Predictors of N-Acetyltransferase Activity: Should Caffeine Phenotyping and NAT2 Genotyping Be Used Interchangeably in Epidemiological Studies?1

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Abstract
To determine whether NAT2 genotyping could be used interchangeably with caffeine phenotyping in assessing N-acetyltransferase activity in epidemiologic studies, sources of interindividual variability in N-acetyltransferase activity were assessed among 90 subjects of various ethnic backgrounds in Hawaii. Forty-three subjects were patients with in situ colorectal cancer treated by polypectomy, and 47 were healthy population controls. Subjects were administered a lifestyle questionnaire and were evaluated for N-acetyltransferase activity by caffeine phenotyping. NAT2 genotype was also assessed by PCR amplification of peripheral leukocyte DNA for the M1, M2, and M3 variant alleles. Fifty-four percent of the overall variation in acetylation activity was explained by the three genotype categories (homozygous variant, heterozygous, and homozygous wild-type). This proportion was reduced to 42% when genotype was modeled using only two categories ("slow" being homozygous variant; "rapid" being all others). Use of gout medications (probenecid or allopurinol), consumption of heavily browned fish, and P450IA2 activity (also measured by caffeine phenotyping), together explained another 11% of the variance. No association was found between acetylation activity and sex; race; age; education; smoking; physical activity; weight; consumption of coffee, alcohol, red meat, processed meat, and cruciferous vegetables; or use of menopausal estrogens, after taking genotype into account. Results were similar for colorectal cancer patients and controls. Considerable variation in acetylation activity was observed within the homozygous wild-type group. This study suggests that the use of genotyping, instead of phenotyping, to assess the association of acetylation with cancer risk is unlikely to introduce major misclassification or bias, especially when the three genotype categories are modeled and the sample size is large. However, when the rapid acetylation phenotype is the at-risk group (e.g., when studying colon cancer), phenotyping appears judicious given the variability in acetylation activity within this group.

Introduction
The recent associations of the slow N-acetylation phenotype with bladder cancer in arylamine-exposed workers (1) and of the rapid acetylator phenotype with colorectal cancer (2) urgently need confirmation in large population-based studies because this genetic polymorphism may be useful in characterizing individual cancer risk. Because the world-wide distribution of the slow acetylation phenotype varies greatly, from 5% in Canadian Eskimos, to 10–20% in Japanese, 50–60% in Caucasians, and 90% in Northern Africans (3), these associations may also clarify unexplained risk patterns, such as the high colorectal cancer risk of Japanese Americans (4).

N-acetyltransferase is under autosomal dominant genetic control, and its activity is coded for by two distinct genes, NAT1 and NAT2, located on chromosome 8 (5). The latter gene exhibits polymorphisms arising from point mutations in both coding and noncoding regions that can result in decreased expression, low activity, or enzyme instability. The presence of two germline copies of any of several mutations in the NAT2 gene produces a slow-acetylator phenotype. Although NAT1 has also been shown to be polymorphic in humans (6), the investigation of its association with disease has only recently begun (7).

Most human studies of N-acetylation have used metabolic phenotyping methods using drugs, such as isoniazid, sulfamethazine and dapsone, or, more recently, caffeine (8). A urine sample is collected a few hours after dosage and analyzed by HPLC.4 The ratio of urine metabolite(s) to the unmetabolized parent compound permits an assignment to the phenotype. The caffeine method is attractive because the sampling time and dose do not need to be very precise, and the metabolic ratio appears to be independent of urine flow and kidney function. However, although the enzyme is generally regarded as

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1 The abbreviations used are: HPLC, high-performance liquid chromatography; AFMU, 5-aceetylamo-6-formylamino-3-methyluracil; MET, metabolic equivalent; HAA, heterocyclic aromatic amine; PAH, polycyclic aromatic hydrocarbon.
noninducible (1), it is possible that the disease process, its treatment, or some xenobiotics, such as medications or alcohol (9), may distort the phenotyping. In studies of its association with disease, this may result in increased variability (i.e., loss of statistical power), or in bias if the distorting factors are also associated with the disease. Thus, the phenotyping approach may present logistical difficulties in population studies.

PCR-based methods have recently been developed for detecting the main mutations associated with the slow acetylation phenotype in DNA from blood or fixed-tissue (10, 11). Initially, three different point mutations (M1, M2, and M3) in the NAT2 gene were described. The genotyping method is relatively straightforward, and the results are independent from the disease status of the subject and not influenced by external variables. Moreover, in epidemiological investigations, it is usually easier to obtain biological material for genotyping analysis than it is to perform lengthy phenotyping protocols. Indeed, recent studies have used the genotyping to investigate associations of NAT2 polymorphism with bladder and breast cancer (12, 13).

Several reports have shown an excellent concordance between the phenotyping and genotyping methods used in the classification of slow and rapid acetylators. This concordance has been 90–98% in Japanese (14), Caucasians (10, 11, 15, 16), and Chinese (17). However, it has been shown that, depending on the prevalence of the at-risk genotype, even a small rate of genotyping misclassification may result in a substantial attenuation of the risk estimates (18). Also, it remains unclear whether the genotype, a categorical variable, carries as much information as the phenotype, a continuous variable, which has the advantage of integrating both genetic and epigenetic influences on the activity of the enzyme.

We report here on studies aimed at characterizing the main constitutional, lifestyle, and laboratory predictors of N-acetyltransferase activity to determine whether NAT2 genotyping can be used instead of caffeine phenotyping in assessing cancer risk in epidemiological studies.

Materials and Methods

Subjects. Participants were described previously (19) and included patients with in situ adenocarcinoma of the large bowel and healthy population controls. Eligible patients were all those diagnosed with this condition in the main medical centers of Oahu between July 1989 and October 1991 and whose treatment did not include colectomy. The controls were selected from among the participants in a population-based survey conducted by the Hawaii State Department of Health and were matched to each patient on sex, age (within 5 years), and ethnicity. Overall, 72.4% of the eligible patients and 71.6% of the eligible controls were interviewed. A total of 43 cases and 47 controls (73.4% of the eligible interviewed subjects) agreed to the biological component of the study, of whom 45 were Japanese, 15 Caucasian, 12 Filipino, 12 Hawaiian/part-Hawaiian, and 6 Chinese. The mean age of the participants was 65.6 years.

Questionnaire Data. A detailed questionnaire on demographics, diet, and lifestyle was administered at the participants’ homes by trained interviewers. The dietary information collected consisted of a quantitative diet history (20) focusing on usual intake during the year before diagnosis for cases and during the year preceding the interview for controls. It also included questions on browning of meat and fish surfaces, 5 years before interview, as well as frequency of consumption of cruciferous vegetables (cabbage, broccoli, Brussels sprouts, and watercress) during the week before the caffeine test. Daily tobacco, alcohol, coffee, and soda consumption, as well as medication and supplement use, was ascertained for the 2-week period preceding the caffeine test. Subjects were also asked to specify the time spent at various levels of physical activity during a typical workday and weekend day (21). These data were summarized over an average 24-h period and converted into METs (21). Weight was measured in light clothing.

Dosing Regimen and Specimen Collection. Subjects were asked to refrain from consuming any foods or beverages (except water) from 10:00 pm to the time of the blood draw the following morning. After the blood draw, the subjects drank a cup of coffee made of two packets of Maxwell House instant coffee (57 mg caffeine/packet). Then they voided 4 h after coffee consumption and provided a 1-h urine specimen at the end of the 5th hour after dosing. No other source of caffeine was consumed during the 5-h period. The urine samples were acidified with ascorbic acid, aliquoted, and stored at −70°C until analysis.

Intraindividual Variability Study. An additional and independent sample of 16 healthy, nonsmoking individuals of various ethnic backgrounds participated in the reproducibility portion of the study. Eight male and eight female volunteers recruited from among the staff of our institution were administered caffeine as described above once weekly for 4 weeks. No recommendation was given with regard to diet or lifestyle; however, we varied the day of the week for the dosing to optimize the possible effect of daily variations in lifestyle.

Phenotyping. We quantified urinary levels of AFMU1-methylxanthine (1X) to determine the N-acetyltransferase phenotype, as well as the urinary ratios of [1,7-dimethyluric acid (17U) + 1,7-dimethylxanthine (17X)]/caffeine (137X) to assess P450IA2 activity. Urine samples were quickly thawed and extracted (200 μl) according to a slight modification of the method of Butler et al. (22) by adding 120 mg ammonium sulfate and 120 μl 4-acetamido-phenol (160 μg/ml) as an internal standard to the urine in a 15-ml centrifuge tube followed by mixing for 2 min. Caffeine and its metabolites were partitioned by the addition of 6 ml chloroform/isopropanol (19:1). The organic phase was removed after centrifugation, evaporated to dryness, then resuspended in 100 μl of the HPLC mobile phase A consisting of 0.045% aqueous acetic acid containing 9% methanol. Chromatography was carried out with a Supelcosil LC-18 5 μm (4.6 × 250 mm) reverse-phase HPLC column using a Beckman System Gold chromatograph. Samples were eluted at 1.1 ml/min using linear gradients of mobile phases A and B (methanol) as follows: 0–3 min (0% B); 3–5 min (2% B); 5–6 min, (2–20% B); 6–16 min, (20% B); 16–21 min (60% B); 21–35 min (0% B). The identification of the metabolites was done by comparing their retention times and absorption spectra (Beckman Model 168 diode array detector) with those of authentic standards.

NAT2 Genotyping. Polymorphisms in the NAT2 gene were determined using the DNA purified from lymphocytes obtained from the subjects. Lymphocytes were digested with SDS (0.5% final) and proteinase K (200 μg/ml) overnight at 37°C, followed by extraction with phenol and chloroform/isoo-amyl alcohol (24:1) the next day (23). The DNA was precipitated using isopropanol, and the final DNA pellet was dissolved in TE (10 mM Tris.HCl pH 8.0, 1 mM EDTA pH 8.0). The amount of DNA was quantified spectrophotometrically by measurement of absorbance at 260 nm. The ratio of absorbance at 260 and 280 nm indicated its purity.

The NAT2 gene was PCR amplified using primers N4:
Fig. 1. Probit plot of the metabolic ratio AFMU/1X.

5'TCTAGCATGAATCCTGCG3' and N5: 5'GGAACAAATTGGACTTGG3'. The Ampliwaix TM PCR GEM100 beads and protocol for Hot Start PCR from Perkin-Elmer Cetus were used in this amplification. A 1091-bp fragment was obtained using 500 ng of genomic DNA, 50 pmol of each primer, 200 μmol of each dNTP, 1 unit of AmpliTag DNA polymerase, and a buffer (1.25X PCR buffer II, Perkin-Elmer Cetus) composed of 12.5 mM Tris.HCl (pH 8.3), 3 mM MgCl2, and 62.5 mM KCl in a total volume of 80 μl. In a Perkin-Elmer Cetus PCR 480 system thermal cycler, the reaction was denatured at 94°C for 4 min and then subjected to 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min. A final extension at 72°C for 5 min was performed. After PCR, 20-μl aliquots were subjected to digestion overnight with restriction enzymes KpnI, TaqI, and BamHI (Promega) according to the manufacturer’s recommendations. These digestions are diagnostic for the alleles M1, M2, and M3, respectively (11). Taq digests were electrophoresed on 4% 3:1 NuSieve agarose, and the KpnI and BamHI digests were resolved on a 3% 3:1 NuSieve agarose (FMC BioProducts, Inc.).

Data Analysis. The objective of the analysis was to study the variability of N-acetyltransferase activity, as assessed by the caffeine test. The metabolic ratio was log transformed, as log(x + 1), for its distribution to approximate normality. In the intraindividual variability study, the coefficient of variation, averaged across individuals, was taken as a measure of variability, as was the intraclass correlation coefficient, computed from a one-way random-effects ANOVA model (24). The coefficient of variation was also used to measure variability in laboratory measurement.

A probit model and a probit plot (25) of the AFMU/1X ratio were used to determine a cutoff point for the distributions of slow and rapid metabolizers. Lastly, analysis of covariance was used to examine how much variability was accounted for by the NAT2 genotype and other covariates. A stepwise procedure was executed to determine the importance of other variables after genotype was controlled for. The potential covariates selected for the regression were those shown to affect levels of N-acetyltransferase or other xenobiotic metabolizing enzymes and/or to be associated with cancer risk. These variables have the potential not only to increase the background variability in N-acetyltransferase activity but also to act as confounders of an association between acetylation and cancer.

Results

Intraindividual and Laboratory Variability. Among the 16 subjects phenotyped with caffeine once a week for 4 weeks, no misclassification occurred upon repeated measurement when the subjects were classified as “slow” or “rapid” acetylators (see below for definition). However, when treated as a continuous variable, the mean coefficient of variation for the urinary metabolic ratio (AFMU/1X) was 24% (range, 9–55%), and the intraclass correlation coefficient for the four repeated measurements was 0.73. Analyses of duplicate samples in our laboratory resulted in good reproducibility of the measurement of AFMU/1X, with intra- and interassay coefficients of variation of 7.9 and 12.8%, respectively. These intraindividual and laboratory variations are comparable to published data (17, 22, 26, 27).

Acetylator Phenotype. No caffeine metabolites could be detected in the urine of 2 of the 90 subjects. They were excluded from subsequent analyses involving phenotype. The distribution of N-acetyltransferase activity, expressed as log(AFMU/1X + 1), among the 88 remaining subjects phenotyped with caffeine is presented in a probit plot in Fig. 1. Visual inspection and probit analysis suggested that a cutoff point of 0.4 [0.34 on a log(x + 1) scale] could be used to distinguish slow acetylators (14.9%) from rapid acetylators (85.1%). This cutoff point for our multiethnic population was intermediate between that of 0.3 found for Japanese (28) and Chinese (29) and that of 0.6 observed in a mainly Caucasian population from Arkansas (22). In agreement with past studies (28), the proportion of slow acetylators (AFMU/1X < 0.4) was 9.1%.
Table 1: Acetylation activity by sex, age, and lifestyle among in situ colorectal cancer cases and population controls

<table>
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* Adjusted by covariance analysis for race, and case-control status when appropriate.

Table 1 Continued

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in Japanese. The corresponding proportion for Caucasians (35.7%) was somewhat low compared to previous studies (22), in part because of the lower cutoff point used for our sample. There was no statistically significant difference in the proportion of rapid acetylators (P = 0.23) or in race-adjusted mean metabolic ratio (P = 0.64) between cases and controls. The crude odds ratio for in situ colorectal cancer for rapid compared to slow acetylators was 2.1 (95% confidence interval, 0.6-7.4).

The association of sex; age; education; smoking; alcohol, coffee, red meat, chicken and fish, processed meat, and cruciferous vegetable intakes; color of meat and fish surfaces; weight; physical activity; gout medications; and P450IA2 activity with N-acetylbtransferase activity is shown in Table 1. The results were similar between the case and control groups in showing associations with alcohol, consumption of fish with browned surfaces, and use of gout medications (probenecid or allopurinol). In this univariate analysis, alcohol and gout medications were associated with a lower metabolic ratio, and consumption of browned fish was associated with a higher ratio. No association was also found between the metabolic ratio and use of vitamin A, vitamin E, or β-carotene supplements; use of aspirin, menopausal estrogens, acetaminophen, or nonsteroidal antiinflammatory agents; family history of colorectal cancer; or personal history of diabetes (data not shown).

**Comparison of Genotype and Phenotype.** Using the criterion that both alleles in an individual must be mutated to produce the slow phenotype, there was a 94% overall concordance between genotype and phenotype, corresponding to a sensitivity of 100% and specificity of 93.2% for the genotype in correctly classifying slow acetylators. Five individuals had a rapid acetylator phenotype despite being homozygous variants. These "misclassified" subjects were all homozygous with two different mutations (M1/M2 or M2/M3) and were either Caucasian (n = 2), Filipino (n = 2), or Japanese (n = 1). Samples for these five subjects were reanalyzed using the same protocol by an independent laboratory, which confirmed the genotypes of four of the five subjects. The genotype of one Filipino subject was reclassified from M2/M3 to WT/M3, for a new overall concordance rate of 95% between phenotype and genotype. In the subsequent analyses, this individual was classified as WT/M3.
NAT2 Genotype. The distribution for all detected NAT2 genotypes in the 90 genotyped subjects is presented in Table 2 and was consistent with the Hardy-Weinberg equilibrium (Table 2; Fig. 2). Among the covariates competing in the stepwise regression, the only three genotype categories because such a model explained 12% more of the variance in acetylation activity. Moreover, it would allow for the examination of a genetic dose-response.

Consistent with the commonly accepted notion that N-acetyltransferase is largely noninducible, few environmental factors among the many assessed in this study were found to correlate with its activity. Only use of medications for gout and consumption of browned fish were found to be correlated with caffeine metabolism. After taking genotype into account, these factors were responsible for a modest 9% of additional variation in activity. The association of N-acetyltransferase activity with P4501A2 activity probably reflects the fact that, along the metabolic pathway of caffeine, the steps involving P4501A2 take place earlier than those involving N-acetyltransferase (31). To our knowledge, this is the first time that probenecid and allopurinol, two drugs used in the treatment of gout, were found to be related to caffeine metabolism. Because these results were based on a small number of users (n = 7) and were obtained in a cross-sectional study, they need confirmation with a larger sample size and a prospective design. Nevertheless, they suggest that certain drugs may distort the caffeine phenotyping of N-acetyltransferase. An alternative interpretation of our finding is that slow acetylators may be more susceptible to gout.

We also found that an indicator of past browning of fish (color of fish surface) was directly associated with acetylation activity, after adjusting for NAT2 genotype and P4501A2 activity. Dietary factors other than alcohol (see below) have not previously been associated with N-acetyltransferase activity. Procainocinogens, such as HAAs and PAHs, are formed on the surface of protein-rich foods, such as meat and fish, when they are cooked at high temperatures. Thus, our finding is suggestive of an association between a diet rich in these procainocinogens and acetylation activity. This is in contrast with the lack of change in N-acetyltransferase activity observed by Sinha et al. (32) in subjects who consumed meat cooked at high temperatures containing high amounts of HAAs for 1 week. In their study, exposure to PAHs did not increase with the intervention diet. Thus, the possibility that N-acetyltransferase is induced by PAHs but not HAAs would be consistent with the intervention study, as well as ours. The most likely explanation, however, is that our findings have no biological basis and merely reflect a chance statistical correlation between the rapid acetylation phenotype and this particular diet in our population.

Predictors of N-Acetyltransferase Activity. The results of the stepwise regression showed that the three genotype categories considered above explained 54% of the variation in the acetylation ratio (Table 4). However, no statistically significant difference in metabolic ratio by specific allele combinations within each of the three genotype categories (Table 2). As shown in Fig. 2, there was a substantial overlap in metabolic ratio between the heterozygous group and the homozygous wild-type group. As in other populations (11, 15-17), the latter group showed considerable variation (30-fold) in acetylation activity. The association of N-acetyltransferase activity with P4501A2 activity probably reflects the fact that, along the metabolic pathway of caffeine, the steps involving P4501A2 take place earlier than those involving N-acetyltransferase (31).

Among the covariates competing in the stepwise regression, only three were selected for inclusion into a model that already contained the three genotype categories. These are modeled using two categories (homozygous mutant and heterozygous) for the analysis of N-acetyltransferase activity. An alternative interpretation of our finding is that slow acetylators may be more susceptible to gout.
Predictors of N-Acetyltransferase Activity

Potential covariates included sex; age; race; years of schooling; daily intake of red meat, chicken and fish, processed meat and cruciferous vegetables; color of meat surface; color of fish surface; cigarettes/day; alcoholic drinks/day; cans of soda/day; physical activity (METs); use of vitamin A, vitamin E, and β-carotene supplements; use of aspirin, menopausal estrogens, acetaminophen, nonsteroidal anti-inflammatory agents, and gout medications; family history of colorectal cancer; personal history of diabetes; weight; and [(17U + 17X)/137X]. Variables listed in order of entry into the stepwise regression. Genotype was "Log(AFMU/1X)."

The study suggests that using genotyping (without phentotyping) to assess the association of acetylation with cancer is unlikely to introduce a sizable misclassification in exposure or outcome. Our results are in agreement with past reports showing no association of acetylation activity with adult age (1, 17, 31), smoking (17, 31, 33), physical activity (31), and weight (17, 34), although body weight has previously been associated with the acetylation of sulphasemethazine (35), isoniazid (36), and caffeine (31). Acute alcohol ingestion has also been shown to increase acetylation activity, possibly because of the increased availability of acetate, a metabolite of ethanol (9). In our data, usual alcohol intake during the two preceding weeks was (negatively) associated with acetylation activity in the univariate analysis but not after adjusting for genotype.

We failed to find an association between cruciferous vegetable intake and acetylation activity. This is in agreement with an intervention study conducted in nine individuals by Vistisen et al. (31), in which consumption of 500 g of broccoli per day for 10 days did not affect caffeine acetylation.

Genotype, gout medication, and diet accounted for almost two-thirds of the variance in acetylation activity. A certain degree of genotypic misclassification may explain part of the remaining variance. We were able to document misclassification on genotype in our sample for four individuals who had two variant alleles but were found to have higher metabolic ratios than expected, possibly because of unrecognized molecular differences either in the gene or in the regulation of the expression of the gene, RNA, or protein. The existence of unrecognized NAT2 mutant alleles associated with the slow phenotype was not an issue, however, because the three slow alleles recently identified are known to occur at very low frequency in Caucasians and Orientals (11, 37) and because the M1, M2, and M3 mutations assessed in our study accounted for all of the slow acetylator phenotypes present in our sample. Finally, the genotype classification was unable to capture the large variation in metabolic ratio observed among individuals who were homozygous wild-type. This opens the possibility that a gradient in cancer risk exists within this genotype category, a possibility that certain unidentified allelic forms may be associated with an increased risk and carry a high risk.

Fig. 2. Distribution of the metabolic ratio (AFMU/1X) by NAT2 genotype.
bias in the risk estimates, especially when all three genotype categories are modeled and the sample size is large. Assessing certain dietary factors (i.e., brewing of meat and fish surfaces) and therapeutic drugs (i.e., gout medications) may modestly improve the precision of the risk estimates. However, as previously pointed out by Rothman et al. (17), when the rapid acetylation phenotype is the at-risk category (e.g., when studying colon cancer), it would seem judicious to phenotype because of the large variability in acetylation existing within this group and the importance of identifying a potential subgroup of individuals at very high risk.

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Predictors of N-acetyltransferase activity: should caffeine phenotyping and NAT2 genotyping be used interchangeably in epidemiological studies?

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