Polymorphism in the N-Acetyltransferase 1 (NAT1) Polyadenylation Signal: Association of NAT1*10 Allele with Higher N-Acetyltransferase Activity in Bladder and Colonic Tissue

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Laboratory of Biochemical Risk Analysis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709; Division of Molecular Epidemiology, National Center for Toxicological Research, Jefferson, Arkansas 72079; University of Arkansas Medical Sciences, Arkansas Cancer Laboratory of Biochemical Risk Analysis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709; Division of Polymorphism in the N-Acetyltransferase 1 (NAT1) Polyadenylation Signal: colorectal cancer. The aromatic amines and their metabolites, a class of carcinogen implicated in these exposures, can be N- or O-acetylated by the NAT1 and NAT2 enzymes. Acetylation may result in activation to DNA-reactive metabolites or, in some cases, detoxification. Many studies have focused on genetic variation in NAT2 and its potential as a risk factor in bladder and colorectal cancer; however, NAT1 activity is higher in bladder and colonic mucosa than NAT2, and the NAT1 enzyme also exhibits phenotypic variation among human tissue samples. We hypothesized that specific genetic variants in the polyadenylation signal of the NAT1 gene would alter tissue levels of NAT1 enzyme activity and used a PCR-based method to distinguish polymorphic NAT1 alleles in samples obtained from 45 individuals. When the NAT1 genotype was compared with the NAT1 phenotype in bladder and colon tissue samples (p-aminobenzoic acid activity), we observed a ~2-fold higher NAT1 enzyme activity in samples from individuals who inherited a variant polyadenylation signal (NAT1*10 allele). This is the first observation relating a genetic polymorphism in NAT1 to a rapid/slow NAT1 phenotype in humans.

Abstract

Exposures to carcinogens present in the diet, in cigarette smoke, or in the environment have been associated with increased risk of bladder and colorectal cancer. The aromatic amines and their metabolites, a class of carcinogen implicated in these exposures, can be N- or O-acetylated by the NAT1 and NAT2 enzymes. Acetylation may result in activation to DNA-reactive metabolites or, in some cases, detoxification. Many studies have focused on genetic variation in NAT2 and its potential as a risk factor in bladder and colorectal cancer; however, NAT1 activity is higher in bladder and colonic mucosa than NAT2, and the NAT1 enzyme also exhibits phenotypic variation among human tissue samples. We hypothesized that specific genetic variants in the polyadenylation signal of the NAT1 gene would alter tissue levels of NAT1 enzyme activity and used a PCR-based method to distinguish polymorphic NAT1 alleles in samples obtained from 45 individuals. When the NAT1 genotype was compared with the NAT1 phenotype in bladder and colon tissue samples (p-aminobenzoic acid activity), we observed a ~2-fold higher NAT1 enzyme activity in samples from individuals who inherited a variant polyadenylation signal (NAT1*10 allele). This is the first observation relating a genetic polymorphism in NAT1 to a rapid/slow NAT1 phenotype in humans.

Introduction

The human NAT13 enzyme can carry out N-acetylation of carcinogenic aromatic amines (1, 2). NAT1 also catalyzes the O-acetylation of N-hydroxylarylamines and an intramolecular N/O-acetyltransfer reaction that converts aroylhydroxamic acids to mutagenic acetoxy esters (1-3). The NAT1 enzyme is expressed in bladder epithelial tissue and in cell lines derived from bladder epithelium (4, 5). Recent in vitro studies have suggested that NAT1 may have an important role in the metabolism of some bladder carcinogens (2, 6); thus, variation in NAT1 activity among human populations might affect risk of carcinogen-induced carcinoma of the bladder as well as that of the colon.

The NAT2 enzyme has long been known to be polymorphic (reviewed in Ref. 7), and recent studies have described the molecular genetic basis for the rapid/slow acetylation phenotype (8-12). In contrast, the NAT1 enzyme has generally been considered to be monomorphic (reviewed in Ref. 13) for two reasons: (a) until very recently, only one form of the NAT1 enzyme had been isolated; and (b) human population studies of NAT1 phenotypic variation appeared to show a unimodal distribution. However, some recent studies have suggested the potential for high and/or low activity alleles of NAT1 (14, 15). These studies measured NAT1 enzyme activity in whole blood using PABA or p-aminosalicylic acid (14, 15). A preliminary report also has suggested that there are some rare NAT1 alleles that lack activity (16). In addition, Vatsis et al. (17) have demonstrated that sequence polymorphisms in the 3' untranslated region of NAT1 occur relatively frequently in human populations. However, as yet, there have been no comparisons between NAT1 phenotype and NAT1 genotype for the known alleles. Thus, it was unknown if differences in tissue levels of NAT1 among humans are related to specific sequence differences in the NAT1 structural gene. In this study, we measured the in vitro NAT1 enzyme activity of bladder and colon tissue samples using PABA (a NAT1 specific substrate) and found that higher PABA activity is associated with a specific NAT1 polyadenylation signal sequence variant (NAT1*10).

Materials and Methods

Human urinary bladder (n = 26) and colorectal (n = 19) mucosa were obtained from the U.S. Cooperative Tissue Network, the Sir Charles Gairdner Hospital (Perth, Western Australia), and the John L. McClellan Memorial Veterans Medical Center (Little Rock, AR). Information on subjects concerning gender, age, medications, tobacco use, alcohol consumption, or lifestyle was not available. Following collection, samples were immediately frozen in liquid nitrogen and stored at ~80°C until used. Homogenates of thawed tissue were prepared as described elsewhere (18) to allow preparation of both tissue cytosols and DNA. DNA was isolated from 10,000 X g pellets, using a modified phenol extraction procedure (19). The supernatants of the 100,000 X g centrifugation was used as a source of tissue cytosol. DNA (20) and protein (21) content were determined as described. In vitro acetylation of PABA to N-acetyl-PABA (a specific indicator of NAT1 activity) was measured under linear conditions (acetyl-CoA, 2 mM) essentially as described (22, 23).

Statistical analyses (Mantel-Haenszel x2, Fisher's exact test, t test of log transformed data, Mann-Whitney test, normality tests, and box-and-whisker plots) were carried out using the SigmaStat (Jandel Corp., San Rafael, CA) and Epistat software packages (Finnish Institute of Occupational Health). Probit transformations of the data were carried out by plotting the enzyme activity against the corresponding percentage under the normal probability curve, applying the method of Butler et al. (24).

For NAT1 genotype analysis by PCR, we used a mutant primer PCR-RFLP method to discriminate between three sequence variants in the NAT1 gene (25). These alleles are distinguished by changes in the 3' region of the NAT1 gene and around the putative mRNA polyadenylation signal at nucleotide 1086 ("AAATAA"; Fig. 1). The alleles identified by this method were: (a) NAT1*4, the common allele4 and presumably, the "wild type" allele that contains a "T" at nucleotide 1088 and a "C" at nucleotide 1095; (b) NAT1*10, a variant in which nucleotide 1088 = "A" and nucleotide 1095 = "A" (the change at

4 See Ref. 26 for an explanation of NAT1 allele nomenclature.
change shifts the position of the putative polyadenylation signal. NAT*4 differs from NAT*1 at two nucleotide positions (nucleotides 1088 and 1095). The nucleotide 1088 position of the 9-bp deletion cannot be determined, but it is possible that NAT*1 is a T > G (Ser > Ala) change at nucleotide 640. The sequence variants are reversion of NAT*0 (they share the nucleotide 1095 C > A change). The position of sequence ('AAT') occurring immediately upstream of nucleotide 1088 with a nucleotide 1088 results in a shift in the mRNA polyadenylation signal; (c) can be distinguished by observation of a 9-bp mobility shift of the 131-bp band NAT*4 homozygote). Digest of a NAT*10 allele results in fragments of 131, cut the PCR product into fragments of 105, 71, 45, and 26 (Fig. 2, Lane 1, and 1 unit Taq DNA polymerase were added to 100 ng of genomic DNA in a volume of 30 μl; and the PCR was heated to 94°C for 4 mm and then subjected to 35 cycles of 94°C for 30 s, 42°C for 30 s, and 72°C for 45 s. A final 72°C extension for 5 min was performed.5 Following MboII digestion and electrophoresis on a high resolution agarose gel (4% Metaphor; FMC BioProducts, Rockland, ME), genotypes containing the NAT*4 and NAT*11 alleles produced distinct band patterns (Fig. 2). The MboII digestion of the NAT*4 allele cut the PCR product into fragments of 105, 71, 45, and 26 (Fig. 2, Lane 1, NAT*4 homozygote). Digest of a NAT*10 allele results in fragments of 131, 75, 45, and 26 bp (Fig. 2, Lane 3, NAT*10 homozygote). The NAT*11 allele can be distinguished by observation of a 9-bp mobility shift of the 131-bp band to a 122-bp band (Fig. 2, Lanes 4, 5, and 6, heterozygote and homozygote). PCR fragment digestion patterns, along with the genotypes, are shown in Fig. 2. We have confirmed these genotypes using a second allele-specific PCR method for nucleotide 1088 (data not shown; method described in Ref. 25). For ~80 individuals participating in other studies, we have confirmed PCR-RFLP genotypes by direct sequencing of the polymorphic region (data not shown). More information with regard to NAT1 alleles and genotyping can be found in Vatsis et al. (26) and Bell et al. (25).

Results and Discussion

Probit plots of NAT1 activity in bladder and colon tissue samples are shown in Fig. 3 and demonstrate that there is a relatively large sample-to-sample variation within each tissue type. We observed an ~8-fold variation among bladder tissue samples and a 53-fold variation among colon tissue samples. Average NAT1 activity in colon tissue was about 8-fold higher than in bladder tissue (Table 1). The sample-to-sample variation observed in Figs. 3 and 4 (particularly the very low activity for some samples) may be caused, in part, by degradation of the NAT1 enzyme. Instability of NAT1 has been observed in other studies (1, 2); however, the relationship between activity and genotype strongly suggests that there might be a molecular genetic determinant to the variation in NAT1 activity.

In both tissue types, NAT*10 alleles were significantly overrepresented among individuals with high NAT1 activity relative to low NAT1 activity. Within the bladder tissue samples (Fig. 3A), the frequency of NAT*10 alleles (7 of 12) among the 12 individuals with “high” NAT1 activity (>2.2 nmol/min/mg protein) was significantly greater relative to the frequency of NAT*10 alleles (1 of 13) among the 13 lowest activity individuals (Mantel-Haenszel χ² = 7.1; P = 0.008). One individual was heterozygous for the NAT*11 allele (9-nucleotide deletion and nucleotide 640 Ser > Ala) and was excluded from the statistical analysis. In colon tissue (Fig. 3B), the frequency of NAT*10 alleles among the 8 individuals with “high” NAT1 activity (7 of 8; >20 nmol/min/mg protein) was significantly higher relative to the frequency of NAT*10 alleles among the 11 lowest activity individuals (4 of 11; Mantel-Haenszel χ² = 4.7; P = 0.037). Among all samples, only one individual was homozygous for the NAT*10 allele (in the colon tissue samples). Thus, in sample sets from both tissues, there were significant associations between the NAT*10 allele and higher NAT1 activity. Although the high/low grouping used in this analysis is somewhat arbitrary and the size of these sample sets is small, the differences we observed are highly suggestive of a phenotypic effect for the NAT*10 allele.

In Table 1, the mean NAT1 activities in bladder and colon tissue are shown for samples from individuals with the NAT*10 allele compared with those homozygous for NAT*4, and these data are displayed graphically in box-and-whisker plots. Bladder tissue samples from subjects with the NAT*10 allele (all heterozygotes) had a mean NAT1 activity of 4.6 ± 2.6 nmol/min/mg protein (n = 8); while those homozygous for NAT*4 (n = 17) had mean NAT1 activity of 2.3 ± 1.6 pmol/min/mg protein. This 2-fold difference was significant (t-test of log transformed data, t = −2.74, df = 23, P = 0.012). Colon mucosa samples from subjects with the NAT*10 allele also had higher mean NAT1 activity (28.6 ± 14.1 nmol/min/mg protein versus 16.6 ± 13.1 nmol/min/mg protein), but this difference was of borderline significance (t-test on log transformed data, t = −2.04, df = 17, P = 0.057). The limited number of samples in both bladder and colon tissue data sets and the high degree of variation within genotype groups indicate that these results should be interpreted cautiously. As can be seen in the plots in Figs. 3 and 4, there are a few samples with NAT*10 alleles that have low activity and a few NAT*4 alleles among the high activity samples.

The degree of variation within groups and across tissue types suggests that there are other determinants of bladder and colon NAT1 activity in addition to the effect of the altered polyadenylation signal that is present in the NAT*10 allele. Deacetylase may have an important role in benzidine metabolism in human liver (27) and expression of deacetylase varies among humans (28). However, it is unlikely that there is enough deacetylase activity in cytosol preparations from bladder and colon tissues to impact the levels of acetylated PABA. Transcriptional effects, particularly tissue-specific factors, may also be important in determining variability in NAT1 activity among individuals and between tissue types.

The recognition of the polyadenylation signal by RNA polymerase during transcription initiates cleavage of the growing mRNA chain followed by the subsequent addition of the poly(A) sequence to the transcript. Polyadenylation state [length of the poly(A) tail] can influence the degradation or stability of a mRNA molecule (29), and there are numerous examples where turnover of protein and mRNA are coordinately regulated (29–31). The putative polyadenylation signal of the NAT1 gene is altered in the NAT*10 allele (T>A change at nucleotide 1088; Fig. 1). This change creates a new consensus polyadenylation signal that is shifted three nucleotides in the 5’ direction and is flanked on the 3’ side by an adenine triplet (AATAAA AAA). We speculate that the new polyadenylation signal present in
the NAT1*10 allele produces a more stable mRNA, which in turn impacts NAT1 enzyme levels. Alternative mechanisms may also be possible. For example, the NAT1*10 allele might be in linkage disequilibrium with other mutations in the coding or regulatory region of NAT1. However, preliminary sequencing studies of the coding regions of the NAT1*10 allele do not support this possibility (18). Moreover, the possibility of linkage between NAT1*10 and upstream regulatory mutations has not been investigated. We have initiated studies that may reveal the relationship between specific NAT1 alleles and mRNA levels, mRNA stability, polyadenylation status, and enzyme activity. It is interesting that the association between NAT1*10 and high PABA activity appeared to be stronger in urinary bladder than in colon tissue. This is consistent with our epidemiological studies comparing NAT1*10 genotypes and risk of colorectal and bladder cancer (25, 32). We reported recently on preliminary evidence showing that the NAT1*10 allele was associated with a 2.9- to 26-fold increased risk among bladder cancer patients who were also smokers (32), whereas NAT1*10 was associated with only a 1.9-fold risk among colorectal cancer patients (25). Considering that mean NAT1 activity was about 8-fold lower in bladder relative to colon (Table 1), the NAT1*10 allele (which presumably effects mRNA stability) may have more impact in tissues where NAT1 mRNA levels are low. Furthermore, we also have data suggesting that NAT1 activity in bladder is correlated with DNA adduct levels in this tissue (18).

This work is the first demonstration that a DNA sequence polymorphism in the 3' untranslated region of the NAT1 gene is associated with differences in NAT1 enzyme activity in human tissues. Tissue samples from individuals carrying the NAT1*10 allele had about 2-fold more NAT1 enzyme activity than those homozygous for the NAT1*4. However, preliminary sequencing studies of the coding regions of the NAT1*10 allele do not support this possibility (18). Moreover, the possibility of linkage between NAT1*10 and upstream regulatory mutations has not been investigated. We have initiated studies that may reveal the relationship between specific NAT1 alleles and mRNA levels, mRNA stability, polyadenylation status, and enzyme activity.

Table 1 Mean NAT1 enzyme activity among NAT1 genotypes as measured by PABA acetylation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bladder Activity</th>
<th>n</th>
<th>Colon Activity</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT1 (all genotypes)</td>
<td>2.9 ± 2.3</td>
<td>26</td>
<td>23.6 ± 14.6</td>
<td>19</td>
</tr>
<tr>
<td>NAT1*10</td>
<td>4.6 ± 1.6</td>
<td>8</td>
<td>28.6 ± 14.1d</td>
<td>11</td>
</tr>
<tr>
<td>NAT1*4</td>
<td>2.3 ± 2.6</td>
<td>17</td>
<td>16.6 ± 13.1</td>
<td>8</td>
</tr>
<tr>
<td>NAT1*11</td>
<td>3.3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Values represent mean ± SD pmoles N-acetyl-PABA/mg protein/min. b Heterozygous NAT1*10/NAT1*4 genotypes (except one colon sample was a NAT1*10/NAT1*11 homozygote). c t test on log transformed data, t = -2.74, P = 0.012. d t test on log transformed data, t = -2.04, P = 0.057.
NAT1 genotype and phenotype

more common NAT1*4 allele. Given that the NAT1 enzyme can mediate activation and detoxification pathways for numerous carcinogens, this gene may have an important role in modulating cancer risk. If these preliminary observations can be confirmed by further studies, then the NAT1 genotype will need to be considered as a genetic risk factor in cancer epidemiology.

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References

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