Acetylation as an Indicator of Risk

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Aromatic amine acetylation has been recognized for many years as an important metabolic polymorphism in humans because of its relationship to disease. This system serves as a model in risk assessment because of its role in drug and carcinogen activation and detoxification and because of the ease with which it is measured. However, possible interactions of NAT1–NAT2 phenotypes or genotypes illustrate the complexity of xenobiotic metabolism pathways. Moreover, the use of such information for risk assessment is further complicated by the association of the rapid phenotype with increased risk in colon cancer and the slow phenotype with increased risk in urinary bladder cancer. Before this biomarker can be effectively utilized as a significant predictor of individual risk, it will be necessary to identify specific sources of aromatic amine exposure and to characterize further the substrate specificity of NAT1 and NAT2 in relation to the multiplicity of enzyme variants occurring in human populations. — Environ Health Perspect 105(Suppl 4):763–766 (1997)

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Definition of Marker

To be useful, a biomarker of susceptibility must differentiate between the low- and the high-risk groups, have low false positive and low false negative rates, be easy to perform, and be simple to interpret. Acetyltransferases (NAT1 and NAT2, EC 2.3.1.5) meet these needs in many respects. Since the recognition of the NAT2 acetylation polymorphism from its effects on iso- niazide toxicity (1), it has been studied in relation to several diseases in hope of finding a useful marker of susceptibility. By the use of different substrates, acetylation activity could be separated into NAT1 (p-aminobenzoic acid [PABA]) and NAT2 (sulfamethazine [SMZ]) activity. Until recent reports of variations in PABA metabolism (2), NAT1 was thought to be monomorphic while the polymorphism was ascribed to the NAT2 activity. The identification of the genetic basis for the phenotypic variations was identified in 1990 (3) for NAT2 and in 1993 (4) for NAT1. Other investigators quickly reported the identification of ethnic variations (5) when the most frequent variant alleles seen in Caucasians differed in Asian and African-American populations.

Investigators have found exposure to xenobiotics plus NAT2 acetylation to indicate risk for some diseases but not others. The relationship between NAT2 acetylation phenotype and exposure to certain xenobiotics has been discussed by Cartwright et al. (6). Slow phenotype subjects who were exposed to compounds such as aromatic amines had the greatest risk of urinary bladder cancer. The use of acetylation as a risk marker has been evaluated in a group of lung cancer cases and controls with no difference identified between the groups (7). Two groups working independently have reported a relationship between rapid acetylation phenotype and risk of development of colon cancer (8,9). Both groups reported a 2- to 3-fold odds ratio for colon cancer in the group that was rapid acetylator phenotype. Increased risk of colon cancer has also been associated with the combination of rapid acetylator (NAT2) and the rapid form of CYP1A2 in one report (10) and with the rapid acetylator genotype of NAT1 (NAT1*10) when it occurred in association with the rapid NAT2 genotype (NAT2*4) (11) in another report. A recent report found an increased frequency of the rapid NAT2 genotype (NAT2*4) with a specific breast cancer, lobular carcinoma (12). Finally, Ambrosone et al. report a greater than 5-fold increased risk for premenopausal smokers who are rapid acetylator phenotype and thin (13).

The impact of acetylation phenotype in these studies points out the need for information on the tissue metabolism involved in the carcinogenic pathway.

Tissue distribution and substrate specificity help explain the epidemiology results noted above. NAT1 and NAT2 occur in the liver, with NAT2 being 2 to 10 times the level of NAT1, depending on the NAT2 status of slow or rapid acetylator, respectively. The predominant extrahepatic form is NAT1, regardless of the acetylator phenotype. NAT activity has been found in many extrahepatic tissues including colon mucosa, placenta, and leukocytes (14–16). NAT1 and NAT2 have a broad substrate specificity (17,18) with catalytic activity for amines, N-hydroxyarylamines and N-hydroxy-N-aryldiazamines. However, there is little activation of the foodborne heterocyclic amines until these compounds undergo N-oxidation by the polymorphic cytochrome P4501A2. These data suggest, and investigators found, that subjects at greatest risk for a colon neoplasm from exposure to heterocyclic amines in the diet would be those individuals exposed at the highest level who have the most efficient metabolism of these compounds to proximate carcinogens (10). This model is also compatible with the finding of a role for NAT1 polymorphism in colon cancer risk (11). Development of similar understanding in other malignancies awaits further epidemiology and metabolism studies. While acetylation can serve as a risk marker for some diseases, the application of this information will not be trivial.

Use of Acetylation as a Biomarker of Susceptibility

Identification of At-Risk Group

The primary advantage of a biomarker such as metabolic phenotype is to allow the identification of a group at risk for a given disease. As mentioned in reference to bladder and colon cancer, the power of phenotype or genotype information is improved when combined with exposure data. Use of phenotype or genotype...
information to predict the at-risk group also requires a biologically plausible mechanism for the disease being studied.

**Identification of Exposure Risk**

Epidemiology is the tool used to identify at-risk lifestyles. However, development of a biologically plausible model requires detailed metabolic studies of the liver as well as of the tissues or organs at risk for the disease of interest. Understanding the mechanism of the risk may require the investigator to use both susceptibility and exposure biomarkers such as acetylation phenotype and the presence of carcinogen–protein or carcinogen–DNA adducts. Examples include adduct and metabolism studies performed with liver, colon, and bladder tissue, together with identification of hemoglobin and DNA adducts present in cases and controls (17,19–23).

Armed with information about the status of a genetic risk (acetylation status) and the lifestyles that provide exposure linked to the risk observed, one should be better prepared to suggest behavioral changes that may reduce the risk, e.g., avoidance of heterocyclic amine exposures by limiting consumption of meat cooked at high temperature. There may be better individual adherence when recommendations are based on individual specific measurement of genetic, environmental, and dietary risk factors than has occurred with the use of generic warnings.

**Adjustment of Treatment Dosage to Maximize Effect and Minimize Risk**

Acetylation phenotype has been studied in the pediatric area to help determine drug dosage of a treatment regimen (24). Where the substrate specificity is known to involve the acetylation pathway, the use of metabolic phenotyping or genotyping should provide more precise information for determining effective therapeutic dose for drugs undergoing acetylation. The alternative is to monitor for toxicity and to adjust the dose after toxicity is diagnosed. The latter is commonly used to adjust dosage for a wide range of chemicals. Determining the functional state of a given pathway has the obvious advantage of allowing dosage adjustment before toxicity develops.

**Study of Ethnic Group Relationships**

Many of the ethnic variations in disease incidence and mortality may have as a basis differences in metabolic pathways as well as differences in environmental and dietary exposure. Investigators have reported a wide range of values for acetylation activity in different groups (25–28).

**Advantages and Limitations of Acetylation**

**Noninvasive, Low Risk**

This susceptibility marker requires only an urine sample if metabolic phenotyping is performed. If genotyping is used, a small blood sample is usually taken and the individual risk is usually regarded as minimal.

**Phenotyping and Genotyping Methods**

Earlier phenotyping methods utilized sulfamethazine, isoniazid, or dapsone as the surrogate drug (8,29,30). Both blood and urine samples were required. A rare but severe allergic reaction can occur following the administration of sulfamethazine, and isoniazid-induced hepatitis and polynuertits and dapsone neuropathy have also been reported (31–33). Current phenotyping involves administration of the surrogate drug, caffeine, which has a much lower frequency and severity of adverse drug reaction. Any adverse reaction to caffeine is rare and requires a much higher dose than is used (100 mg caffeine); most people who are sensitive can be identified and excluded by asking about sensitivity in advance of administration. Sensitivity in our studies has been <1%, and no person has had an adverse reaction to caffeine whether administered as coffee, a soft drink (Diet Coke) or a NoDoz tablet (Table 1).

**Enzyme Expression and Assay Variability**

Expression of the enzyme activity over time is an important factor. This permits scheduling of the study to be adjusted to accommodate the subjects’ lifestyle. Day-to-day variations in environmental and dietary exposures have little impact on the acetylation activity (34). In addition, the caffeine metabolites in the urine specimen collected can be stabilized by acidification with ascorbic acid and subsequent refrigeration.

**Correlation between Phenotype and Genotype**

Many (>15) alleles of the NAT2 gene have been identified in the human population. Phenotyping can provide a simple overview of activity level without the necessity of checking for every possible mutation that has been identified (35). Discordance can indicate the possibility of a yet unidentified genetic mutation (36) or the possibility of interactions at the gene or enzyme level.

At least two studies have reported interactions between NAT1 and NAT2 activity that may complicate the use of acetylation activity in risk assessment. Through the use of phenotyping assays with caffeine and PABA, Cribb et al. found a correlation (r = 0.8, p = 0.0002) between AFMU/1X and PABA activity in subjects who were slow NAT2 (low AFMU/1X ratio) (37). The second report uses genotyping to demonstrate increased risk of colon cancer when the rapid NAT1 variant allele (NAT1*10) occurs with the rapid NAT2 (NAT2*4) allele (OR 2.8; 95% CI, 1.4–5.7; p = 0.003) (11), suggesting a gene–gene interaction. The importance of the NAT1 polymorphism is further supported by a report of a doubling of urinary bladder NAT1 activity and of DNA adduct levels when the heterozygous genotype (NAT1*10/NAT1*4) is compared to the homozygous genotype (NAT1*4/NAT1*4) (38). These studies illustrate some of the difficulty encountered in approaching risk assessment through the measurement of a single polymorphism.

**Ease of Data Analysis**

While phenotyping produces a continuous variable (with caffeine this is the urinary molar ratio of AAMU/1X or AFMU/1X), most investigators find conversion of this number to a dichotomous variable more convenient. Plotting the data on a log-probability scale gives a clear break between rapid and slow phenotype that can be used to assign slow or rapid phenotype (34).

**Cost**

The phenotyping assay requires measurement of the metabolites of a surrogate drug, caffeine. This information comes

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Table 1. A comparison of assay methods.

| Surrogate drug | Assay method | Sample required | Cost | Sample throughput | Risk | Status |
|----------------|--------------|-----------------|------|-------------------|------|--------|
| Sulfamethazine | HPLC         | Urine/blood     | Moderate | Low | Low | Proven |
| Caffeine       | HPLC         | Urine           | Moderate to high | Moderate | Very low | Proven |
| Caffeine       | ELISA        | Urine           | Low | High | Very low | Unproven |
ACETYLATION AND RISK ASSESSMENT

from a high-pressure liquid chromatography (HPLC) assay performed on a sample of urine collected after caffeine dosing. The use of an HPLC system that includes a diode array detector is important to assure correct peak assignment. This equipment is expensive ($40,000 U.S.) and the system is not trivial to maintain.

The reverse-phase columns used in the assay seem to have a significant variation in their performance level. Because of this variation, columns should be purchased from the same lot number if at all possible. Even in the same lot, some columns give poor separation of caffeine metabolites and cannot be used, further adding to the expense.

Sample preparation requires extraction steps that are expensive in personnel time. This cost is reduced somewhat by batch processing of frozen, stored samples. The low throughput of the system (20–40 samples/24 hr) is costly in the amount of operator time required to produce a given result. This limit has been approached by trying to shorten the time per chromatogram (solvent gradient programs at about 45 min) by varying column and gradient conditions. Moreover, the newer, computerized systems have markedly improved the analysis of the chromatogram by automating the identification of the peaks and by automating the calculation of values from standard curves.

Approximately 5% of the urine samples have interfering substances that require reanalysis of the sample. Efforts to identify these substances have been unsuccessful to date.

For the ratio of AFMU1/IX to reflect NAT2, the sample is usually collected for 1 hr from the fourth to the fifth hour after caffeine dosing. No other caffeine is permitted between dosing and sample collection. Food and liquids are permitted to allow adequate urine volume to prevent interference with the excretion of metabolites and therefore the phenotyping assay.

Conclusions
Because of the large number of mutations identified in the NAT2 gene, the use of phenotype is still an important technique. Phenotype gives the investigator a simpler classification system (rapid vs slow) than is easily achieved with a system of 15 or more alleles combined in many possible ways. The ability of NAT2 phenotype to predict risk has been shown in colon cancer and in bladder cancer (6,8,10,39–43). Failure to identify an association with other diseases may be due to a lack of understanding of the mechanism of the disease and its link to NAT2. This could result because there is no link or because the link is more complex than the investigator recognizes. One example of the latter is provided by the identification of meat cooking, CYP1A2, and NAT2 as risk factors for development of colon neoplasia (10). The interaction of NAT1 and NAT2 is another important example (11,37).

A method that is less expensive and has more rapid sample throughput would be very desirable (Table 1). A recent report details a rapid, low-cost, enzyme-linked immunosorbent assay (ELISA) for use in phenotyping for NAT2 (44) and studies are underway to compare this method with HPLC and with genotyping.

Recommendations
Phenotyping should be continued along with specific genotyping as we gain experience with larger numbers, better assay methods, and the interpretation of more complex relationships among the enzymes involved in xenobiotic metabolism. The current understanding of xenobiotic metabolism, the measurement of that metabolism, and the evaluation of exposure are all still relatively incomplete. Therefore, the use of this information to assign specific individual risk, while desirable, has not yet been validated in a cohort- or population-based study. The next step will be to use this information as an intervention tool to reduce risk by reducing exposure to environmental and dietary factors proposed to be linked to a disease. The effect of these actions must then be evaluated to determine whether or not arisk behavior change occurs and whether or not risk reduction can be implemented.

Acetylation continues to be an interesting metabolic step because it is easily and safely measured, and it seems to play an activation role in colon cancer and a detoxification role in bladder cancer. This system is likewise important in drug toxicity as well as in other chronic diseases. Finally, there are well recognized interindividual variations that may explain some of the known disease variability among different ethnic groups. All of these areas need further exploration before we will be able to prescribe individual specific prevention strategies.

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