

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

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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 *NAT1*³ enzyme can carry out *N*-acetylation of carcinogenic aromatic amines (1, 2). *NAT1* also catalyzes the *O*-acetylation of *N*-hydroxyarylamines and an intramolecular *N,O*-acetyltransfer reaction that converts aryhydroxamic 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 life style 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 $10,000 \times g$ pellets, using a modified phenol extraction procedure (19). The supernatants of the $100,000 \times g$ 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 χ^2 , 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, Madera, CA) and EpiTest 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 ("AATAAAA"; Fig. 1). The alleles identified by this method were: (a) *NAT1*4*, the most 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

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³ The abbreviations used are: *NAT1* and *NAT2*, *N*-acetyltransferase 1 and 2, PABA, *p*-aminobenzoic acid.

⁴ See Ref. 26 for an explanation of *NAT1* allele nomenclature.

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      1062                1088      1095
      |                |          |
NAT1*4  TCA AAT AAT AAT AAT AAT AAT AAT AATAAA TGT CT
NAT1*3  TCA AAT AAT AAT AAT AAT AAT AAT AAT AAA TGT AT
NAT1*10 TCA AAT AAT AAT AAT AAT AAT AAT AAA AAA TGT AT
NAT1*11 TCA AAT AAT AAT AAT AAT . . . . . AAA TGT AT

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Fig. 1. Sequence of *NAT1* alleles in the 3' untranslated region. *NAT1*10* differs from *NAT1*4* at two nucleotide positions (nucleotides 1088 and 1095). The nucleotide 1088 change shifts the position of the putative polyadenylation signal. *NAT1*11* differs from *NAT1*4* by a 9-bp deletion in the 24-bp trinucleotide repeat sequence (nucleotides 1065–1088), a C > A change at nucleotide 1095, and a T > G at nucleotide 640. The exact position of the 9-bp deletion cannot be determined, but it is possible that *NAT1*11* is a reversion of *NAT1*10* (they share the nucleotide 1095 C > A change). The position of PCR primer N1536R is shown; it contains a mismatch at nucleotide 1098, which generates a partial *MboII* site.

nucleotide 1088 results in a shift in the mRNA polyadenylation signal); (c) *NAT1*11*, a variant that contains a 9-bp deletion in the trinucleotide repeat sequence ("AAT") occurring immediately upstream of nucleotide 1088 with a T > G (Ser > Ala) change at nucleotide 640. The sequence variants are illustrated in Fig. 1.

To differentiate the *NAT1*10* allele from the *NAT1*4* allele, the *NAT1* reverse primer contained a mismatched base (T > G) at nucleotide 1098. Subsequent PCR amplification of the *NAT1*4* allele with this primer resulted in a PCR product containing a new *MboII* restriction site. PCR conditions for the *NAT1* PCR-RFLP method were the same as in (25). Briefly, *NAT1*-specific primers N1306F (5' cta ttt aga ata agg agt aa) and N1536R (5' aca ggc cat ctt tag aa) (15 pmol each), 2.0 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate, and 1 unit Taq DNA polymerase were added to 100 ng of genomic DNA in a volume of 30 μ l; and the PCR was heated to 94°C for 4 min 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 *NAT1*4* and *NAT1*11* alleles produced distinct band patterns (Fig. 2). The *MboII* digestion of the *NAT1*4* allele cut the PCR product into fragments of 105, 71, 45, and 26 (Fig. 2, Lane 1, *NAT1*4* homozygote). Digest of a *NAT1*10* allele results in fragments of 131, 75, 45, and 26 bp (Fig. 2, Lane 3, *NAT1*10* homozygote). The *NAT1*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, *NAT1*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 *NAT1*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 *NAT1*10* alleles (1 of 13) among the 13 lowest activity individuals (Mantel-Haenszel $\chi^2 = 7.1$; $P = 0.008$). One individual was heterozygous for the *NAT1*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 *NAT1*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 *NAT1*10* alleles among the 11 lowest activity individuals (4 of 11; Mantel-Haenszel $\chi^2 = 4.7$; $P = 0.037$). Among all samples, only one individual was homozygous for the *NAT1*10* allele (in the colon tissue samples). Thus, in sample sets from both tissues, there were significant associations between the *NAT1*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 *NAT1*10* allele.

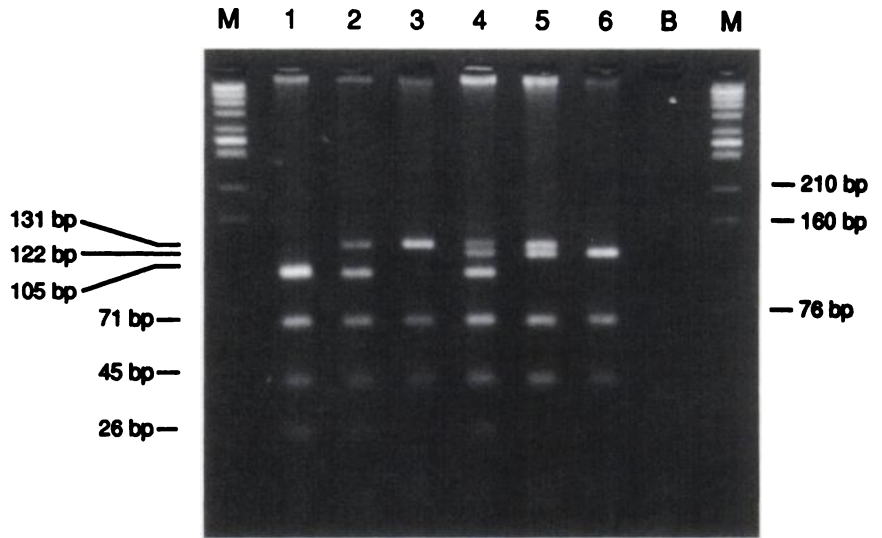
In Table 1, the mean NAT1 activities in bladder and colon tissue are shown for samples from individuals with the *NAT1*10* allele compared with those homozygous for *NAT1*4*, and these data are displayed graphically in box-and-whisker plots. Bladder tissue samples from subjects with the *NAT1*10* allele (all heterozygotes) had a mean NAT1 activity of 4.6 ± 2.6 nmol/min/mg protein ($n = 8$); while those homozygous for *NAT1*4* ($n = 17$) had mean NAT1 activity of 2.3 ± 1.6 nmol/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 *NAT1*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 *NAT1*10* alleles that have low activity and a few *NAT1*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 *NAT1*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 *NAT1*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

⁵ Recently, we have also used a new forward primer N1208F (5' gac tct gag tga ggt aga aat a; annealing at 55°C), which has increased specificity for *NAT1*.

Fig. 2. Gel electrophoresis PCR products from the possible NAT1 genotypes. M, marker ($\phi\chi 174$ HindIII); Lane 1, NAT1*4/NAT1*4; Lane 2, NAT1*4/NAT1*10; Lane 3, NAT1*10/NAT1*10; Lane 4, NAT1*4/NAT1*11; Lane 5, NAT1*10/NAT1*11; Lane 6, NAT1*11/NAT1*11; B, blank, no DNA negative control; M, marker ($\phi\chi 174$ HindIII). In Lane 4, the 131-bp band is caused by poor MboII digestion of the NAT1*4/NAT1*11 heteroduplex PCR product.



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

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

	Bladder		Colon	
	NAT1 activity ^a	n	NAT1 activity	n
NAT1 (all genotypes)	2.9 ± 2.3	26	23.6 ± 14.6	19
NAT1*10 ^b	4.6 ± 1.6 ^c	8	28.6 ± 14.1 ^d	11
NAT1*4	2.3 ± 2.6	17	16.6 ± 13.1	8
NAT1*11	3.3	1		

^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*10 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.

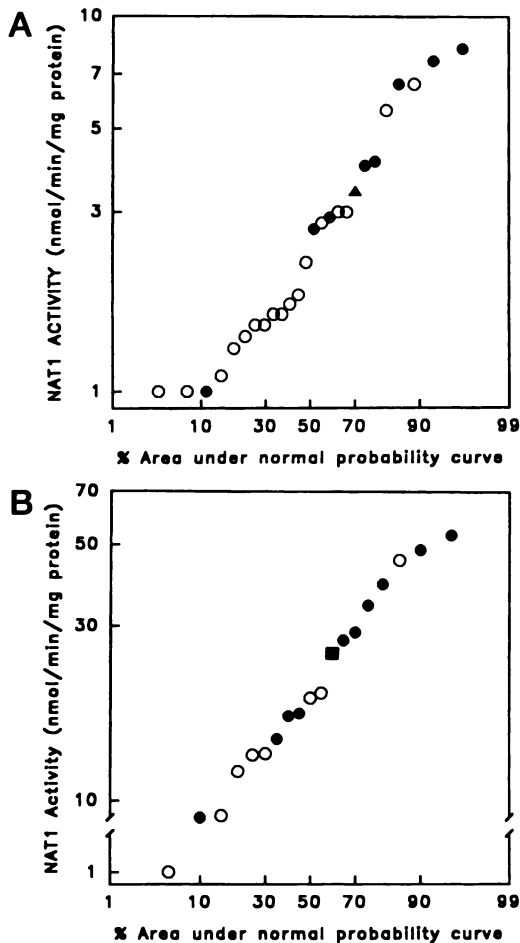


Fig. 3. Probit plots of NAT1 activity in bladder (A) and colon (B) tissue as measured by N-acetyl-PABA. ○, NAT1*4/NAT1*4 homozygotes. ●, the subjects who were heterozygous for the NAT1*10 allele. ■, the single NAT1*10/NAT1*10 homozygote. ▲, a NAT1*4/NAT1*11 heterozygote. NAT1*10 alleles were overrepresented among individuals with high NAT1 activity.

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),⁶ 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

⁶ J. A. Taylor, D. Umbach, E. Stephens, T. Castranio, D. Paulson, C. Robertson, J. L. Mohler, and D. A. Bell. Role of N-acetylation polymorphism at NAT1 and NAT2 in smoking-associated bladder cancer, manuscript in preparation.

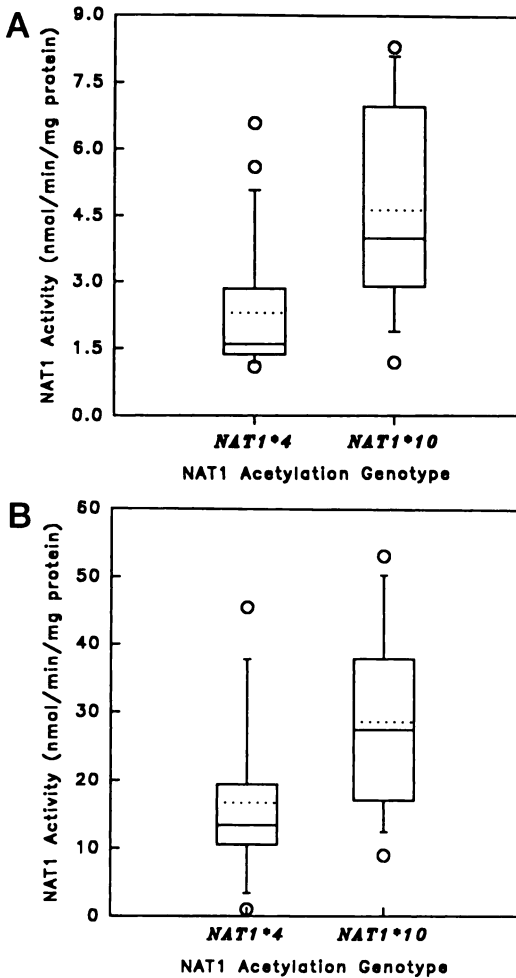


Fig. 4. A. bladder tissue cytosol NAT1 activity for NAT1*4 genotypes versus NAT1*10 genotypes. B. colon mucosa tissue cytosol NAT1 activity for NAT1*4 genotypes versus NAT1*10 genotypes. Box-and-whisker plots of bladder and colon tissue NAT1 activity. . . . , mean value; —, median; the box, 25th and 75th percentile; capped line, 10th and 90th percentile; O, data values beyond the 10th and 90th percentile.

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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