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SHORT COMMUNICATION

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Re-investigation of the concordance of human *NAT2* phenotypes and genotypes

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Abstract A comparative study of *N*-acetyltransferase 2 (*NAT2*) genotyping and phenotyping (caffeine test method) was performed on 211 persons to elucidate apparent discrepancies in the assignment of *NAT2*12* and *NAT2*13* alleles which occur in the literature. The study used the standard procedures of genotyping (two PCR runs and application of seven restriction enzymes) and phenotyping (determination of the two caffeine metabolites 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-methylxanthine (1X)), as documented in detail and validated by the Deutsche Forschungsgemeinschaft. The data were consistent with an AFMU/1X molar ratio of 0.85 as cut-off point (antimode) between phenotypically slow and rapid acetylators. Under this provision, several R/S allele combinations did not comply, either fully or partly, with their associated phenotypes. In particular, there was a wide phenotypic overlap of the alleged rapid allele combination groups (i) *NAT2*12A*/**5A*; *NAT2*12C*/**5D*; *NAT2*4*/**5B*, (ii) *NAT2*13*/**6B*; *NAT2*4*/**6A*, and (iii) *NAT2*13*/**7A*; *NAT2*4*/**7B*. These groups obviously contained both phenotypically rapid and slow acetylators. If one assumes that the presence of one "wild type" allele *NAT2*4* defines a rapid acetylator the assignment of the alleles *NAT2*12A*, *NAT2*12C*, and *NAT*13* as determinants of a rapid acetylator phenotype must be questioned. This refers in particular to the nucleotide changes $A^{803}G$ (*NAT2*12A*, *NAT2*12C*) and $C^{282}T$ (*NAT2*13*). Based on discussions in the literature and the data presented here, there is accumulating evidence that current assignments of the *NAT2*12* and *NAT2*13* alleles as determinants of a rapid acetylator state should be reconsidered.

Keywords *N*-Acetyltransferase 2 · *NAT2* · Genotyping · Phenotyping · Caffeine test

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Introduction

Human *N*-acetyltransferase (*NAT*) polymorphisms are important for explaining interindividual differences in response to drugs (Meisel 2002) and industrial chemicals (Golka et al. 2002). Human *NAT* polymorphisms are primarily due to single-nucleotide polymorphisms (SNP) in the coding region of *NAT* genes (Pompeo et al. 2002). Variant alleles of *NAT2* have been identified and assigned to slow and rapid acetylator phenotypes (Grant et al. 1997). A unified gene nomenclature as a consensus has been established (Vatsis et al. 1995; Hein et al. 2000a) (http://www.louisville.edu/medschool/pharmacology/NAT.html).

The concordance and/or discordance of *NAT2* genotypes and phenotypes has been a matter of discussion (Butler et al. 1992; Hickman et al. 1992; Bell et al. 1993; Cascorbi et al. 1995; Golka et al. 1996; Gross et al. 1999), and a potential for mis-classification has been noted (Butcher et al. 2002). Specifically, the classifications of the *NAT2*12A* and *NAT2*13* alleles have been challenged. Both alleles are generally addressed as determining a rapid acetylator phenotype (Hein et al. 2000b). However, Gross et al. (1999) have pointed to inconsistencies and concluded that these assignments require further re-evaluation. In this context, they emphasized the need of further investigations of the coherence of *NAT2* phenotype and genotype assignments.

In principle, assignment problems may arise from both *NAT2* genotyping (Cascorbi and Roots 1999) and phenotyping (see Discussion section). In order to

comply with the need for validation of methods in this field, the Deutsche Forschungsgemeinschaft (DFG) has assessed current methodologies and issued a recent volume that contains established and validated methods on genotyping and phenotyping of polymorphic genes/enzymes of xenobiotic metabolism, among others of *NAT2* (DFG 2004a, 2004b). On the basis of these validated methods for routine use, we have re-investigated the coherence of *NAT2* genotypes and phenotypes to elucidate the apparent differences. The genotyping procedure (DFG 2004a) was based on the methods of Cascorbi et al. (Cascorbi et al. 1995; Cascorbi and Roots 1999). For phenotyping, the caffeine test of Grant et al. (1984) was used, as validated by the DFG (2004b).

Materials and methods

This study of comparative *NAT2* genotyping and phenotyping was performed on 211 persons attending the Unit of Clinical Occupational Medicine of the Institute of Occupational Physiology at the University of Dortmund. Most of these persons suffered from transitional cell carcinoma of the urinary bladder and there was suspicion of an occupational disease (in Germany: BK 1301) caused by aromatic amines. In these persons, *NAT2* genotyping and phenotyping was part of the medical expertise on the individuals that had to be provided by the institute. This pre-selection caused an over-representation of slow acetylators, compared with the normal central European population (''Caucasians'').

N-Acetyltransferase 2 genotyping

NAT2 genotyping by PCR was performed as described and validated by the DFG (2004a). Whole-blood EDTA samples were stored until processing at –20°C. DNA isolation used commercially available kits, following the manufacturer's instructions.

The method of the DFG is based on the work of Cascorbi et al. (1995) which originally produced three amplificates where one initially amplified *NAT2* DNA fragment comprising 1211 bp served as starting DNA for two further amplificates with sizes of 442 and 420 bp, respectively. Instead, the modified method of the DFG (2004a) uses only two PCR runs, while still applying the usual seven restriction enzymes. The resulting general workflow is as follows: DNA isolation, first PCR with check of success, three restriction enzymes (*MSP* I, *Fok* I, *Dde* I) with gel electrophoresis and second PCR with check of success, three restriction enzymes (*Kpn* I, *Dde* I, *BamH* I) with gel electrophoresis and *Taq* I restriction overnight, gel electrophoresis of the *Taq* I restriction cleavage and evaluation. The evaluation followed the current assignment of *NAT2* alleles (Hein et al. 2000b), as described in detail by the DFG (2004a).

N-Acetyltransferase phenotyping (caffeine test)

The caffeine test method of Grant et al. (1984) was used for *NAT2* phenotyping. The procedure has been validated by the DFG (2004b). The subjects drank 1–2 cups of coffee (sometimes tea or a caffeine-containing soft drink), and spot urine was taken 2 h after caffeine intake. Most of the cases provided a second urine sample after 4 h that was used as an analytical duplicate. No differences were observed in phenotypes based on the duplicate samples, and for duplicate analyses the mean AFMU/1X ratio was taken for further assessment.

The urine was immediately adjusted to pH 3.5 by addition of the required small amounts of 1 mol L^{-1} hydrochloric acid and stored in 10 mL plastic tubes at –20°C until processing. The chromatographic standard 1-methylxanthine (1X) was a commercial product of Sigma (Deisenhofen, Germany) and the standard 5-acetylamino-6-formylamino-3-methyluracil (AFMU) had been synthesised in our group (Röhrkasten et al. 1997). The HPLC procedure followed the method of Grant et al. (1984), as validated by the DFG (2004b). The urinary AFMU/1X molar ratio served as the final quantitative determinant. As outlined previously (Golka et al. 1996), the determination of the cut-off point (antimode) between slow and rapid acetylator phenotypes is crucial. This is addressed in the Results and Discussion sections (v.i.).

Results

Study group, AFMU/1X ratios and genotypic acetylator assignments

The caffeine test for *NAT2* phenotyping was performed on the study group of 211 persons, and the resulting urinary AFMU/1X ratios are compiled in Table 1. Genotypic assignments of slow (S/S homozygotes) and rapid acetylators (R/R homozygotes and R/S heterozygotes) were done according to current procedures and nomenclature (Butcher et al. 2002; Hein et al. 2000b; DFG 2004a, 2004b). The maximum individual AFMU/1X ratio of the homozygous S/S genotypes and the

Table 1 AFMU/1X ratios vs genotypes, based on the current allele assignments according to Hein et al. (2000a) and DFG (2004a). Genotypic acetylator state: S/S homozygously slow, R/S heterozygotes, R/R homozygously rapid

	S/S	R/S	R/R
n	127	83	11
Mean	0.25	1.23	2.22
Standard deviation	0.15	0.83	1.02
Median	0.22	1.045	2.27
Range	0.825	4.80	3.79
Interquartile range	0.16	0.77	1.26
Minimum	0.025	0.10	0.84
Maximum	0.85	4.90	4.63

minimum of the homozygous R/R genotypes was practically identical (0.85 and 0.84, respectively). Hence, a AFMU/1X ratio of 0.85 was subsequently taken as cut-off point (antimode) between slow and rapid acetylators. By doing this, there was some overlap in the group assigned to the R/S genotype (v.i.).

Coincidence of *NAT2* phenotyping and genotyping

The basic results of the genotypings and the corresponding phenotypings (AFMU/1X ratios) are given in Table 2 (median, minimum, maximum values) and in Fig. 1 (graphical presentation of individual values). As mentioned above, the present data are consistent with an AFMU/1X ratio of 0.85 that had been described by Braz Vieira de Silva Pontes et al. (1992) as cut-off point (antimode) between phenotypically slow (AFMU/1X < 0.85) and rapid (AFMU/1X > 0.85) acetylators. If this is accepted, several R/S allele combinations do not comply, either fully or partly, with their assigned phenotypes.

The current view is that "rapid acetylator alleles" *NAT2*4* ("wild type"), *NAT2*12A*, *NAT2*12B*, *NAT2*12C*, and *NAT2*13* dispose to a rapid acetylator phenotype (Grant et al. 1997; Butcher et al. 2002; Hein et al. 2000b). If one disregards the only minor overlap of the "rapid" allele combination designated in Table 2 and Fig. 1 as #10 (*NAT2*4/NAT2*5A*), the data of some heterozygous allele combinations deserve attention. All four persons with the heterozygous allele combination #14 (comprising combinations *NAT2*12B/*5E*; *NAT2*5C/*6A*; *NAT2*5D/*6C*) were phenotypically slow acetylators. This might be ascribed to the alternative allele combinations *NAT2*5C/*6A* and/or *NAT2*5D/*6C* in which only slow alleles are present, and is therefore in line with the current concept.

More difficult to explain is the wide phenotypic overlap of the heterozygous allele combinations #11 (*NAT2*12A/*5A*; *NAT2*12C/*5D*; *NAT2*4/*5B*), #15 (*NAT2*13/*6B* / *NAT2*4/*6A*) and #16 (*NAT2*13/*7A*; *NAT2*4/*7B*). According to the current assignment system, these should be rapid acetylators. However, these groups obviously contain both phenotypically rapid and phenotypically slow acetylators. If one assumes that, in any case, the presence of one "wild type" allele *NAT2*4* defines a rapid acetylator, the assignment of the alleles *NAT2*12A* and/or *NAT2*12C* (#11), and *NAT*13* (#15, #16) as determinants of a rapid acetylator phenotype must be questioned. This refers to the nucleotide changes $A^{803}G$ (*NAT2*12A*, *NAT2*12C*) and $C^{282}T$ (*NAT2*13*). Because of the larger number of persons ($n = 35$) in group #11, this seems especially true for the change $A^{803}G$ ($lys^{268} \rightarrow arg$) that is associated with both *NAT2*12A* and *NAT2*12C*.

Discussion

This study was based on established standard methods of *NAT2* phenotyping and genotyping, as evaluated by the DFG (2004a, 2004b). As far as *NAT2* phenotyping by the "caffeine test" method is concerned, it has previously been discussed that the exact positioning of the cut-off point (antimode) between phenotypically slow and rapid acetylators seems laboratory-dependent (Golka et al. 1996), as discriminating AFMU/1X ratios vary between 0.48 (Grant et al. 1984) and 1.0 (Golka et al. 1996). These discrepancies may be attributed to differences in chromatographic conditions. The Deutsche Forschungsgemeinschaft (DFG 2004b) has pointed out that the stability of AFMU depends on pH, temperature, and storage period, and that AFMU may be degraded to 5-acetylamino-6-amino-3-methyluracil (AAMU) by deformylation. The procedure used for

Table 2 AFMU/1X molar ratios in subgroups assigned to NAT2 allele combinations

Genotype (see Table 1)	Allele comb no.	*NAT2* alleles or possible allele combinations	*N*	Median	Minimum	Maximum
S/S	1	**5A/*5B*	7	0.22	0.18	0.85
	2	**5A/*6A*	1	0.04		
	3	**5A/*6C;*5B/*6A*	51	0.23	0.05	0.69
	4	**5B/*5B*	39	0.26	0.025	0.81
	5	**5B/*5C*	2	0.263	0.256	0.27
	6	**5B/*7B*	10	0.175	0.10	0.36
	7	**5C/*5C*	1	0.26		
	8	**6A/*6A*	15	0.185	0.08	0.52
	9	**6A/*7B*	1	0.065		
R/S	10	**4/*5A*	5	1.04	0.72	2.02
	11	**12A/*5A;*12C/*5D;*4/*5B*	35	0.98	0.16	2.74
	12	**12A/*5D;*4/*5C*	1	0.985		
	13	**12A/*6A;*12B/*6B;*4/*6C*	1	2.54		
	14	**12B/*5E;*5C/*6;*5D/*6C*	4	0.203	0.10	0.60
	15	**13/*6B;*4/*6A*	33	1.24	0.395	4.90
	16	**13/*7A;*4/*7B*	4	0.923	0.20	1.38
R/R	17	**4/*4*	11	2.27	0.84	4.63

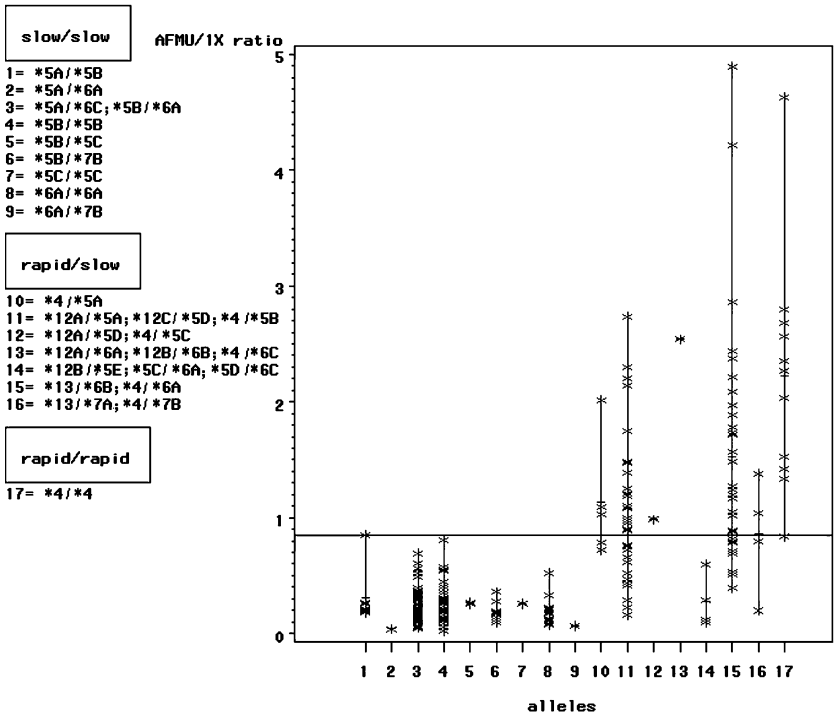

Fig. 1 Individual NAT2 phenotyping data (ratios of urinary AFMU/1X), allocated to genotypic groups of *NAT2* alleles and allele combinations

synthesis of the standard used in our laboratory had been described by Röhrkasten et al. (1997), to which reference has been made by the DFG (2004b).

As far as the current procedures of *NAT2* genotyping are concerned, there is a clear discrepancy in the phenotypic assignment of the *NAT2*12A* ($A^{803}G$) and *NAT2*13* ($C^{282}T$) alleles as determinants of a slow or rapid acetylator state. Originally, it had been suggested that *NAT2*13* was associated with the slow acetylator phenotype (Cascorbi et al. 1995), but later this was ascribed to a genotyping artefact (Cascorbi and Roots 1999). Clearly, different mechanisms for slow acetylator phenotypes must be considered (Leff et al. 1999), and there is a potential of mis-classification of genotypes and deduced genotypes (Butcher et al. 2002).

On one hand, rapid acetylator phenotypes are currently deduced for the *NAT2*12* alleles *A–C* (Butcher et al. 2002). This is based on the argumentation of Hein et al. (2000b) that recombinantly expressed enzymes of the human *NAT2*12* and *NAT2*13* clusters reach *N*-, *O*-, and *N*,*O*-acetyltransferase activities comparable to those of the rapid acetylator *NAT2*4* enzyme (Hein et al. 1994, 1995). However, this is not in line with a comparative study of *NAT2* genotyping and phenotyping by O'Neil et al. (2000) that points, for persons with the *NAT2*12A*, *NAT2*12B* or *NAT2*13* alleles, mostly to either a slow acetylator phenotype or a borderline result.

On the other hand, the specific assignments of *NAT2*12A* and *NAT2*13* as determinants of the slow acetylator phenotype have been challenged by the group of Kadlubar, based on their own data (Gross et al. 1999). In their discussion, they pointed out that the assignment of *NAT2*12A* as leading to a rapid acetylator had been, in first instance, based on data from just two subjects from a single family with *NAT2*5B*/**12A* genotypes (Cascorbi et al. 1996). In addition, the recombinant human *NAT2* activity of the *NAT2*13* is similar to that of the rapid *NAT2*4* allele (Hein et al. 1995). This is explainable because the SNP leading to the *NAT2*13* allele does not implicate an amino acid exchange in the enzyme. Therefore, it is being considered that the expression of *NAT2*12A* and *NAT2*13* could be influenced by additional factors (Gross et al. 1999).

In essence, there is now accumulating evidence that the assignments of the *NAT2*12A* and *NAT2*13* alleles as determinants of a rapid acetylator state should be revisited.

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