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TOXICOGENOMICS

Refinement of the prediction of *N*-acetyltransferase 2 (NAT2) phenotypes with respect to enzyme activity and urinary bladder cancer risk

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Abstract Polymorphisms of *N*-acetyltransferase 2 (NAT2) are well known to modify urinary bladder cancer risk as well as efficacy and toxicity of pharmaceuticals via reduction in the enzyme's acetylation capacity. Nevertheless, the discussion about optimal NAT2 phenotype prediction, particularly differentiation between different degrees of slow acetylation, is still controversial. Therefore, we investigated the impact of single nucleotide polymorphisms and their haplotypes on slow acetylation in vivo and on bladder cancer risk. For this purpose, we used a study cohort of 1,712 bladder cancer cases and 2,020 controls genotyped for NAT2 by RFLP-PCR and for the tagSNP rs1495741 by TaqMan® assay. A subgroup of 344 individuals was phenotyped by the caffeine test in vivo. We identified an 'ultra-slow' acetylator phenotype based on combined *6A/*6A, *6A/*7B and *7B/*7B genotypes containing the homozygous minor alleles of C282T (rs1041983, *6A, *7B) and G590A (rs1799930, *6A). 'Ultra-slow' acetylators have significantly about 32 and 46 % lower activities of caffeine metabolism compared with other slow acetylators and with the *5B/*5B genotypes, respectively ($P < 0.01$, both). The 'ultra-slow' genotype showed an association with bladder cancer risk in the univariate analysis (OR = 1.31, $P = 0.012$) and a trend adjusted for age, gender and smoking habits (OR = 1.22, $P = 0.082$). In contrast, slow acetylators in general were not associated with bladder cancer risk, neither in the univariate (OR = 1.02, $P = 0.78$) nor in the adjusted (OR = 0.98, $P = 0.77$) analysis. In conclusion, this study suggests that NAT2 phenotype prediction should be refined by consideration of an 'ultra-slow' acetylation genotype.

Keywords Isoniazid metabolism · NAT2 *5B haplotype · NAT2 *6A haplotype · rs1041983 · rs1799930 · rs1801280

Introduction

Large meta-analyses have shown an association between NAT2 slow acetylation genotypes and increased urinary bladder cancer (UBC) risk (García-Closas et al. 2005; Sanderson et al. 2007). Although the association was significant in meta-analyses, the results are controversial at the level of individual studies. For example, 35 of 46 studies included into a meta-analysis did not amount to statistical significance (Moore et al. 2011). Possible explanations for the lack of significance at the level of individual studies are differences in exposure to bladder carcinogens and ethnicity (Golka et al. 2002; Selinski 2012; Bolt 2013a, b), as well as the relatively low case numbers and statistical power limitations.

A further aspect may limit the studies on NAT2 genotypes with bladder cancer risk, namely the technique of NAT2 genotyping and phenotype prediction (Deitz et al. 2004). Conventionally, haplotype pairs of seven NAT2 exon SNPs (rs1801279, rs1041983, rs1801280, rs1799929, rs1799930, rs1208 and rs1799931) have been

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used to derive slow, intermediate and rapid genotypes (Grant et al. 1997; Hein 2006) according to the consensus nomenclature (Hein 2002; Hein et al. 2008, http://www.louisville.edu/medschool/pharmacology/NAT.html). Recently, a tagging SNP rs1495741 (Rothman et al. 2010, García-Closas et al. 2011) and a 2-SNP genotype (Selinski et al. 2011) have been recommended. This fuelled again the debate on the optimal technique of NAT2 genotyping and phenotype prediction (Selinski et al. 2011; Agúndez 2003; He et al. 2012; Hein and Doll 2012a, b, Suarez-Kurtz et al. 2012a, b). A remaining crucial problem is the understanding of the relevance of different slow haplotypes (Cascorbi et al. 1995; Gross et al. 1999; Bolt et al. 2005; Hein 2006, 2009; Golka et al. 2008; Ruiz et al. 2012).

In the present study, we used the *IfADo* UBC case–control series, consisting of 1,712 cases and 2,020 controls (Golka et al. 2009; Lehmann et al. 2010; Selinski et al. 2011, 2012, 2013a; Schwender et al. 2012). In this case–control series, neither the 7-SNP-based genotype, nor the tagging SNP nor the 2-SNP genotype was significantly associated with bladder cancer risk. Interestingly, we could identify an 'ultra-slow' genotype based on comparison with in vivo caffeine metabolism.

In contrast to solely discriminating between slow and rapid genotypes, the consideration of this 'ultra-slow' genotype showed an association with bladder cancer risk. If confirmed in independent cohorts, the 'ultra-slow' genotypes should be used for refinement of NAT2 phenotype prediction in future.

Materials and methods

Patients

We used eight case–control series, in total 1,712 UBC cases and 2,020 controls from Germany (1,268/1,693), Hungary (226/76), Pakistan (106/61) and Venezuela (112/190) as described earlier (Golka et al. 2009; Selinski et al. 2012, 2013a; for details, see Supplementary Tables S1A-C, Supplementary Figure S1, Supplementary Materials and Methods). NAT2 7-SNP genotypes were determined in 1,692 cases and 1,995 controls, the NAT2 tagSNP genotype was determined in 1,702 cases and 1,957 controls. A subgroup of 344 Caucasians was phenotyped for NAT2 using the caffeine test (for details see Selinski et al. 2011). The sample collection was approved by the local ethics committee and by the IRB (institutional review board). Exclusion criteria were a missing written informed consent, age of less than 20 years and, in case of the German and Hungarian study groups, non-Caucasian descent.

Genotyping and phenotyping

The A/G substitution of the NAT2 tagSNP (rs1495741) on chromosome 8p23, position 18,272,881, was detected via TaqMan® SNP Genotyping Assay (Applied Biosystems, Darmstadt, Germany). Genotyping of the NAT2 polymorphisms G191A (rs1801279), C282T (rs1041983), T341C (rs1801280), C481T (rs1799929), G590A (rs1799930), A803G (rs1208) and G857A (rs1799931) was performed using PCR- and RFLP-based methods (Blaszkewicz et al. 2004). All SNPs were in Hardy–Weinberg equilibrium (HWE; Supplementary Table S2). NAT2 phenotyping was performed using the caffeine test according to standard methods determining the ratio of two caffeine metabolites 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-methylxanthine (1-MX) in urine by liquid–liquid extraction, HPLC separation and UV detection (Blaszkewicz 2004). The person under investigation was given two cups of coffee containing caffeine—if possible after complete evacuation of the bladder. Urine was sampled routinely 2 and 4 h after the administration of caffeine. For quality control, five test persons provided urine samples 2, 4 and 6 h after caffeine intake. The results showed no deviation in the phenotyping, indicating that the time of sampling played a minor role. The influence of a full or empty bladder before the administration of caffeine was investigated on seven persons. Deviations in phenotyping were not observed. During the evaluation of the method, urine samples were taken without regard to food intake or medication. All these variations in the sampling procedures gave no indication of interferences of the results in these different aspects. For the classification, a cut-off of 0.85 was used to differentiate between slow (<0.85) and rapid acetylators (≥0.85). For details, see Supplementary Materials and Methods.

Statistical methods. Deviations from HWE were checked for each of the eight SNPs in each study group separately in cases and controls using chi-square tests. The NAT2 haplotype pairs were determined from the seven NAT2 SNPs using PHASE, v2.1.1 (Stephens et al. 2001; Li and Stephens 2003; Stephens and Donnelly 2003) as described earlier (Agúndez et al. 2008; Selinski et al. 2011, 2013a), and the 7-SNP genotype was derived according to the nomenclature of the *N*-acetyltransferases (Hein 2002; Hein et al. 2008, http://www.louisville.edu/medschool/pharmacology/NAT.html). The A/A, A/G and G/G genotypes of the NAT2 tagSNP rs1495741 were considered as slow, intermediate and rapid, respectively (García-Closas et al. 2011). The 2-SNP genotype was defined as the sum of variant alleles of the two NAT2 SNPs rs1041983 (C282T) and rs1801280 (T341C) as described earlier (Selinski et al. 2011). Associations of genetic markers with UBC were evaluated using asymptotic chi-square tests, odds ratios (OR) and 95 % confidence intervals (CI) when expected cell frequencies

were ≥5; otherwise, we used exact tests and 95 % CIs. For combined study groups, the method of Mantel–Haenszel was used to adjust for the different study regions if homogeneity of the ORs could be assumed according to the Breslow–Day test ($P \geq 0.05$). In case of inhomogeneity, random effect models were used (DerSimonian and Laird 1986). Adjusted ORs and 95 % confidence intervals as well as Wald tests adjusted for age, cigarette smoking, gender and study region in case of combined study groups were calculated using logistic regression. This analysis was repeated stratifying for smoking habits. Effects of exposure on NAT2 risks were investigated using exact chi-square tests in a stratified analysis. Genotype differences in the in vivo activity were tested using the Wilcoxon or the Kruskal–Wallis test in case of equal and Welch's *t* test in case of unequal variances. Global differences were checked using the *F* test; pairwise comparisons were made using the Tukey test. Equality of the variances was tested using the Levene test; normality could not be assumed according to the Kolmogorov–Smirnov test. The level of significance was $\alpha = 0.05$ for all tests and confidence intervals. The software R, version 2.15.0 (R Development Core Team 2008), was used to test for HWE and fit the random effect models. For all further calculations, we used the software package SAS/STAT®, version 9.2 (SAS/STAT® software, version 9.2. Copyright© 2002–2008).

Results

Identification of an 'ultra-slow' genotype

In a first step, we studied a possible association of traditionally used NAT2 genotypes with bladder cancer risk in the *IfADo* UBC case–control series. Neither the 7-SNP genotype, nor the tagging SNP as well as the 2-SNP genotype showed a significant association with bladder cancer risk (Table 1; Supplementary Tables S3-S5). This was the case for the univariate as well as the multivariate analysis adjusted for smoking habits, age, gender and study group. Stratifying for smoking habits yielded a similar result. Next, we studied the seven individual SNPs that are usually applied to derive the 7-SNP genotype. Interestingly, two of them showed a borderline significant association with bladder cancer risk, namely C282T ($P = 0.0319$) and G590A ($P = 0.0763$) (Table 2A, B; Supplementary Tables S6-S7).

To understand the relationship between C282T and G590A and NAT2 activity, we studied a group of 344 individuals who have been phenotyped by the caffeine test. The homozygous minor alleles of both, C282T and G590A, showed lower activities than those of all other individual SNPs (at least 37 % reduction, $P < 0.0001$, Table 3). Note that we can distinguish two groups of SNPs: homozygous minor alleles of C282T, G590A and G857A (mainly present in the *6 and *7 haplotype clusters) usually cannot be observed together with homozygous minor alleles of T341C, C481T and A803G (mainly present in the *5 haplotype cluster; Table 4; Supplementary Table S8).

Next we studied how C282T and G590A are linked to haplotypes. The C282T variant is mainly present in the slow *6A and *7B haplotypes (Table 4; 98.6 %). G590A is mainly present in the *6A haplotypes (99.9 %). The homozygous minor allele of C282T corresponds mainly to *6A/*6A, *6A/*7B and *7B/*7B (95.6 %), whereas the homozygous minor allele of G590A corresponds to *6A/*6A (100 %). The *6A/*6A genotypes showed significantly lower activities than *5B/*5B (0.17 vs. 0.28; $P = 0.0001$; Table 5A, B). Also, the combination of *6A/*6A and *6A/*7B (for *7B/*7B no in vivo data were available) resulted in lower activities than *5B/*5B (0.15 vs. 0.28; $P < 0.0001$; Table 5A, C). The concept that *6A and *7B define 'ultra-slow' haplotypes is further supported by the observation that an increasing number of *6A and *7B haplotypes is associated with a decreasing activity of caffeine metabolism (Table 5A). Pairwise comparison showed significantly lower activities of the *6A/*6A genotypes compared to *5B/*6A ($P = 0.0186$) and *5B/*5B ($P = 0.0001$). Also, the combined *6A/*6A and *6A/*7B resulted in lower activities than the combined *5B/*6A and *5B/*7B genotypes ($P = 0.0081$) as well as the *5B/*5B genotypes ($P < 0.0001$). In conclusion, in vivo the combined *6A/*6A and *6A/*7B define a genotype that is associated with a particularly low activity of caffeine metabolism.

Association of the 'ultra-slow' genotype with bladder cancer risk

The aforementioned 'ultra-slow' genotype (combined *6A/*6A, *6A/*7B, *7B/*7B) was associated with bladder cancer risk in the combined study cohort of 1,692 cases and 1,995 controls (OR = 1.31, $P = 0.0123$) (Table 6A, B). After adjusting for age, gender and smoking habits, a trend was obtained (OR = 1.22; $P = 0.0816$). Analysis of individuals with reported high levels of occupational exposure to bladder carcinogens did not reveal an increased relevance of the 'ultra-slow' genotype in this subgroup (Supplementary Table S12A, B).

Discussion

Genotyping NAT2 is of high relevance, since many drugs but also urinary bladder carcinogens are substrates of this enzyme (Golka et al. 1996, 2002; Hengstler et al.

Table 1 Distribution of the NAT2 7-SNP and tagSNP genotype and UBC risk in all study groups combined stratified by smoking habits

	Cases	Controls	Unadjusted			Adjusted		
			P	OR	95 % CI	*P*	OR	95 % CI
7-SNP genotype								
All combined								
Rapid	0.60	0.58	Reference					
Intermediate	0.35	0.37						
Slow	0.05	0.05	0.7844	1.02	0.89–1.17	0.7732	0.98	0.85–1.13
Total	1,692	1,995						
Non-smokers								
Rapid	0.55	0.54	Reference					
Intermediate	0.41	0.40						
Slow	0.05	0.05	0.8835	0.98	0.75–1.28	0.5974	0.93	0.71–1.22
Total	365	885						
Former smokers								
Rapid	0.60	0.60	Reference					
Intermediate	0.36	0.35						
Slow	0.04	0.05	0.7056	1.08	0.72–1.63	0.7689	1.034	0.83–1.30
Total	767	691						
Current smokers								
Rapid	0.62	0.61	Reference					
Intermediate	0.32	0.35						
Slow	0.06	0.04	0.6395	1.07	0.81–1.41	0.5509	1.09	0.82–1.46
Total	548	397						
tagSNP genotype								
All combined								
Rapid	0.61	0.58	Reference					
Intermediate	0.35	0.37						
Slow	0.04	0.05	0.4636	1.05	0.92–1.21	0.8114	1.02	0.88–1.18
Total	1,702	1,957						
Non-smokers								
Rapid	0.57	0.55	References					
Intermediate	0.39	0.40						
Slow	0.04	0.04	0.6303	1.07	0.82–1.39	0.8538	1.03	0.78–1.35
Total	365	867						
Former smokers								
Rapid	0.62	0.60	Reference					
Intermediate	0.34	0.35						
Slow	0.04	0.05	0.9034	1.01	0.81–1.26	0.6286	1.06	0.84–1.33
Total	773	679						
Current smokers								
Rapid	0.63	0.62	Reference					
Intermediate	0.33	0.34						
Slow	0.05	0.05	0.7291	1.05	0.80–1.38	0.6915	1.06	0.79–1.42
Total	552	396						

The 2-SNP genotype yields the same results as the 7-SNP genotype. The UBC risk of genotypic slow acetylators is compared to the baseline risk of rapid and intermediate genotypes (reference). A detailed analysis of the individual study groups is given in Supplementary Tables S3–S5 for all three phenotype predictors

OR odds ratio, 95 % CI 95 % confidence interval

Unadjusted *P* values ORs and 95 % CIs were estimated using the method of Mantel–Haenszel to account for the different study groups if not indicated otherwise. Adjusted *P* values, ORs and 95 % CIs were estimated by logistic regression models adjusting for age, gender, smoking habits and study groups

1998; Vineis et al. 2001; Hung et al. 2004; García-Closas et al. 2005; Hein 2006, 2009; Sanderson et al. 2007; Agúndez 2008; Moore et al. 2011; Cai et al. 2012). In this study, we further refined NAT2 phenotype prediction by identification of an 'ultra-slow' genotype. This 'ultra-slow' genotype is defined by a combination of *6A/*6A, *6A/*7B, *7B/*7B haplotype pairs and corresponds mainly to the homozygous minor alleles

Table 2 Homozygous minor alleles of C292T and G590A show highest risks for urinary bladder cancer among all seven measured NAT2-SNPs in the combined study group of 1,692 cases and 1995 controls with known NAT2 genotype

A. Frequencies and *P* values assuming different modes of inheritance for all 7 SNPs[a]

SNP	AA		AB		BB		*P*	*P*	*P*
	Case	Control	Case	Control	Case	Control	Genotype difference	AB/BB versus AA	BB versus AA/AB
G191A	0.9953	0.9985	0.0047	0.0015	0.00	0.00	0.0263	0.0263	–
C282T	0.45	0.46	0.42	0.44	0.13	0.10	0.0440	0.8798	0.0161
T341C	0.32	0.30	0.49	0.51	0.19	0.19	0.2469	0.1063	0.9148
C481T	0.34	0.32	0.48	0.51	0.18	0.17	0.1483	0.1150	0.5507
G590A	0.50	0.51	0.39	0.41	0.10	0.08	0.0913	0.9601	0.0368
A803G	0.33	0.34	0.49	0.49	0.17	0.17	0.8876	0.8049	0.6324
G857A	0.93	0.93	0.07	0.07	0.0024	0.0025	0.5759	0.2948	–

B. C282T and G590A confer higher UBC risks than the NAT2 genotype, especially in current smokers (for a detailed analysis of the individual study groups see Supplementary Tables S6 A-D, S7 A-D)[b]

		Cases	Controls	Unadjusted			Adjusted		
				P	OR	95 % CI	*P*	OR	95 % CI
C282T (rs1041983) genotype									
All combined	Rapid (CC)	0.13	0.10	Reference					
	Intermediate (CT)	0.42	0.44						
	Slow (TT)	0.45	0.46	**0.0161**	**1.30**	**1.05–1.60**	0.0882	1.22	0.97–1.52
	Total	1692	1995						
Non-smokers	Rapid (CC)	0.12	0.09	Reference					
	Intermediate (CT)	0.42	0.44						
	Slow (TT)	0.46	0.47	0.2189	1.31	0.85–2.02	0.2994	1.27	0.81–1.98
	Total	365	885						
Former smokers	Rapid (CC)	0.11	0.11	Reference					
	Intermediate (CT)	0.43	0.45						
	Slow (TT)	0.46	0.44	0.7985	1.05	0.74–1.47	0.6316	1.09	0.77–1.55
	Total	767	691						
Current smokers	Rapid (CC)	0.16	0.12	Reference					
	Intermediate (CT)	0.41	0.43						
	Slow (TT)	0.43	0.45	**0.0415**	**1.49**	**1.01–2.19**	0.1010	1.41	0.94–2.11
	Total	548	397						
G590A (rs1799930) genotype									
All combined	Rapid (GG)	0.10	0.08	Reference					
	Intermediate (GA)	0.39	0.41						
	Slow (AA)	0.50	0.51	**0.0368**	**1.28**	**1.01–1.62**	0.1084	1.23	0.96–1.57
	Total	1692	1995						
Non-smokers	Rapid (GG)	0.10	0.07	Reference					
	Intermediate (GA)	0.41	0.39						
	Slow (AA)	0.49	0.54	0.3375	1.25	0.79–1.98	0.4543	1.20	0.74–1.94
	Total	365	885						
Former smokers	Rapid (GG)	0.09	0.08	Reference					
	Intermediate (GA)	0.40	0.43						
	Slow (AA)	0.52	0.49	0.5480	1.13	0.76–1.67	0.4537	1.17	0.78–1.74
	Total	767	691						

Table 2 continued

B. C282T and G590A confer higher UBC risks than the NAT2 genotype, especially in current smokers (for a detailed analysis of the individual study groups see Supplementary Tables S6 A-D, S7 A-D)[b]

		Cases	Controls	Unadjusted			Adjusted		
				P	OR	95 % CI	*P*	OR	95 % CI
Current smokers	Rapid (GG)	0.13	0.09	Reference					
	Intermediate (GA)	0.39	0.53						
	Slow (AA)	0.49	0.49	0.0710	1.48	0.96–2.28	0.1506	1.40	0.89–2.20
	Total	548	397						

Significant *P* values ($P < 0.05$) were indicated in bold

AA homozygous major alleles; AB heterozygous genotypes; BB homozygous minor alleles

OR odds ratio, 95 % CI 95 % confidence interval

[a] *P* values adjusted for study region according to the method of Mantel–Haenszel are given. The results for G191A are based on 1 case from Hungary (0.44 %), 7 cases (6.25 %) and 3 controls (1.61 %) controls from Venezuela

[b] Unadjusted *P* values ORs and 95 % CIs were estimated using the method of Mantel–Haenszel to account for the different study groups. Adjusted *P* values, ORs and 95 % CIs were estimated by logistic regression models adjusting for age, gender, smoking habits and study groups

Table 3 C282T and G590A are associated with particularly low NAT2 activity analysed in vivo by the caffeine test

SNP	Genotype	N	Min	1st Q	Median	3rd Q	Max
G191A	GG	313	0.02	0.20	0.40	1.23	6.55
	GA	0					
	AA	0					
C282T	CC	143	0.03	0.29	0.93	1.53	6.55
	CT	137	0.02	0.17	0.32	0.97	4.90
	TT	**33**	**0.06**	**0.10**	**0.15**	**0.23**	**0.34**
T341C	TT	101	0.06	0.20	1.10	1.85	6.55
	TC	162	0.02	0.18	0.40	1.06	3.45
	CC	50	0.03	0.21	0.27	0.38	0.69
C481T	CC	110	0.06	0.20	0.93	1.78	6.55
	CT	158	0.02	0.18	0.39	1.66	3.45
	TT	45	0.03	0.21	0.28	0.38	0.69
G590A	GG	156	0.02	0.29	0.89	1.49	6.55
	GA	130	0.04	0.17	0.32	0.92	4.90
	AA	**27**	**0.06**	**0.10**	**0.17**	**0.22**	**0.34**
A803G	AA	105	0.06	0.19	1.02	1.78	6.55
	AG	168	0.02	0.19	0.38	1.05	3.50
	GG	40	0.03	0.21	0.28	0.38	0.69
G857A	GG	294	0.03	0.21	0.42	1.27	6.55
	GA	19	0.02	0.10	0.17	0.44	1.78
	AA	0					

For details on mutually exclusive SNP genotypes and test results, see Supplementary Table S8 A, B

Results for the homozygous variant alleles of C282T and G590A are indicated in bold

Min: minimum, 1st Q: 25 % quantile, 3rd Q: 75 % quantile, Max: maximum

The activity of NAT2 in vivo (caffeine test) was measured by the AFMU/1-MX ratio of two caffeine metabolites. A threshold of 0.85 discriminates between phenotypically slow (<0.85) and rapid acetylators. Homozygous variant alleles correspond to slow acetylators

of C282T (rs1041983 T/T) and G590A (rs1799930 A/A). Individuals with the 'ultra-slow' genotype show much lower activities of caffeine in vivo metabolism compared to all other slow genotypes (0.15 vs. 0.22; $P < 0.0001$).

Interestingly, the 'ultra-slow' genotype but not the common slow NAT2 genotype was associated with increased urinary bladder cancer risk in the *IfADo* UBC case–control series. This suggests that individuals with extremely low NAT2 activity may be particularly susceptible to urinary bladder carcinogens. Discrimination between these 'ultra-slow' and other slow metabolizers may be superior over conventional pooling of all slow acetylators. Regarding the reduction in genotyping effort, the C282T-T341C 2-SNP genotype suggested by Cascorbi et al. (1995) and Selinski et al. (2011) also yields this discrimination between slow

Table 4 NAT2 haplotypes according to the latest update of the NAT2 nomenclature (July 22, 2011), amino acids exchanges and frequency in the studied cases and controls

rs identifier Nucleotide exchange		rs1801279 G191A	rs1041983 C282T	rs1801280 T341C	rs1799929 C481T	rs1799930 G590A	rs1208 A803G	rs1799931 G857A	Cases		Controls	
Phenotype	Haplotype											
Rapid	*4	G	C	T	C	G	A	G	736	0.22	909	0.23
Slow	*5A	G	C	**C**	**T**	G	A	G	86	0.03	124	0.03
Slow	*5B	G	C	**C**	**T**	G	**G**	G	1335	0.39	1566	0.39
Slow	*5C	G	C	**C**	C	G	**G**	G	56	0.02	83	0.02
Slow	*6A	G	**T**	T	C	**A**	A	G	1011	0.30	1127	0.28
Slow	*6B	G	C	T	C	**A**	A	G	1	0.00	1	0.00
Slow	*7B	G	**T**	T	C	G	A	**A**	120	0.04	143	0.04
Rapid	*12A	G	C	T	C	G	**G**	G	21	0.01	17	0.00
Rapid	*12C	G	C	T	**T**	G	**G**	G	1	0.00	0	0.00
Rapid	*13	G	**T**	T	C	G	A	G	9	0.00	17	0.00
Slow	*14B	**A**	**T**	T	C	G	A	G	4	0.00	3	0.00
Slow	*14C	**A**	C	**C**	**T**	G	**G**	G	4	0.00	0	0.00
Total									3384		3990	
Position (37.3)		18257704	18257795	181257854	18257994	18258103	18258316	18258370				
Function		Missense	Synonymous	Missense	Synonymous	Missense	Missense	Missense				
Mutation site		*14	*13	*5	*11	*6	*12	*7				
Amino acid exchange		R64Q	Y94Y	I114T	L161L	R197Q	K268R	G286E				

Variant alleles are indicated in bold

and 'ultra-slow' haplotypes. In Caucasians and similar populations, C282T may serve as a tagSNP for the *6A and *7B haplotypes and T341C indicates the *5 haplotype cluster. Our results correspond well to previous studies of Cascorbi et al. (1995) and Ruiz et al. (2012), demonstrating a notably decreased NAT2 activity in vivo in *6/*6 compared to *5/*5 genotypes. Similarly large and recent studies on UBC indicate similar effects of *5B, *6A and *7B haplotypes (García-Closas et al. 2005) or no effects of the homozygous variant alleles of the NAT2 SNPs that are present in these haplotypes (Moore et al. 2011). Interestingly, the well-known enhanced risk of anti-tuberculosis drug-induced hepatotoxicity in slow acetylators (Cai et al. 2012) seems to be more pronounced for *6/*6 genotypes in European (Leiro-Fernandez et al. 2011) and Asian (Huang et al. 2002; Lee et al. 2010; An et al. 2012) study groups, though the slow *5 haplotypes are less common in East Asian populations (Sabbagh et al. 2011), as well as in several large Brazilian studies (Possuelo et al. 2008; Teixeira et al. 2011) but not all (Santos et al. 2013). A meta-analysis of 14 studies indicates that tuberculosis patients with NAT2*6/*6 genotypes may have an even higher risk of anti-tuberculosis drug-induced hepatotoxicity than *5/*5 tuberculosis patients (all slow vs. rapid genotypes OR = 3.05, *6/*6 vs. rapid OR = 3.61, *5/*5 vs. rapid OR = 2.45; Selinski et al. 2013b).

A limitation of the current study is that the 'ultra-slow' genotype was significantly associated with bladder cancer risk in the univariate analysis, whereas only a trend was obtained in the multivariate regression adjusted for age, gender and smoking habits. It should also be noted that the current literature concerning the identification of differences between slow haplotypes regarding velocity and substrates remains controversial (Cascorbi et al. 1995; Gross et al. 1999; Bolt et al. 2005; Hein 2006, 2009; Golka et al. 2008; Ruiz et al. 2012). In particular, Hein (2006) showed the lowest acetylation speed in vitro for the *5B haplotypes containing the slow T341C, C481T and A803G SNPs. Generally, G191A (*14 haplotypes), T341C (*5 haplotypes) and G590A (*6 haplotypes) are associated with reductions in N- and O-acetylation capacity in a number of different bacterial and eukaryotic expression systems (Zang et al. 2007; Walraven et al. 2008). The effect of G857A (*7 haplotypes) seems to be substrate dependent (Zang et al. 2007; Walraven et al. 2008). These four missense SNPs result in a reduction in protein. In case of G191A, G590A and G857A but not T341C, this seems to be related to the NAT2 protein stability. The silent SNPs

Table 5 Activity of NAT2 in vivo (caffeine test) suggests that *6A/*6A and *6A/*7B genotypes are particularly slow

A. In vivo activity in NAT2 genotypes

Phenotype	Genotype	N	Min	1st Q	Median	3rd Q	Max
Rapid	*4/*4	20	0.20	1.51	2.04	2.80	6.55
Intermediate	*4/*5A	3	0.74	0.74	0.79	1.04	1.04
	*4/*5B	68	0.17	0.93	1.20	1.63	3.45
	*4/*5C	2	0.59	0.59	0.69	0.79	0.79
	*4/*6A	42	0.36	0.92	1.42	1.97	4.90
	*4/*7B	4	0.97	1.01	1.13	1.49	1.78
	*6A/*12A	2	0.57	0.57	2.04	3.50	3.50
Slow	*5A/*5B	10	0.18	0.21	0.27	0.29	0.47
	*5A/*6A	3	0.08	0.08	0.14	0.18	0.18
	*5B/*5B	35	0.03	0.21	0.28	0.38	0.69
	*5B/*6A	70	0.04	0.16	0.21	0.32	0.72
	*5B/*5C	5	0.09	0.20	0.26	0.27	0.63
	*5B/*7B	9	0.02	0.10	0.14	0.20	0.44
	*5C/*6A	7	0.10	0.14	0.29	0.45	0.60
	*6A/*6A	27	0.06	0.10	0.17	0.22	0.34
	*6A/*7B	6	0.07	0.09	0.11	0.17	0.18
'Ultra-slow'		33	0.06	0.10	0.15	0.19	0.34
All other slow		139	0.02	0.16	0.22	0.34	0.72
Total		313					

B. Pairwise comparisons using the Tukey test indicates that the NAT2 activity of *6A/*6A genotypes is significantly lower compared to *5A/*6A and *5B/*5B genotypes[a]

Genotype	*5B/*5B	*5B/*6A	*6A/*6A
*5B/*5B	–	0.0684	0.0001
*5B/*6A	0.0684	–	0.0186
*6A/*6A	0.0001	0.0186	–

C. Pairwise comparisons using the Tukey test indicate that the NAT2 activity of 'ultra-slow' *6A/*6A, *6A/7B genotypes are significantly lower compared to *5B/*5B and *5B/*6A, *5B/*7B genotypes[b]

Genotype	*5B/*5B	*5B/*6A, *5B/*7B	'Ultra-slow'
*5B/*5B	–	0.0250	<0.0001
*5B/*6A, *5B/*7B	0.0250	–	0.0081
'Ultra-slow'	<0.0001	0.0081	–

Min: minimum, 1st Q: 25 % quantile, 3rd Q: 75 % quantile, Max: maximum, 'Ultra-slow': combined *6A/*6A, *6A/*7B, *7B/*7B genotypes

[a] The F test indicated global differences ($P = 0.0002$); the Levene test indicated equal variances ($P = 0.0715$); *6A/*6A versus all other slow genotypes $P < 0.0001$ (Welch's t test)

[b] The F test indicated global differences ($P < 0.0001$); the Levene test indicated unequal variances ($P = 0.0377$); 'ultra-slow' versus all other slow genotypes $P < 0.0001$ (Welch's t test)

C282T and C481T as well as the missense SNP A803G seem to have no effect on acetylation capacity, protein stability or mRNA levels in vitro (Zang et al. 2007; Walraven et al. 2008). In summary, in vitro studies suggest that *5 haplotypes are particularly slow, whereas in vivo *6 seems to be more important. Therefore, it will be of high interest whether the 'ultra-slow' NAT2 genotype and its association with particularly low caffeine metabolism and increased bladder cancer risk will be confirmed in independent cohorts.

In conclusion, we suggest to further refine NAT2 phenotype prediction by consideration of an 'ultra-slow' genotype based on combined *6A/*6A, *6A/*7B and *7B/*7B genotypes.

Table 6 Slow *6A/*6A genotypes as well as 'ultra-slow' *6A/*6A, *6A/*7B and *7B/*7B genotypes conferred higher UBC risk than other slow genotypes, a detailed analysis of the study groups stratified for smoking habits is given in Supplementary Tables S9–S11

Genotype	Cases	Controls	Unadjusted			Adjusted		
			P	OR	95 % CI	*P*	OR	95 % CI
A. Slow *6A/*6A genotypes compared to other slow and rapid genotypes as well as to all genotypes combined								
Rapid	0.40	0.42	Reference					
Other slow	0.49	0.50	0.8111	0.98	0.85–1.13	0.4655	0.95	0.81–1.10
*6A/*6A	0.10	0.08	0.0568	1.27	0.99–1.63	0.1965	1.19	0.91–1.55
Total	1692	1995						
All other	0.90	0.92	Reference					
*6A/*6A	0.10	0.08	**0.0369**	**1.29**	**1.02–1.63**	0.1084	1.23	0.96–1.57
Total	1692	1995						
B. 'Ultra-slow' NAT2 *6A/*6A, *6A/*7B and *7B/*7B genotypes compared to other slow and rapid genotypes as well as to all genotypes combined[a]								
Rapid	0.40	0.42	Reference					
Other slow	0.47	0.48	0.6523	0.97	0.84–1.12	0.4007	0.94	0.80–1.09
'Ultra-slow'	0.13	0.10	**0.0276**	**1.29**	**1.03–1.62**	0.1764	1.18	0.93–1.50
Total	1692	1995						
All other	0.87	0.90	Reference					
'Ultra-slow'	0.13	0.10	**0.0123**	**1.31**	**1.06–1.63**	0.0816	1.22	0.98–1.54
Total	1692	1995						

OR odds ratio, 95 % CI 95 % confidence interval

Unadjusted *P* values ORs and 95 % CIs were estimated using the method of Mantel–Haenszel to account for the different study groups. Adjusted *P* values, ORs and 95 % CIs were estimated by logistic regression models adjusting for age, gender, smoking habits and study groups

Significant results ($P < 0.05$) were indicated in bold

[a] 'Ultra-slow' are the *6A/*6A, *6A/*7B and *7B/*7B genotypes

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