement over the earlier RFLP approach.

The extensive metabolizer phenotype is strongly associated with lung cancer in the original study [data not shown, see Caporaso et al. (15)]. There was no association between the earlier RFLP marker and lung cancer risk (16), and there was only a weak association with the PCR-determined genotype. An explanation of the differing degrees of association with the different tests requires discussion.

From both a technical and an epidemiologic perspective, accounting for the subjects in whom the genotype and the phenotype do not correspond is of central importance. Two general explanations for a lack of correspondence are: an influence of the disease state or other distorting factors on the determination of phenotype (unlikely, but difficult to exclude owing to the case–control study design) and misclassification error in the genotyping assay.

With regard to the first possibility, the non-correspondence of phenotype and genotype may be due to an effect–cause relationship; that is, in theory, the tumor, tumor products, or tumor treatment (although subjects in this study were untreated) could modulate expression or measurement (i.e., the phenotyping procedure) of the ability to metabolize DBR. While studies have generally found no effect of chemotherapy on the phenotype (27), and the phenotype of subjects after cancer therapy has not been altered (7,11), the possibility is difficult to totally rule out.

### Table 1. Comparison of the merits of phenotyping and genotyping to characterize CYP2D6 in population studies.

| Consideration                | Phenotype                      | Genotype                     |
|------------------------------|--------------------------------|------------------------------|
| **Factors that influence validity** |                                |                              |
| Medications                  | Many drugs influence the phenotype determination (32) | Not affected                 |
| Medical illness              | Abnormal liver or kidney function may distort phenotyping | Not affected                 |
| Degree of subject cooperation | Failure to collect all timed urine or to take drug probe diarrows determination | Informed consent for phlebotomy |
| Foods                        | Watercress (29); others unknown | Not affected                 |
| **Factors that influence feasibility** |                                |                              |
| Exclusions                   | Many, due to time required to collect timed urine; need to administer drug probe; patient safety considerations; patient conflicts; refusals | Few: only greatly ill patients will be unable to donate small blood sample required (also, other sources of DNA may be used: hair follicles, paraffin blocks, etc.); HIV or blood precautions |
| **Test performance**         |                                |                              |
| Sensitivity*                 | Excellent                      | Fair to good, with steady improvement expected |
| Specificity*                 | Fair to very good, depending on how well subjects taking medications are identified and excluded | Excellent                    |
| **Summary**                  |                                |                              |
| Advantages                   | Historical role; true assessment of “biochemical level”; well characterized in many ethnic groups; method of choice in mechanistic, validation, and studies in new ethnic groups | Identifying heterozygotes, look for dose response; simple; free of effect–cause bias; only means to study subjects who cannot halt interfering medications (i.e., patients with Parkinson’s disease, schizophrene) |
| Disadvantages                | Timed urine sample; probe drug (IND needed for use of debrisoquine in the USA); medication interference; careful patient instruction and cooperation required | Mutations still incompletely characterized; ethnic heterogeneity likely |
| Conclusion                   | Declining role but still important in selected settings | Steady increase in use likely in the future |

*Sensitivity = true positives/(true positives + false negatives); in this case a positive is a deficient metabolizer. Here sensitivity does not refer to how well the test is able to identify subjects with the disease, but only how well the test identifies deficient metabolizers. The “gold standard” is phenotyping performed in healthy, fasting subjects, receiving no medications, an administration of debrisoquine, followed by 8-hr urine collection.

**Specificity = true negatives/(true negatives + false positives) or the ability of test to identify nondiseased, where diseased or positives are deficient metabolizers, and negatives are the more common extensive metabolizers. By this definition, the specificity of the genotype test is quite good because all subjects identified with two mutant alleles are phenotypic poor metabolizers.
This problem will be addressed directly in a study currently in progress in which early-stage lung cancer patients are phenotyped prior to treatment and again following surgical treatment of lung cancer. Preliminary results from this study indicate no change in the phenotype following treatment (28). Alternatively, unrecognized medications may have rendered phenotyping inaccurate. Individuals who are true extensive metabolizers have been misclassified in the phenotyping assay because of failure to recognize that these subjects were receiving a medication or dietary item capable of distorting phenotyping or owing to laboratory error. We have carefully reviewed data from questionnaires and medical abstracts used to specifically capture this information, and found that an unrecognized dietary agent (29) or medication (e.g., quinine (30,31)) might in theory account for the findings, but the probability appears small.

The second possibility is that the genotype assay does not yet recognize all the possible mutations that may result in the poor metabolizer phenotype. While perfect correspondence between the genotype and phenotype in extensive and intermediate metabolizers is not expected because family studies have demonstrated incomplete dominance (that is, obligate heterozygotes may have either extensive or intermediate metabolizer phenotypes), the failure to detect mutant alleles consistently in phenotypic poor metabolizers is problematic. Of the nine poor metabolizer subjects, three are homozygous for the mutant genotype, two are heterozygous, and four are wild-type homozygous. The presence of the latter group indicates the shortcomings of the current assay. Further mutations may account for the deficient metabolism phenotype in these subjects. It is likely that elucidation of further mutant alleles will be required before the assay fulfills its promise as the final arbiter of the question of an association with lung cancer.

In conclusion, this preliminary examination of a new approach to genotyping the CYP2D6 locus (DBR phenotype) allows certain conclusions. The genotype and phenotype show a significant association, although complete correspondence is not present. The question of the degree of association with lung cancer is the subject of ongoing study. It would be premature to draw conclusions from the results of the "B" mutation alone, however, it is of interest to note that the odds ratio for risk in extensive metabolizers (pcr genotype) is similar to that which is derived from the published case-control studies in the aggregate [odds ratio for EM is approximately 2 (32)]. If nondifferential misclassification is assumed, the characterization of further mutations (improved sensitivity) should adjust the point estimate upward while improvements in phenotyping (i.e., recognizing and eliminating some currently unappreciated medication which inhibits CYP2D6) will improve specificity and would likely reduce published point estimates, derived from this study. Finally, we list the relative merits of the phenotyping and genotyping approaches as applied to population studies in Table 1. With certain important exceptions, we anticipate that the advantages of genotyping will mandate increasing reliance on this approach.

One further possibility must be mentioned. Implicit in the previous discussion is the assumption that the "real association" must be with the genotype rather than the phenotype. In fact, it may be that the DBR metabolic ratio, ultimately determined in the individual as a complex of genetic and environmental factors, most accurately reflects lung cancer susceptibility itself the consequence of tobacco and other carcinogen exposures, in concert with hereditary predisposition. Table 1 summarizes characteristics of the phenotype and genotype approaches.

This manuscript was presented at the Conference on Biomonitoring and Susceptibility Markers in Human Cancer: Applications in Molecular Epidemiology and Risk Assessment that was held in Kailua-Kona, Hawaii, October 1–November 1991.

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