

Pharmacogenetics: Detecting Sensitive Populations

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Risk assessment models strive to predict risks to humans from toxic agents. Safety factors and assumptions are incorporated into these models to allow a margin of error. In the case of cancer, substantial evidence shows that the carcinogenic process is a multistage process driven by the interaction of exogenous carcinogenic exposures, genetic traits, and other endogenous factors. Current risk assessment models fail to consider genetic predispositions that make people more sensitive or resistant to exogenous exposures and endogenous processes. Several cytochrome P450 enzymes, responsible for metabolically activating carcinogens and medications, express wide interindividual variation whose genetic coding has now been identified as polymorphic and linked to cancer risk. For example, a restriction fragment length polymorphism for cytochrome P4501A1, which metabolizes polycyclic aromatic hydrocarbons, and cytochrome P4502E1, which metabolizes *N*-nitrosamines and benzene, is linked to lung cancer risk. Cytochrome P4502D6, responsible for metabolizing many clinically important medications, also is linked to lung cancer risk. The frequency for each of these genetic polymorphisms vary among different ethnic and racial groups. In addition to inherited factors for the detection of sensitive populations, determining the biologically effective doses for carcinogenic exposures also should quantitatively and qualitatively enhance the risk assessment process. Levels of carcinogen-DNA adducts reflect the net effect of exposure, absorption, metabolic activation, detoxification, and DNA repair. These effects are genetically predetermined, inducibility notwithstanding. The combination of adduct and genotyping assays provide an assessment of risk that reflects recent exogenous exposure as well as one's lifetime ability to activate and detoxify carcinogens.—*Environ Health Perspect* 102(Suppl 11):81-87 (1994)

Key words: cytochrome P450, cancer, genetic polymorphisms, risk assessment

Introduction

The protection of populations from carcinogenic agents (and other toxins) generally requires a risk assessment process before regulation or remedial action. A formal risk assessment involves several steps including a hazard assessment, dose-response assessment, exposure assessment, and risk characterization (1). In general, data from scientific studies are extrapolated to human experience through mathematical modeling that identifies a level of chemical exposure that might predictably result in a specific number of adverse outcomes (e.g., clinical cancer). Mathematical modeling, however, essentially is a substitute for scientifically determined data and is sometimes accepted, for a variety of reasons, without adequate validation. Many risk assessment steps incorporate untested assumptions or have methodological problems such as using

premises that are not consistent with known scientific data (e.g., using multistage models with the number of stages less than that determined from human cancer studies). Moreover, a fundamental limitation of risk assessment is that it usually examines risk for populations rather than for individuals, thereby not considering interindividual variation in response to xenobiotic exposure. Individuals or groups of individuals (e.g., families or ethnicity) might be more sensitive or resistant to particular exposures based on xenobiotic activation or detoxification, DNA repair, genetic structure, etc. Thus, the risk assessment process that does not include the interaction of the environment with interindividual capacities will become increasingly limited.

Carcinogenesis is a multistage process of normal growth, differentiation, and development gone awry (2,3). It is driven by spontaneous and carcinogen-induced genetic and epigenetic events. Carcinogenic agents initiate the process by causing DNA mutations and altered gene expression. These genetic effects, in concert with additional carcinogen exposure and other genetic or epigenetic effects, lead to tumor promotion. Through these stages, cells have selective reproductive and clonal expansion capabilities. Progressive phenotypic changes and genomic instability occur (aneuploidy, mutations, and gene amplification). These genetic changes enhance the probability of

initiated cells transforming into a malignancy; the odds of which are increased during repeated rounds of cell replication. Angiogenesis allows for a tumor to grow beyond 1 or 2 mm in size. Ultimately, tumor cells can disseminate through vessels invading distant tissues and establishing metastatic colonies. Each of these steps can be directly affected by carcinogen exposure. The response to these exposures, however, can vary from individual to individual (4). Note also that the current concepts of initiation, promotion, genetic, and epigenetic effects have been conceptually important but are now considered simplistic and not consistent with current human carcinogenesis models.

The role of protooncogenes and tumor suppressor genes has become increasingly apparent in the multistage model of carcinogenesis (2). Both are important to the regulatory mechanisms of growth, cell cycle control and terminal differentiation (2,5). Activation of protooncogenes enhance the probability of neoplastic transformation, which can either be an early or late event. Tumor suppressor genes code for products that, unlike protooncogenes, enhance the probability of neoplastic transformation when their activity is lost. For example, the *p53* tumor suppressor gene, located on chromosome 17, is the most commonly altered suppressor gene among all tumors so far studied (6). Single base substitutions

This article was presented at the Workshop on Pharmacokinetics Defining the Dose for Risk Assessment held 4-5 March 1992 at the National Academy of Sciences in Washington DC

The author is grateful to Curtis Harris for his review of this manuscript and Dorothea Dudek for her skillful editorial assistance

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can result in loss of function or production of *p53* proteins that either interfere with normal function or otherwise directly enhance neoplastic transformation (7).

The multistage process of carcinogenesis is best exemplified by a model of human colorectal tumorigenesis described by Fearon and others (8). In the early stages, loss or inactivation of *APC* (9,10) and *MCC* genes (11), hypomethylation and genomic instability accompanies the phenotypic appearance of an adenoma. More advanced tumors involve oncogenes and tumor suppressor genes, typically not observed in early adenomas. The combined events are more important than the actual order in which they occur. The genetic abnormalities include *Ki-ras* mutations on chromosome 12, mutation of *p53* tumor suppressor genes on chromosome 17, and a deletion of *DCC*, the putative tumor suppressor gene on chromosome 18q that may be involved in cell-to-cell adhesion and possibly metastasis. Other allelic losses can occur in any of the other chromosomes. Thus, it appears that at least six genetic events occur in the development of colorectal carcinoma.

The method by which carcinogenic agents affect DNA and produce mutational spectra is varied. Chemical carcinogens generally undergo metabolic activation to electrophilic intermediates that form DNA adducts through covalent binding (12). Promutagenic adducts can then cause mutations through mispairing or base substitutions during DNA synthesis. The binding of carcinogens to DNA nucleotides is apparently nonrandom (13-15) and has been shown to affect protooncogenes and tumor suppressor genes. Among the best studied examples of metabolic activation is the epoxidation reactions of polycyclic aromatic hydrocarbons (PAH), of which benzo[*a*]pyrene (BP) is one example (16,17). These compounds are composed of fused benzene rings that are essentially water insoluble but readily absorbed through the lungs and gastrointestinal tract. They are commonly found as combustion products of fossil fuels (e.g., coal, diesel exhaust) and vegetable matter. Consequently, PAHs occur as environmental pollutants. BP becomes metabolically activated in a phase 1 reaction by forming a reactive diol epoxide that can covalently bind to DNA-forming adducts. Initially, cytochrome P450 (CYP)1A1 and epoxide hydroxylase catalyze the conversion of BP to a dihydrodiol. Then, CYP3A4 converts this product to a diol-epoxide (i.e., BP-7,8-diol-9,10-epoxide) that is the reactive form. However, along

this pathway, intermediates might be removed via conjugation (e.g., glutathione transferase), further oxidation, or reduction. These metabolites can then be excreted in urine or feces. It is worthwhile noting that the activity of several enzymes involved in these activation and detoxification processes, as well as DNA repair, varies markedly in individuals, so that reactions leading to the formation and removal of BP-related adducts and mutations can occur at higher or lower rates (4).

On a molecular basis, cells possess the ability to repair DNA damage (18). Smaller alkyl adducts can be excised while larger adducts require the excision of several bases. An extensively studied repair enzyme is the *O*⁶-alkylguanine-DNA-alkyltransferase (19). This enzyme repairs damage from alkylating agents such as tobacco-specific nitrosamines and other *N*-nitrosocompounds. It is a suicide protein in that it transfers the alkyl group to itself and becomes inactivated. Cell cytotoxicity and tumor cell resistance are negatively correlated with the levels of this enzyme (20), and levels vary within organs and among people (21,22). PAH-DNA adducts can be repaired by a nucleotide excision pathway. A unimodal distribution of repair rates of benzo[*a*]pyrene diol-epoxide DNA adducts has been observed using human lymphocytes *in vitro* (23). The interindividual variation was substantially greater than the intraindividual variation, which suggests a role for inherited factors.

Genetic Predispositions to Cancer

Interindividual variation in response to xenobiotics and their potential carcinogenic effects is mediated by inherited predispositions (Table 1). Family cancer syndromes, the most evident expression of inherited predispositions, can lead to up to a 1000-fold increased risk of cancer in family members (24). However, most individuals do not have such an obvious genetic predisposition, and host susceptibility relating to a specific gene is less obvious because these mutations as risk factors are barely detectable above

background. Current studies show that these factors are more common in a family, ethnic, and racial groups.

CYP1A1 has been extensively studied in the metabolism of PAHs. Activity of CYP1A1 varies in lung tissues from different persons (25,26), which is an inherited trait (27) but also can be induced upon exposure to agents such as tobacco smoke (28). Inducibility varies among individuals and is notably higher in lung cancer patients than in noncancer controls (29,30). Levels of aryl hydrocarbon hydroxylase activity also have been correlated with levels of DNA adducts (31) and with prognosis in lung cancer (32). Recently, a restriction fragment length polymorphism has been described, using *Msp* 1 restriction digestion, that is reported to correlate with lung cancer risk in a Japanese cohort (33) and also found to be in genetic disequilibrium with a mutation in the catalytic region of the enzyme (34). Evidence suggests the mutation results in increased metabolic activation (K Kawajiri, personal communication) and the polymorphism was correlated with a 3-fold increase in lung cancer risk (33). Further study showed that the effect was greatest in persons with squamous cell cancer and in persons with the least amount of tobacco smoking history (35). This latter point indicates that the effect of this polymorphism is strongest in persons with less carcinogenic exposure.

The National Cancer Institute-University of Maryland (NCI-UMD) Case-Control Study investigated lung cancer patients and controls (pulmonary disease patients and nonlung cancer patients) for inherited predispositions to lung cancer. The control groups had similar age and smoking status. The *Msp* 1 polymorphism of CYP1A1 and lung cancer risk was studied in 101 persons enrolled in this study, but no association was found with either lung cancer risk or histological lung cancer type. However, there was a statistically significant difference in allelic frequencies for African Americans versus Caucasians. This suggests that on the basis of the

Table 1. Examples of interindividual variation in response to xenobiotics and cancer risk or prognosis

Response	Example	Human cancer type
Metabolic activation	Cytochrome P4501A1	Lung (33)
	Cytochrome P4502D6	Lung (37,38)
	Cytochrome P4502E1	Lung (48)
	<i>N</i> -Acetyltransferase	Bladder/colon (81,82)
Detoxification	Glutathione S-transferase M1	Lung/stomach/colon (81,82)
DNA repair	Excision of UV damage	Skin (83)
Protooncogene	HRAS-1	Lung (84,85)
	<i>L-myc</i>	Lung/gastric/sarcoma (86,87)
Tumor suppressor gene	<i>p53</i>	Li-Fraumeni syndrome (88)

Japanese data, African Americans would be more sensitive to lung cancer. Separate analysis by race did not reveal an association with lung cancer risk although the numbers of each group substantially limited the statistical power of the study. Thus, the study numbers are currently being increased. In a separate Norwegian study (36), no association with the *Msp* 1 RFLP and lung cancer was found. Importantly, this study was severely hindered by not utilizing age- and smoking-matched controls that can result in falsely negative findings. The frequency of the exon 7 mutation in cancer patients and matched controls, and its linkage with the *Msp* 1 RFLP, in American and European ethnic groups is now required.

The study of the cytochrome P4502D6 (*CYP2D6*, also known as the debrisoquine polymorphism) is among the best examples of inheritable interindividual differences in metabolism. This enzyme is responsible for metabolism of several medications including tricyclic antidepressants, beta-blocking antihypertensives, and debrisoquine. Poor metabolizers are at risk of adverse drug reactions. In a cohort of smokers in London, England, a 4-fold higher risk of lung cancer was associated with the extensive metabolic phenotype (37). This association has been confirmed in the NCI-UMD lung cancer case-control study with an odds ratio of six (38). The extensive metabolic phenotype also has been shown to have an interactive effect with occupational exposures to asbestos and PAHs (39).

Genotyping methods for *CYP2D6* have been sought to avoid the requirement for time-consuming urinary phenotyping and the attendant hypothetical risks of drug administration. These methods also would clarify whether the association with lung cancer might be a result of a cancer effect on the phenotypic expression rather than an inherited predisposition that predated the development of cancer. An *Xba* 1 RFLP was described (40) where identifiable alleles were associated with the poor metabolizer phenotype. However, it was soon found that the *EcoR* 1 RFLP only correctly predicted one-third of poor metabolizers (41). Since then, it was found that most of the poor metabolizer phenotypes could be explained by specific mutations in *CYP2D6* (42). These and other mutations have now been designated as A, B, C, and D mutations (Table 2) (40,42-44).

Two polymerase chain reaction (PCR) assays have been published to determine the A and B mutations in *CYP2D6* (42,45).

Table 2 Cytochrome P4502D6 genotypes and phenotypes (42,44)

Type	<i>Xba</i> 1 RFLP ^a	Location	Description	Associated phenotype
Wild-type	29 Kb	NA ^b	Wild-type sequence	Extensive
A	29 Kb	Exon 5	Nucleotide deletion (2737A)	Poor
B	29 Kb	Exon 3	Splice site defect (1934 G→T)	Poor
C	29 Kb	Exon 5	Nucleotide deletions (2702-2706 AGAI)	Extensive
D	11.5 Kb	NA	Deletion of cytochrome P4502D6	Poor

^aResults of Southern blot analysis yielding fragment length shown. ^bNot applicable

The D mutation is identified with the *Xba* 1 restriction digest and Southern blotting (40). The A and B mutations can be identified by using a PCR mismatch assay where primers differ only by the 3-bp base that matches with either the wild-type base or the mutant base (42). The assay begins with a first step that uses primers specific only for *CYP2D6* and not the pseudogenes *CYP2D7* and *CYP2D8*, which are otherwise almost 95% homologous. The second step uses the mismatch primers. The assay requires careful validation to ensure correct priming, sensitivity, and specificity. This method can reportedly characterize over 95% of metabolic phenotypes in large numbers of Europeans. The second method takes advantage of a *Bst* N 1 restriction site at the B mutation and uses an altered primer that introduces a *Dra* 1 restriction site at the A mutation (45) (R Wolf, personal communication). In our laboratory, we have been genotyping individuals using a combined approach that uses the *Bst* N 1 digest for the B mutation with the primers previously identified as not significantly homologous (42) and the mismatch assay for the A mutation. We have found that the frequency of poor metabolizers in Caucasians by genotyping was statistically significantly higher than in African-Americans, suggesting an inherited predisposition to lung cancer in African Americans (P Shields, unpublished results).

Thus far, the mechanistic relationship of *CYP2D6* activity to lung cancer remains unknown. The only known carcinogenic substrate identified for *CYP2D6* is 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol (NNK) but not other tobacco-specific nitrosamines that have been tested (46). Thus, the lung cancer association might relate to altered substrate specificity of the enzyme in extensive metabolizers not studied, unidentified carcinogenic substrates, or a gene that is in linkage disequilibrium with another gene related to cancer risk.

CYP2E1 is responsible for metabolizing a number of potential human carcinogens including benzene and N-nitrosamines. Several genetic polymorphisms have been

identified (47,48). One polymorphism includes two distinct base substitutions in the same area, which are in genetic disequilibrium so that either one can be studied (47). This area is involved in transcription regulation and preliminary studies indicate that one type allows for increased expression of the chloramphenicol acetyl transferase gene in transfected HepG 2 cells. The frequency of the polymorphic alleles is different in Japanese and Americans (S Kato, personal communication, 1992). Whether this polymorphism has a relationship to cancer risk is currently under study. The other polymorphism is at *Dra* 1 restriction enzyme site in intron 6 (M Watanabe, personal communication). While its biological significance is unknown, it has been reported that the distribution of genotypes is significantly different in lung cancer cases and controls (48).

Molecular Dosimetry—Identifying a Combined Carcinogen Exposure and Susceptibility

One indicator for the net effect of exogenous carcinogen exposure and inherited traits for absorption, metabolism, and DNA repair is the carcinogen-DNA adduct. Measurement of adducts can be useful for estimating a biologically effective dose of a carcinogen and the risk for fixed mutations. A variety of assays are available to identify carcinogen-DNA and protein adducts. Enzyme immunoassays (49-58), ³²P-postlabeling and nucleotide chromatography (59-61), fluorescence spectroscopy (62), synchronous fluorescence spectroscopy (SFS) (63-66), gas chromatography and mass spectroscopy (GC/MS) (66,67), and electrochemical detection (68) have been applied to the analysis of human lung samples or a surrogate tissue or cell population.

Central to the studies of DNA adducts is the development of sensitive and specific assays that are required to detect femtomole and attomole levels of adducts in microgram amounts of DNA. Current methods are challenged because of the complexity and multitude of possible exposures in human tissues. The specificity of adduct

assays, and therefore their quantitative reliability, can be enhanced by using micro-preparative techniques. For example, subjecting enzymatically digested lung DNA to high-pressure liquid chromatography (HPLC) followed by the ^{32}P -postlabeling assay, which relies on three different separations (HPLC and two-dimensional thin-layer chromatography) can detect *N*-nitrosamine-related alkyl adducts such as *O*⁶-methyl-2'-deoxyguanosine (69), *O*⁶-ethyldeoxyguanosine (*O*⁶ethylG) (69), *N*-7-methyldeoxyguanosine (*N*⁷methylG) (70) and *N*-7-ethyldeoxyguanosine (S Kato, personal communication) at levels as low as one adduct in 10^7 2'-deoxyguanosine residues. Other laboratories have used HPLC after ^{32}P -postlabeling (71). Immunoaffinity chromatography also has been combined with ^{32}P -postlabeling assay to identify *O*⁶-methyldeoxyguanosine (D Cooper, personal communication) and polycyclic aromatic hydrocarbons (P Shields, unpublished data). In both cases, the level of detection was at least one adduct in 10^7 unmodified deoxyguanosine. Another feature of assays that are dependent upon micro-preparative techniques is that they are developed using authentically synthesized adduct standards, use internal standards, and quantitation based upon calibration curves.

Organ tissue selection, multiple sources of exposures, and other confounders impact upon study design and results. It would be optimal to use easily obtainable body fluids such as blood or urine for risk assessment. However, it remains to be established if blood testing will be a reliable surrogate for other tissues. For example, some data suggest that the determination of PAH adducts in peripheral lymphocyte DNA reflects dietary rather than inhalational exposures (72,73). We also will need to consider cellular differences in life-span (lymphocytes and red blood cells survive longer than granulocytes), DNA repair capacity, and metabolic capacity. It has been reported that the initial adduction of lymphocytes and granulocytes is similar but that adduct levels are more persistent in the latter (74). Our laboratory has used lymphocytes (49-51,63) while others have studied total white blood cells (predominantly granulocytes) (54,72). Oral mucosal cells are another relatively noninvasive source of DNA and can allow for the detection of several types of adducts (57,60).

Multiple types of adducts have been observed in individual lung samples, confirming the complex nature of carcino-

Tabb 3. Genetic polymorphisms and ethnicity.

Polymorphism	Populations compared	At risk ^a
Cytochrome P4501A1	African-Americans vs Caucasians	African-Americans
Cytochrome P4502D6	African-Americans vs Caucasians	African-Americans
Cytochrome P4502E1	Americans vs Japanese	Japanese
L-myc protooncogene	African-Americans vs Caucasians	African-Americans
H-ras protooncogene	African-Americans vs Caucasians	African-Americans
p53	African-Americans vs Caucasians	NA ^b

NA, not applicable. ^aPopulation found to have higher frequency at alleles associated with lung cancer. ^bAllele associated with cancer risk but statistically varies among compared populations.

genic exposures (75). Small alkyl adducts, polycyclic aromatic hydrocarbons adducts, and aromatic amines have been identified. Improved micro-preparative techniques have led to the unambiguous identification of specific polycyclic aromatic hydrocarbons adducts in human lung (63,66). This finding has now been confirmed by combining immunoaffinity chromatography with the ^{32}P -postlabeling assay (P Shields, unpublished data).

Exposures to PAH compounds are associated with an increased risk of lung cancer. Industrial pollution, fossil fuels, and tobacco smoke account for the major environmental sources. Dietary exposures also commonly occur because of overcooked or charcoal-broiled meats. Adduct levels have been correlated with exposure in coke oven workers (54,59,76), tobacco consumption (67), and urban versus rural residence (77), but decreases during vacation from occupational sources (51). Seasonal variation in adduct levels also has been observed (78). Some studies have not found correlations with tobacco consumption but this may be because of other sources of exposure (e.g., diet).

Conclusions

The current data indicate that several inherited genetic traits are associated with cancer risk. Frequencies of these also vary among ethnic populations (Table 3). The interaction of environmental exposures and metabolic capacities suggests that current risk assessment models need to be biologically based and consider the variation in sensitivity among individuals. The multistage model of carcinogenesis further suggests that single low-dose exposures and most genetic traits will likely not be sufficiently strong by themselves to drive the carcinogenic process. Thus, risk assessment models need to incorporate interactive effects (chemical, radiation, viral, and physical agents interacting with each other and with host factors).

The use of genotyping assays and adduct determinations require rigorous

field testing in carefully designed studies. Genotyping assays need to be studied in both case-control studies and prospective studies where confounding variables are carefully considered (e.g., tobacco use, multiple sources of exposure). Genotyping assays are preferable to phenotyping assays because they cannot be altered by the presence of disease. For adduct assays, the importance of the development and validation phases cannot be overstated and need to be meticulously performed prior to their use in large field trials.

NOTE ADDED IN PROOF: Since the preparation of this manuscript in 1992, all of the data cited as personal communication have now been published. There also are a number of more recent reviews. New data has identified several examples of gene-environment interactions where the effects of metabolizing polymorphisms vary depending on exposure, further impacting upon risk assessment procedures and outcomes.

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