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Correlation Between Genotype and Phenotype of the Human Arylamine *N*-Acetyltransferase Type 1 (NAT1)

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ABSTRACT. Arylamine *N*-acetyltransferase 1 (NAT1) conjugates several aromatic amines and their *N*-hydroxylated metabolites by *N*- or *O*-acetylation. NAT1 genotype and phenotype is known to be variable in human populations. In this study, we set out to measure the functional relevance of the frequent NAT1 gene variants for the activity in human red blood cells. Healthy German volunteers (N = 314) were genotyped for *NAT1* alleles **3*, **4*, **10*, **11*, **14*, and **15* using polymerase chain reactions and restriction fragment length pattern analysis, and NAT1 enzyme kinetic parameters were measured in a subset of 105 individuals using *p*-aminobenzoic acid as specific substrate. There was no functional difference between *NAT1* alleles **4* and **10*. In particular, there was no trend of increasing activity from *NAT1*4/*4* to **4/*10* and **10/*10*. Carriers of the *NAT1*11* and **14* alleles had a statistically significant lower enzyme activity compared with carriers of the **3*, **4*, or **10* alleles. Compared with the wild-type genotype *NAT1*4/*4*, activity of the *NAT1*11/*11*, *NAT1*11/*10*, and *NAT1*11/*4* genotypes was reduced by 20.7%, 35.7%, and 31.5%, respectively. Activity of the *NAT1*10/*14* and *NAT1*4/*14* genotypes was reduced by 49.8% and 55.6%, respectively. The difference in NAT1 activity between the **4/*11* and **4/*14* genotypes was also significant ($P < 0.01$). The carrier of the *NAT1*15/*15* genotype had no detectable enzyme activity. In conclusion, functional consequences of *NAT1* mutations were tested in a large population. Activity in carriers of *NAT1* alleles **3*, **4*, and **10* did not differ, alleles *NAT1*11* and **14* appeared to be low activity alleles, and allele *NAT1*15* had no activity. BIOCHEM PHARMACOL **58**;11:1759–1764, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. pharmacogenetics; polymorphism; arylamine *N*-acetyltransferase; NAT1; acetylation; phenotype

In humans, there are two arylamine *N*-acetyltransferase enzymes, NAT1§ and NAT2, which have differential although partially overlapping substrate specificity. Isoniazide, sulfamethazine, dapsone, 2-aminofluorene, and 4-aminobiphenyl are predominantly acetylated by NAT2, whereas PAS, PABA, and sulfanilamide are acetylated by NAT1 [1]. The NAT2 polymorphism is functionally and genetically well characterized [2, 3], with genetic variants recently being found in *NAT1* as well. In total, 24 different human *NAT1* alleles have been identified [2, 4–8], but some are very rare. Those with high or intermediate frequency in Caucasian populations are summarized in Table 1. There are only few data on the relation between the *NAT1* genotype and enzyme activity [4, 6, 9–12]. A study with wild-type and mutant NAT1 proteins expressed in *Escherichia coli* showed a lower enzyme activity of NAT1 14 and defect activity of NAT1 15 [11]. When expressed in *E. coli*, NAT1 11 had lower activity compared with the wild-type NAT1 4, but the difference was statistically not significant [11]. Comparative NAT1 genotype–phenotype studies in human samples showed a lower activity in *NAT1*4/*14* individuals compared to the homozygous wild-type [4, 6]. The activity of one *NAT1*14/*15* individual was extremely low. No data are yet available on the enzyme activity of homozygous *NAT1*15* subjects, and there are few data in the literature concerning the functional relevance of the relatively frequent *NAT1*11* allele. A lower NAT1 activity was observed in erythrocyte lysates of *NAT1*4/*11* individuals [4, 10]. Whether the *NAT1*10* allele possesses any functional difference compared with the wild-type allele is currently a matter of debate [4, 9, 11, 13]. *Para*-Aminobenzoic acid (PABA) was found to be a substrate which is highly specific for NAT1 [14]. NAT1 protein expression was detected in hepatocytes, mononuclear leukocytes, erythrocytes, placenta, and bladder and colorectal epithelial cells [9, 14–18].

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§ *Abbreviations:* Hb, hemoglobin; NAT, arylamine *N*-acetyltransferase; PABA *para*-aminobenzoic acid; PAS, *para*-aminosalicylic acid; and NAcPABA, *N*-acetyl-*para*-acetamidobenzoic acid.

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TABLE 1. Nucleotide and protein changes in frequent *NAT1* allele variants found in the German population

Allele	Mutation	Amino acid changes
*NAT1*4*	none (wild-type)	none
*NAT1*3*	$C \rightarrow A_{1095}$	none
*NAT1*10*	$T \rightarrow A_{1088}$; $C \rightarrow A_{1095}$	none
*NAT1*11*	$C \rightarrow T_{-344}$; $A \rightarrow T_{-40}$; $G \rightarrow A_{445}$, $G \rightarrow A_{459}$; $T \rightarrow G_{640}$; Δ 9 between 1065–1090; $C \rightarrow A_{1095}$;	$Val \rightarrow Ile_{149}$; $Ser \rightarrow Ala_{214}$
*NAT1*14*	$G \rightarrow A_{560}$; $T \rightarrow A_{1088}$; $C \rightarrow A_{1095}$	$Arg \rightarrow Gln_{187}$
*NAT1*15*	$C \rightarrow T_{559}$	$Arg \rightarrow Stop_{187}$

The aim of this study was to further characterize the functional relevance of *NAT1* polymorphism in humans. Therefore, we measured the NAT1 enzyme kinetic parameters K_m and V_{max} in the erythrocytes from 105 healthy male volunteers with the substrate PABA.

MATERIALS AND METHODS

Volunteers

Venous blood samples were taken from 314 non-smoking healthy German male volunteers (19–48 years of age) after written informed consent. For the phenotyping assay, whole blood samples were drawn into 10-mL vials with ethylene diamine tetraacetic acid as anticoagulant and stored at −80°. Representative subsets of the frequent genotypes **4/*4* and **4/*10* were analyzed. All samples from all other genotypes were included, except a few from which not enough blood was available. The person performing the phenotyping measurements was blinded for the genotypes.

Reagents

The AmpliTaq was purchased from Perkin Elmer and restriction enzymes were from New England Biolabs. *Para*-Aminobenzoic acid (sodium salt, 99%), *N*-acetyl-*para*-acetamidobenzoic acid (NAcPABA), acetamidophenol, acetyl CoA (sodium salt, 95%), carnitine acetyltransferase, acetyl-L-carnitine (hydrochloride, 99%), dithiothreitol, triethanolamine (free base), and all other chemicals for the phenotyping assay were purchased from Sigma. Solvents for the HPLC were from Baker. Hemoglobin was quantified by the colorimetric method with Drabkin's reagent using the Merckotest™ kit (Merck AG).

Genotyping Assay

Leukocyte DNA was isolated from 10-mL venous blood samples with standard phenol-chloroform extraction. *NAT1* alleles **3*, **4*, **10*, **11*, **14*, and **15* were identified as described earlier [9, 19]. The nomenclature of Vatsis *et al.* [20] for *NAT1* and *NAT2* alleles was used.

Phenotyping Assay

After thawing for 60 min at room temperature, the hemolysate was centrifuged (4°, 20,000 *g*, 30 min), and the supernatant was used for the assay. The phenotyping assay was performed as described earlier [15], with modifications as follows: 2-mL Eppendorf vials contained 375 μL 0.1 M triethanolamine–HCl buffer (pH 7.5) with 4.5 mM dithiothreitol and 4.5 mM EDTA, 25 μL of hemolysate, 200 μL of an acetyl CoA regenerating system (20 mM L-acetylcarnitine and 2 U/mL carnitine acetyltransferase, dissolved in the same buffer), and 200 μL of acetyl CoA (450 μM in water). After a 5-min preincubation at 37°, the reaction was started by addition of the substrate solution tempered at 37° (10, 20, 50, and 100 μM PABA, respectively, in water). After thorough mixing, the incubation was performed at 37° in an Eppendorf™ thermomixer for 10 min. Two measurements were performed for each concentration. Two substrate-free samples from each individual (water instead of PABA) were analyzed under the same conditions. The incubation was stopped by addition of 333 μL of 15% perchloric acid. After centrifugation (15,000 *g*, 10 min, room temperature), 900 μL of the supernatant was mixed with 100 μL of 500 μM acetamidophenol serving as an internal standard and 50 μL of this mixture was injected into the HPLC system using an autosampler (AS 2000 A from Merck). Formation of NAcPABA was quantified by an HPLC method [21] with an octadecylsilane-coated silica column (250 × 4.6 mm, 5-μm particle size, Hypersil™, Shandon). The mobile phase (1 mL/min) contained 15% acetonitrile v/v in 0.1 M citrate buffer, pH 3.5, with detection at 270 nm using an L-4500 diode array detector (Merck). Under these conditions, retention times were: internal standard 4.6 min, PABA 5.6 min, and NAcPABA 7.3 min. Peak heights were used for quantification. Calibration was performed each day. Limit of detection was 1.4 μM (5.6 pmol/min/μL) for PABA and 0.57 μM (2.3 pmol/min/μL) for NAcPABA.

To record the precision of the complete activity measurements, hemolysate of an *NAT1*4/10* individual was stored in 100-μL portions at −80°. The intra-day precision was determined by measurement of a series of samples with 50 μL hemolysate at day one. The intra-day coefficient of variation was 4.7% (mean 33.3 pmol NAcPABA/min/μL hemolysate, N = 12) at a substrate concentration of 50 μM

TABLE 2A. *NAT1* genotypes in an unselected group of 314 healthy German subjects

NAT1 genotype	N	Frequency %	95% confidence limits
**3/*3*	3	0.95	0.02–3.65
**3/*4*	10	3.19	0.92–7.01
**3/*10*	3	0.95	0.02–3.65
**4/*4*	163	51.91	43.39–59.90
**4/*10*	87	27.70	20.54–35.34
**10/*10*	13	4.14	1.48–8.31
**4/*11*	12	3.82	1.29–7.88
**10/*11*	5	1.59	0.19–4.68
**11/*11*	2	64.00	0–3.10
**4/*14*	9	2.87	0.75–6.56
**10/*14*	5	1.59	0.19–4.68
**4/*15*	1	0.32	0–2.49
**15/*15*	1	0.32	0–2.49

TABLE 2B. *NAT1* alleles in an unselected group of 314 healthy German subjects

NAT1 allele	N	Frequency (%)	95% confidence limits
**3*	19	3.03	1.47–5.25
**4*	445	70.86	65.73–75.45
**10*	126	20.06	15.95–24.52
**11*	21	3.34	1.69–5.65
**14*	14	2.23	0.93–4.22
**15*	3	0.48	0.02–1.71

PABA. To evaluate inter-day precision, two samples with 25 μL hemolysate of this individual were incubated with 100 μM PABA each day the assay was performed. The inter-day coefficient of variation was 9.1% (mean 24.8 pmol NAcPABA/min per μL hemolysate, N = 11).

In a former study it was shown that the intra-individual variation of NAT1 activity is consistent over time [12]. In this study on inter-individual differences, we also measured the intra-individual variation over time. Accordingly, we tested the NAT1 activity with 25 μL hemolysate of the same **4/*10* individual at several days over a period of 4 months. The intra-individual coefficient of variation was 11.9% (mean 21.9 pmol NAcPABA/min/μL, N = 7) with 100 μM PABA. The coefficient of variation of the V_{max} values was 11.7% (mean 28.2 pmol NAcPABA/min/μL). K_m and V_{max} values were estimated by non-linear regression analysis using the Origin 4.1™ program. Data were fitted to the Michaelis–Menten equation $v = (V_{max} \cdot [S]/(K_m + [S])$. Because the activity in various genotypes did not follow a normal distribution, data were analyzed non-parametrically with minimum/maximum values and medians. The Mann–Whitney U-Test was performed for statistical analysis using the SYSTAT 7.0™ program (SPSS Inc.).

RESULTS

Table 1 shows the *NAT1* alleles which were tested in our population. The frequencies of the *NAT1* genotypes and alleles detected in our population are given in Table 2a and b. The genotype frequencies (Table 2a) were not significantly different from the predictions based on the allele frequencies (Table 2b) according to the Hardy–Weinberg theorem. The allele frequencies were also not significantly different (tested by Fisher's exact test) from frequencies in other large Caucasian populations tested [11, 19]. Lin *et al.* [8] described further amino acid polymorphism in NAT1, but these were not found in the German population. Figure 1 illustrates the extent to which the phenotypical variability is explained by the tested *NAT1* genotypes.

A summary of the enzyme kinetic constants dependent on the *NAT1* genotypes is given in Table 3. All statistical comparisons performed here were based on the reaction velocities adjusted to hemoglobin, but almost identical conclusions could be drawn from the data adjusted to volume of hemolysate; both data are presented in Table 3. An explorative analysis of all observed differences in maximum reaction velocity is given in Table 4. There were no differences between genotypes with the *NAT1* allele **10* and wild-type allele **4* with respect to the enzyme activity of NAT1. In particular, there was no trend of increasing activity from *NAT1*4/*4* to **4/*10* and **10/*10* as illustrated in Fig. 1. This fact is further illustrated in Fig. 2, which shows the concentration-dependent PABA acetylation rate for the **4/*4*, **4/*10*, and **10/*10* genotypes. In addition, when the Mann-Whitney U-test was employed, no significant differences between the V_{max} data of these 3 groups were found (Table 4). We could not detect any significant differences between the *NAT1*4/*10* or *NAT1*10/*10* genotypes and individuals with the *NAT1*4/*3* allele, as illustrated in Table 4. Carriers of the

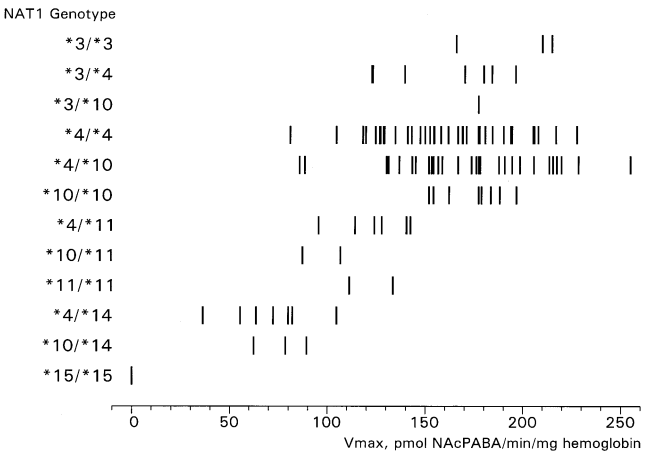

FIG. 1. Maximum *N*-acetylation velocity (V_{max}) stratified for the different genotypes of *NAT1*. The median V_{max} values of the 12 genotypes are given in Table 3 and a statistical analysis is provided in Table 4. No acetylated metabolite was formed in the sample with genotype *NAT1*15/*15*, and activity was decreased in carriers of the *NAT1*11* and *NAT1*14* alleles.

TABLE 3. Enzyme kinetic characterization of PABA *N*-acetylation in 105 subjects dependent on the *NAT1* genotype

Genotype	N	V_{max} [pmol NAcPABA/min/μL hemolysate]	V_{max} [pmol NAcPABA/min/mg Hb]	K_m [μmol/L]
**3/*3*	3	30.9 (28–33)	210.2 (166–215)	20.1 (19–46)
**3/*4*	7	25.2 (20–34)	170.3 (123–197)	18.0 (16–22)
**3/*10*	1	27	177	18
**4/*4*	37	24.1 (12–34)	158.1 (81–228)	20.6 (8–50)
**4/*10*	28	27.6 (13–34)	175.0 (86–255)	22.6 (9–48)
**10/*10*	8	25.1 (23–29)	178.1 (152–197)	21.3 (17–34)
**4/*11*	6	16.5 (15–22)	126.0 (96–143)	20.0 (13–26)
**10/*11*	2	15.5 (14–17)	97.1 (87–107)	32.1 (29–35)
**11/*11*	2	19.1 (18–20)	122.5 (111–134)	12.9 (12–14)
**4/*14*	7	10.7 (7–15)	72.3 (36–105)	25.9 (13–49)
**10/*14*	3	12.1 (9–15)	78.6 (62–89)	20.8 (17–49)
**15/*15*	1	0	0	not measurable

Median values and range of V_{max} and K_m data are presented.

homozygous *NAT1*3/*3* genotype had activities in the upper range (Fig. 1). There was a marginally significant difference between *NAT1*3/*3* and *NAT1*4/*4* ($P = 0.08$), but this value was obtained without adjustment for multiple testing and there was an increase in V_{max} from *NAT1*4/*4* to *NAT1*3/*4* and *NAT1*3/*3*. *NAT1 allele *11* includes a valine to isoleucine exchange at position 149 and a serine to alanine exchange at position 214, as well as several nucleotide polymorphisms with putative effects on mRNA stability (Table 1). Two homozygous carriers of this allele had lower activities compared with **3/*3*, **4/*4*, **4/*10*, and **10/*10*. Two heterozygous carriers of the *NAT1*11/*10* genotype and the subjects with the *NAT1*4/*11* genotype had lower activities compared with **3/*3*, **4/*3*, **4/*4*, **4/*10*, and **10/*10* (Tables 3 and 4). By comparing the medians of the V_{max} values, activity of the *NAT1*11/*11*, *NAT1*11/*10*, and *NAT1*11/*4* genotypes was reduced by 20.7%, 35.7%, and 31.5%, respectively, compared with the homozygous wild-type allele. *NAT1*14* allele has an arginine to glutamine amino acid exchange at position 187. The carriers of one *NAT1*14* allele had a significantly lower activity compared with carriers of the homozygous wild-type genotype or carriers of the *NAT1*4/*10*, **10/*10*, **3/*4*, and **3/*3*, and with the **4/*11* genotypes as well. By comparing the medians of the V_{max} values, the activity of the *NAT1*14/*10* and *NAT1*14/*4* genotypes was reduced by 49.8% and 55.6%, respectively, in comparison with the wild-type. This finding was highly significant even when taking adjustment for multiple testing into account. There was one homozygous carrier of the *NAT1*15* allele. In this subject, there was no PABA *N*-acetylation measurable. In all samples, the frequent genotypes of the second arylamine *N*-acetyltransferase known in humans, namely *NAT2*, were also measured. There was, however, no influence of the *NAT2* genotype on PABA *N*-acetylation measured in these 105 samples (details not shown). The carrier of the rare genotype *NAT1*15/*15* was genetically slow with respect to NAT2.

In conclusion, allele *NAT1*15* is associated with a complete lack of acetylation activity, *NAT1*14* had the second lowest activity, and *NAT1*11* had a slightly lower activity compared with **3*, **4*, and **10*.

DISCUSSION

In our study, there was no significant difference between the activity of the **4/*4*, **4/*10*, and **10/*10* individuals using PABA as substrate. Since *NAT1*10* does not code for any amino acid exchange, this finding should be similarly

TABLE 4. Comparison of the phenotypical differences in PABA *N*-acetylation dependent on the *NAT1* genotype

Genotype	**3/*3*	**3/*4*	**4/*4*	**4/*10*	**10/*10*	**4/*11*	**10/*11*	**11/*11*	**4/*14*
**3/*3*	—	—	—	—	—	—	—	—	—
**3/*4*	NS	—	—	—	—	—	—	—	—
**4/*4*	0.08	NS	—	—	—	—	—	—	—
**4/*10*	NS	NS	NS	—	—	—	—	—	—
**10/*10*	NS	NS	NS	NS	—	—	—	—	—
**4/*11*	0.02	NS	0.01	<0.01	<0.01	—	—	—	—
**10/*11*	0.08	0.04	0.03	0.04	0.04	0.10	—	—	—
**11/*11*	0.08	NS	0.1	0.07	<0.01	NS	NS	—	—
**4/*14*	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	0.08	0.04	—
**10/*14*	0.05	0.02	<0.01	0.01	0.01	0.02	NS	NS	NS

V_{max} values adjusted for hemoglobin were used for this analysis. Significance of activity differences was tested by the Mann-Whitney U-test. $P > 0.10$.

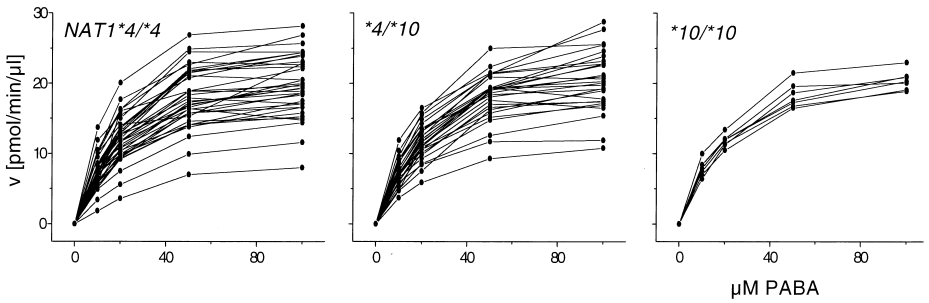

FIG. 2. Concentration-dependent reaction rates for the homozygous wild-type (N = 37), heterozygous carriers of *NAT1*4/*10* (N = 28), and homozygous carriers of the **10* allele (N = 8).

valid for all other substrates of NAT1, in contrast to the amino acid polymorphisms which may have differential impact with different substrates. The results for *NAT1*10* described in earlier studies are conflicting. Badawi *et al.* [13] measured NAT1 activity using PABA as substrate in the cytosol of urinary bladder mucosa cells from a total of 26 subjects. The activities were between 1.0 and 8.2 nmol NAcPABA/min/mg protein. When the activities of their seventeen **4/*4* individuals and eight **4/*10* individuals were compared, the latter group had a significant higher enzyme activity ($P = 0.026$). Similar differences were found by analysis of NAT1 activity in colorectal tissue [9]. In that study, activities ranged between 1.0 and about 50 nmol NAcPABA/min per mg protein and were significantly higher in the eleven individuals with **4/*10* compared with eight **4/*4* individuals. It was hypothesized that the mutations resulting in a longer polyadenylation sequence in *NAT1* allele **10* produce a more stable mRNA. Compared with our data, the power of the studies of Bell *et al.* [9] and Badawi *et al.* [13] is lessened because of a lower sample size and an obviously larger scatter in the bladder and colon tissue measurements, which may be explained by a variable proportion of epithelial cells in the different biopsies compared with the relatively homogenous blood samples. However, it cannot be ruled out that *NAT1*10* mutations have different effects in different tissues because of tissue-dependent differences in transcription factors or RNA-degrading enzymes. For erythrocytes, Payton and Sim [4] recently published data similar to those presented herein, observing no significant difference in the NAT1 activities of *NAT1*4/*4* and *NAT1*4/*10* individuals using PABA as substrate. Hughes *et al.* [11] compared the NAT1 genotype of 144 individuals with the NAT1 function predicted by the urinary PAS acetylation ratio, probably reflecting NAT1 expression in the liver. The means of the log urinary AcPAS/PAS ratios of the **4/*4*, **4/*10*, and **10/*10* individuals were not significantly different.

In our study, all heterozygous and homozygous carriers of allele *NAT1*11* had activities in the lower range (Fig. 1), although the magnitude of impairment of the NAT1 activity was only moderate and there was no continuous trend comparing the homozygous **11/*11* genotypes with the heterozygous **4/*11* and **10/*11* genotypes. Nevertheless, when all 10 carriers of one or two alleles **11* were compared, this allele had lower activity compared with the wild-type *NAT1* allele with high statistical significance ($P < 0.001$; Mann-Whitney U-test). These data on the *NAT1*11* allele are in agreement with those of Payton and Sim [4], who observed a lower NAT1 activity in erythrocyte lysate of one **4/*11* individual compared with **4/*4* (N = 8) and **4/*10* (N = 10) individuals using PABA as substrate. Moreover, Risch *et al.* [10] found a low level of NAT1 activity in erythrocytes of one heterozygous *NAT1*4/*11* person. The *in vitro* data of Hughes *et al.* [11], using NAT1 variants expressed in *E. coli*, consistently showed a lower activity of allele *NAT1*11* (914 nmol/min/unit) compared with *NAT1*4* (968 nmol/min/unit) after adjustment for the amount of immunologically quantified protein, although this difference was not significant.

Our data for the *NAT1*14* and *NAT1*15* alleles are highly consistent with earlier publications. Butcher *et al.* [6] measured the NAT1 activity in peripheral mononuclear blood cells of 85 individuals, 3 of whom had the *NAT1*4/*14* genotype. Their mean V_{max} values (9.1 nmol NAcPABA/min/mg protein) were significantly lower than the V_{max} values of the **4/*4* individuals (mean: 19.7 nmol NAcPABA/min/mg, N = 78). This 53.8% reduction is in agreement with the results of our study, where the activity of the *NAT1*14/*10* and *NAT1*14/*4* genotypes was reduced by 49.8% and 55.6%, respectively, in comparison with the wild-type. One individual with *NAT1* genotype **4/*14* in the study by Payton and Sim [4] also had a lower NAT1 activity than the **4/*4* and **4/*10* individuals. Hughes *et al.* [11] tested the NAT1 activity in whole blood lysate of 20 individuals using *p*-aminosalicylic acid as substrate. The V_{max} value (0.26 nmol NAcPAS/min per mL blood lysate) of the **14/*15* individual was 93-fold lower than the mean values of the other subjects. There was no detectable activity of a recombinant NAT1 15 protein, which is in agreement with the lack of any detectable enzyme activity in the **15/*15* individual of our study and is explained by the fact that the mutation in position 559 (C → T) produces a truncated protein.

In conclusion, the *N*-acetylation capacity of *NAT1* allele **10* is not different compared with that of the wild-type allele. The arginine$_{187}$ to glutamine variant *NAT1*14* had lower activity compared with *NAT1*3*, *NAT1*4*, and *NAT1*10*. The valine 149 to isoleucine plus serine 214 to

alanine variant *NAT1*11* had moderately reduced activity, while the enzyme truncated at amino acid 187 (*NAT1*15*) had no activity.

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