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Short communication

Concordance between the deduced acetylation status generated by high-speed Real-time PCR based *NAT2* genotyping of seven single nucleotide polymorphisms and human NAT2 phenotypes determined by a caffeine assay

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Abstract

Background: The utility of typing single nucleotide polymorphisms (SNPs) for the determination of the *N*-acetyltransferase 2 (NAT2) acetylation status is a matter of debate.
Aims of the study: Evaluation of the concordance between deduced genotype results of seven human *NAT2* SNPs generated by Real-time PCR analysis and human NAT2 phenotypes.
Methods: NAT2 phenotypes of 38 Caucasian workers were determined using a suitable caffeine test method. Genomic DNA aliquots were used for the determination of seven human *NAT2*-specific SNPs (G191A, C282T, T341C, C481T, G590A, A803G, G857A).
Results and conclusions: Phenotypic results based on the molar ratio of 5-acetylamino-6-formylamino-3-methyluracil (AFMU)/(AFMU+1-methlyuric acid (1U)+1-methylxanthine (1X)) calculated from excreted caffeine metabolite levels in urine samples with 0.3 as a cut-off point between slow (<0.3) and rapid acetylators (≥0.3). Twenty-seven samples belonged to the slow (mean 0.13; range: 0.03–0.25), 11 to the rapid (mean: 0.41; range: 0.34–0.48) acetylators. LightCycler™ analyses revealed 11 different *NAT2* variant combinations, whereby *5B/*5B and *5B/*6A or *5A/*6C (each 21%), were the most frequent. The deduced acetylation status of the seven *NAT2* SNPs matched perfectly with the 38 results determined by phenotyping. This study showed a 100% concordance between NAT2 phenotypes and the deduced *NAT2* genotypes and the suitability of the high-speed *NAT2*-specific LightCycler™ analysis in a Caucasian population.

Keywords: Acetylation status; *NAT2* genotyping; NAT2 phenotyping; LightCycler

1. Introduction

Human *N*-acetyltransferase 2 (NAT2) phenotyping is based on the quantification of urinary caffeine metabolites and the calculation of metabolic ratios after the intake of a standardized caffeine (1,3,7-trimethylxanthine) dose. The most analytical procedures reported so far are based on the initially work of Grant et al. [1] including a liquid–liquid extraction of urinary samples prior to the analysis by high-performance liquid chromatography (HPLC). In this study the modified method of Nyéki et al. [2] was used, which represents a substantial improvement, since a single analysis and minimal urine sample treatment enables the simultaneous quantification of five caffeine metabolites including 5-acetylamino-6-formylamino-3-methyluracil (AFMU), 5-acetylamino-6-amino-3-methyluracil (AAMU), 1-methylxanthine (1X), 1-methyluric acid (1U), 1,7-

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dimethyluric acid (17U) necessary for the phenotyping of NAT2, cytochrome P450 isoenzyme 1A2 (CYP1A2) and xanthine oxidase (XO).

NAT2 is a key enzyme in metabolising carcinogenic aromatic and heterocyclic aromatic amines [3]. On the DNA level the *NAT2* gene has been established as the site of the human acetylation polymorphism and comprises two exons whereby the gene product is exclusively encoded by the 2nd exon, which is highly polymorphic, has a length of 870 bp, and carries 11 single nucleotide polymorphisms (SNPs) with varying allelic variants in different populations [4,5]. Seven SNPs are commonly found in human populations. A panel of four (nucleotide positions 191, 341, 590, 857) result in an amino acid exchange yielding in a significant decrease of the acetylation capacity. In contrast, changes in one of the three remaining positions 282, 481, and 803 have no influence on the phenotype but are necessary for an enhanced typing accuracy.

There are a lot of former studies focusing on the relationship between *NAT2* genotype and disease risk using PCR-based assays that detect only three SNPs (C481T, G590A, and G857A) to infer the NAT2 acetylation status. When none of these SNPs were present, the wild-type allele *NAT2*4* corresponding to a rapid NAT2 isoform was designated [6]. Although, numerous *NAT2* SNPs are in linkage disequilibrium, the assessment of only three SNPs lead to several misclassifications [7]. The present study aimed to reconsider whether the rapid determination of seven *NAT2*-specific SNPs by LightCycler™ technology is suitable to properly deduce the acetylation status when compared with an up to date caffeine test method [2] in a Caucasian population.

2. Materials and methods

After approval by the ethic commission and written informed consent prior to the study, NAT2 phenotyping and *NAT2* genotyping was performed in samples of male workers with a mean age of 39 years (range: 25–54 years), a mean height of 177 cm (range: 160–190 cm) and a mean weight of 84 kg (range: 56–120 kg), who were participants of a PAH biological monitoring study in German industries [8]. 30 workers (79%) were current smokers. The smoking status was determined by urinary cotinine measurements with a cut-off of 100 μg/L for smoking. The mean of the measured cotinine concentration was 1724 μg/L with a range between 36.2 and 5072 μg/L. In five out of 38 workers the smoking status was not experimentally determined due to volume limits of the available urine samples and, therefore, based on the information from a questionnaire filled in by each participant.

All participants were instructed to abstain methylxanthine-containing substances overnight. After the agreement of each participant, 33 workers received a 200 mg caffeine tablet (Coffenium N 0.2, Merck Darmstadt, Germany) and five workers an equivalent of dissolved instant coffee (Nescafé Gold, Nestlé, Vevey, Switzerland). After shift (7–8 h) a 5 mL urine sample was taken from each participant and instantaneously diluted with 1 mL citrate buffer to stabilize the caffeine metabolites. After thorough mixing, the samples were stored at 4 °C until analysis. For the determination of caffeine metabolites by high-performance liquid chromatography (HPLC), the following metabolites were measured: 5-acetylamino-6-formylamino-3-methyluracil (AFMU), 5-acetylamino-6-amino-3-methyluracil (AAMU), 1-methyluric acid (1U), 1-methylxanthine (1X) and 1,7-dimethyluric acid (17U). 1,9-dimethyluric acid (19U) served as an internal standard. All compounds are commercially available except AFMU and AAMU which were synthesized. The NAT2 activity was expressed as the ratio calculated as AFMU/(AFMU + 1U + 1X) in accordance to Nyéki et al. [2]. A correct determination of AFMU requires that no or at best trace amounts of AAMU are detectable in the urine sample. AAMU was determined by detection of the corresponding HPLC peak at 260 nm.

NAT2 genotyping was performed with aliquots of genomic DNA extracted from whole blood EDTA samples using the DNA isolation kit from PUREGENE (Biozym, Hessisch-Oldendorf, Germany) or the QIA amp DNA mini kit (QIAGEN, Hilden, Germany). *NAT2*-specific amplification was performed with the help of the new artus™ amplfication system (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. Before pipetting the DNA samples into the LightCycler glass capillaries, the cooling block together with the included adapters has to be pre-cooled to 4 °C. Before the analysis of seven *NAT2*-specific SNPs (G191A, C282T, T341C, C481T, G590A, A803G, G857A), four positive controls (controls A–D) and one negative control (PCR water) per run (included in the kit) have to be considered. After putting the necessary number of capillaries into the cooled block, for each DNA sample one capillary has to be filled with 16 μL RealArt master A, B, C, or D, and 2 μL RealArt-Mg solution (both included in the kit). The resulting master-mixes A, B, C, and D were mixed and centrifuged (400×*g*) to the bottom of the capillary. After the addition of 2 μL genomic DNA per capillary, mixing and centrifugation, the Real-time PCR was performed with the LightCycler™ system (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacture's recommendations. After that, the melting curves of the *NAT2* wild-type (*NAT2*4*) and the corresponding variants were analysed in channels F2 (640 nm) and F3 (705 nm) of the LightCycler™, whereby the master-mix A was used for the detection of the SNP in *NAT2* nucleotide (nt) position 191 and read out in channel F2, master-mix B was used for the SNP detection in *NAT2* nt positions 282 (read out in F2) and 857 (read out in F3), master-mix C for the SNP detection in *NAT2* nt-positions 803 (read out in F2) and 590 (read out in F3), and master-mix D for the analysis of *NAT2* nt positions 341 (read out in F2) and 481 (read out in F3). The evaluation of the melting curves of the seven SNPs described above allowed the rapid determination of the corresponding alleles in only 75 min and finally the prediction of the slow and rapid acetylation status.

3. Results and discussion

Recently, a comprehensive polycyclic aromatic hydrocarbon (PAH) biological monitoring study in German industries has been completed [8]. During this study it was possible to analyse the phenotypic CYP1A2 activity by means of the caffeine test in