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N-Acetyltransferase 2 (NAT2) in Tunisian Population: Correlation Between Acetylation Phenotype and Genotype

Emna Gaies[a], Maher Kharrat[b], Nadia Jebabli[a]*, Basma Ben Njima[b], Anis Klouz[a], Habiba Chaabouni[b] and Mohamed Lakhal[a]

[a]Service de Pharmacologie Clinique - Centre National de Pharmacovigilance de Tunis - Tunisie

[b]Laboratoire de Génétique Humaine, Faculté de Médecine de Tunis - Tunisie

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Abstract. One hundred tuberculous patients were studied during 2004-2005 to determine acetylation phenotype, frequent mutations of NAT2 gene and to compare acetylation phenotype with NAT2 genotype in Tunisian population. Acetylation phenotype was determined by determination of acetylation index. Five mutations of NAT2 gene were evaluated by PCR/RFLP. Results show bimodal distribution of acetylation SA and RA phenotype, 75% and 25% and genotype 56% and 44%, respectively. Ten NAT2 alleles were found, NAT2*4 being the major one. Thirty-two different genotypes were found (9 RA and 23 SA). The major one was NAT2*6 B/NAT2*4. The concordance value was 79%. A good sensibility (98, 2%) of acetylation test for SA detection was found. Thus, acetylation phenotype in SA is predicted with poor error risk.

Keywords: Tunisian population, phenotype, genotype, N-acetyltransferase 2, polymorphism

Introduction

The acetylation polymorphism was discovered over 40 years ago, following isoniazid toxicity differences observed in patients treated for tuberculosis (Huges *et al*., 1954). Subsequently, the differences in isoniazid toxicity were attributed to genetic variability in N-acetyltransferases which are important for transformation of many drugs (isoniazid, dapsone, procainamide, sulfamethazine) (Weber, 1987) and carcinogens (2-naphtylamine, 2-aminofluorene, 4-aminobiphenyl and benzidine) (Hein *et al*., 1992; Grant *et al*., 1991).

There are two functional human arylamine N-acetyltransferases. The first is NAT1 which was originally thought to be monomorphic (Zhao *et al*., 1998; Blum *et al*., 1990), the second is polymorphic NAT2 (Grant *et al*., 1989).

In humans, NAT1, NAT2 and a pseudogene N-acetyltransferase (NATP) are located on chromosome 8p213 (Sim *et al*., 2008; Hickman *et al*., 1994; Blum *et al*., 1990) and are producted on single intronless protein coding exons of 870 pb (Graf *et al*., 1992, Grant *et al*., 1991). Gene expression is autosomal and codominant (Blum *et al*., 1990, Vincent-Viry *et al*., 1994).

In human population, 26 alleles have been reported for NAT2 (Gross *et al*., 1999). Several studies (Vatsis *et al*., 1995; Bell *et al*., 1993; Deguchi *et al*., 1990; Blum *et al*., 1990) admitted that NAT2 contains seven different mutations: five mutations led to amino acid changes and two exerted no influence on the amino acid sequence.

*Author for correspondence; E-mail: nadiascience@voila.fr

Generally mutant alleles are known to show slow acetylation activity (Hein *et al*., 1994). A slow acetylator phenotype is frequently observed in subjects with two germ line copies of alleles containing any of several single nucleotide substitutions. Rapid acetylators have at least one wild type NAT2*4 (Vatsis *et al*., 1995). Distinction between rapid acetylator and slow ones can be easily determined using phenotypic tests by administration of a probe drug as isoniazid, dapsone, caffeine, sulfamethazine (Kita *et al*., 2001; Attitallah *et al*., 2000; Meisel *et al*., 1997; Queiroz *et al*., 1997; Grant *et al*., 1984). The aim of this study was to determine the acetylation phenotype, to identify the frequent mutations of the NAT2 gene and to compare acetylation phenotype with NAT2 genotype in a Tunisian population.

Materials and Methods

The prospective study (July 2004 - April 2005) included 100 tuberculosis patients (suffering for pulmonary tuberculosis, lymph nodes tuberculosis, urogenital tuberculosis, cerebral tuberculosis and peritoneal tuberculosis).

Determination of acetylation phenotype. The acetylation phenotype was determined in 100 subjects by determination of acetylation index, 3 h after isoniazid administration according to Vivien method (Vivien *et al*., 1973).

Blood samples were collected 3 h after administration of isoniazid (5 mg/kg). For 2 mL of serum saturated with 2 g ammonium sulphate, 3 methylbutanol and trichloroacetic acid were added. Then the mixture was shaken and centrifuged.

After that a second extraction was made with chloridric acid, and finally the organic phase was resuspended with vanillin solution. After a rest of 5 min, absorbance of the obtained solution was measured by spectrophotometric method at 360 nm.

A calibration curve has been made in order to determine, in each subject, the concentration of isoniazid after 3 h of isoniazid administration, acetylation index (I_3 = isoniazid administered dose mg/kg per day/isoniazid concentration after 3 h + 0.6) (Vivien *et al*., 1973), therapeutic range (minimal dose = $1.6 \times$ weight/I_3 and maximal dose = $2.6 \times$ weight/I_3) and as a result, the determination of the recommended dose.

Conventionally, when I_3 was higher than 0.65, the subject was considered as a slow acetylator and when I_3 was lower than 0.65 the subject was considered as a rapid acetylator.

Molecular genetic studies. ***PCR amplification.*** Fifteen milliliter of blood was collected from each subject in EDTA sterile tubes for genomic analysis. Genomic DNA was prepared from peripheral blood leukocytes by standard procedures (Miller *et al*., 1988).

Amplifications of 1211 bp fragment containing the coding region were performed in a volume of 100 µL containing 0.1 µg of genomic DNA, 100 ng of each primer, 0.20 mM of each deoxynucleotide triphosphate, 2 mM of $MgCl_2$, 1X PCR Buffer (Eurobio, Paris, France) and 2.5 U of Taq DNA polymerase (Eurobio) (Table1). Thirty cycles of amplification were achieved, each one on a denaturation step for 30 sec at 95 °C, annealing step for 30 sec at 56 °C and extension step for 30 sec at 72 °C. Amplification products were analysed on 1% agarose gel electrophoresis in presence of ethidium bromide stain.

Detection of NAT2 mutations. The analysis was limited to five more common point mutations of the NAT2 gene: R64Q (G to A at nucleotide (nt) position 191), I114T (T to C at nt position 341), R197Q (G to A at nt position 590), K268R (A to G at nt position 803) and G286E (G to A at nt position 857) (Cascorbi *et al*., 1995). The two silent mutations at nucleotide positions 282 and 481 are not included because they did not have any influence on enzyme activity (Cascorbi *et al*., 1995; Hein *et al*., 1994).

Restriction enzyme digestion of PCR-amplification DNA was used to detect the presence of R64Q, R197Q, K268R and G286E mutations. The I114T mutation was detected by allele-specific PCR method.

Analysis of R64Q and G286E mutations. For analysis of these two mutations, the amplification of 1211 bp fragment of NAT2 gene was carried out using the primers P_1 and P_2 (Table 1) and followed by digestion with appropriate restriction enzymes according to the manufacturer's protocol (Table 2).

Analysis of R197Q and K268R mutations. For analysis of these two mutations, a nested PCR was performed in the 1211 bp fragment using primers P_3 and P_4 for R197Q mutation and P_5 and P_6 for K268R mutation (Table 1). The nested PCR products were digested separately with appropriate restriction enzymes according to the manufacturer's protocol (Table 2).

Analysis of I114T mutation. For identifying this mutation, allele-specific PCR was performed allowing amplification of an 816 bp fragment. The amplification was made separately in two PCRs, one with primer P_7 containing the wild-type base (T) at the 3′-end (Table 2), and the other with primer P_8 containing the mutant base (C) at the 3′-end (Table 2).

Heterozygous analysis. For determining allelic localisation of different mutations in subjects having at least two different mutations, DNA was purified in order to analysis mutations as described by (Cascorbi *et al*., 1995). Products obtained from the amplification of the 1211 bp were incubated with the restriction enzyme to determine the first mutation. After

Table 1. PCR primers used for amplification of the NAT2 gene, primers for allele-specific PCR to evaluate I114T mutation and amplification of small fragments containing nt positions 590 and 803

Primer	Specificity	Primer sequence
P_1	NAT2 3′	5′-GTTTTCTAGCATGAATCACTCTGC
P_2	NAT2 5′	5′-GTCACACGAGGAAATCAAATGC
P_3	TaqI 3′	5′-GCAAGGAACAAAATGATGTGG
P_4	TaqI 5′	5′-CCTGGACCAAATCAGGAGAG
P_5	DdeI 3′	5′-ACACAAGGGTTTATTTTGTTCC
P_6	DdeI 5′	5′-GTGGGCTTCATCCTCACCTA
P_7	Wild type 341T 5′	5′-CCTGCAGGTGACCAT
P_8	Mutatant 341C 5′	5′-CCTGCAGGTGACCAC

Table 2. Restriction enzymes used for the detection of fourth mutations of the NAT2 gene

Mutations	Restriction enzyme	Cleavage site*
R64Q	AluI/MspI	96 189 379
		96 - 379
G286E	BamHI	856
		-
R197Q	TaqI	588
		-
K268R	DdeI	776 -
		776 803

*first line = wild type; second line = mutation type.

separation on agarose gel, fragments containing the second mutation were cut out using a sterile scalpel and purified using Kit Wizard® SV Gel and PCR Clean Up System (Promega). Then determination of the second mutation was made using direct digestion with the appropriate restriction enzyme or allele specific PCR followed by digestion.

Statistics analysis. Alleles and genotypes percent frequencies are given, together with 95% confidence limits. Concordance values (percentages) and κ statistics are reported as measures of agreement between genotypes and phenotypes (Cohen, 1960). Sensitivity, specificity and predictive values were calculated for acetylation test.

Results and Discussion

Age of the patients under study varied from 15 to 84 years with 40 years as median age. The sex ratio M/F was 1.7. Forty nine per cent of the patients were treated for pulmonary tuberculosis and 40% for ganglionic one. The median dose of isoniazid administrated was 267 mg per day. Hepatic toxicity was observed in two cases and pruritus in four cases.

The results showed a bimodal distribution of acetylation phenotype with 75% slow acetylators (SA) and 25% rapid acetylators (RA). The frequency of the tested point mutations demonstrate that I114T, K268R and R197Q mutations were the predominant ones, with allelic frequency in the range of 37.5%, 34% and 31.5%, respectively, whereas, the G286E mutation was present only in 1.5% of cases.

Ten NAT2 alleles were found in the population (Table 3), NAT2*4 was the major allele accounting for 24.5% of the total (IC 95%: 18.54 - 30.46).

Table 3. Frequency of NAT2 alleles

Mutations	Alleles	Number	Frequency founded	IC à 95%
Absence de mutation	NAT2*4	49	24.5	18.54 ; 30.46
K268R, I114T	NAT2*5C	32	16	10.92 ; 21.08
I114T	NAT2*5D	25	12.5	7.92 ; 17.08
R197Q, I114T	NAT2*5E	11	5.5	3.89 ; 7.11
R197Q	NAT2*6B	38	19	13.57 ; 24.43
R197Q, K268R	NAT2*6C	2	1	- 0.37 ; 2.37
G286E	NAT2*7A	2	1	- 0.37 ; 2.37
K268R	NAT2*12A	27	13.5	8.77 ; 18.23
R64Q	NAT2*14A	4	2	0.06 ; 3.94
R64Q, R197Q	NAT2*14D	2	1	- 0.37 ; 2.37
I114T, K268R, G286E	-	1	0.5	- 0.47 ; 1.47
I114T, R197Q, K268R	-	7	3.5	0.96 ; 6.04
Total	200	100		

NAT2*6B, NAT2*5C, NAT2*12A and NAT2*5D were present, at a rate of 19%, 16%, 13,5% and 12.5%, respectively. Each of the remaining alleles accounted for 1 - 5.5%.

Evidence for two new alleles was found, the first contained substitutions at nt 341, 803 and 857, the second contained substitutions at nt 341, 803 and 590.

Thirty two different genotypes were found in the study (Table 4), 9 different rapid acetylator genotype and 23 different slow acetylators. Distribution of rapid (heterozygotes and homozygotes) and slow acetylator genotypes was 44% and 56%, respectively.

The major genotype was NAT2*6B/NAT2*4 at a level of 15% (IC 95%: 8.01 - 21.99%). The next most frequent genotype was NAT2*5C/ NAT2*12A which accounted for 12%.

Table 4. Frequency of NAT2 genotypes

	Genotype	Number	Frequency founded	IC à 95%
Rapid genotype	NAT2*4/NAT2*4	5	5	0.73.% ; 9.27%
	NAT2*5C/NAT2*4	4	4	0.16% ; 7.84%
	NAT2*5D/NAT2*4	7	7	2% ; 12%
	NAT2*6B/NAT2*4	15	15	8.01% ; 21.99%
	NAT2*6C/NAT2*4	1	1	- 0.95% ; 2.95%
	NAT2*12A/NAT2*4	2	2	0.26% - 7.55%
	NAT2*5E/NAT2*4	6	6	2.4% - 13.51%
	NAT2*14A/NAT2*4	1	1	- 0.95% ; 2.95%
	I114T, R197Q, K268R/NAT2*4	3	3	- 0.34% ; 6.34%
Slow genotype	NAT2*5C/NAT2*5C	1	1	- 0.95% ; 2.95%
	NAT2*6B/NAT2*5C	8	8	2.69% ; 13.31%
	NAT2*7A/NAT2*5C	1	1	- 0.95% ; 2.95%
	NAT2*12A/NAT2*5C	12	12	5.64 % ; 18.36%
	I114T, R197Q, K268R/NAT2*5C	1	1	- 0.95% ; 2.95%
	NAT2*5C/NAT2*5D	2	2	- 0.74% ; 4.74%
	NAT2*5D/NAT2*5D	2	2	- 0.74% ; 4.74%
	NAT2*12A/NAT2*5D	4	4	0.16% ; 7.84%
	NAT2*5C/NAT2*5E	1	1	- 0.95% ; 2.95%
	NAT2*5E/NAT2*5E	1	1	- 0.95% ; 2.95%
	I114T, R197Q, K268R/NAT2*5E	1	1	- 0.95% ; 2.95%
	NAT2*5D/NAT2*6B	8	8	2.69% ; 13.31%
	NAT2*5E/NAT2*6B	1	1	- 0.95% ; 2.95%
	NAT2*6B/NAT2*6B	2	2	- 0.74% ; 4.74%
	NAT2*6C/ NAT2*6B	1	1	- 0.95% ; 2.95%
	I114T, K268R, G286E/NAT2*7A	1	1	- 0.95% ; 2.95%
	NAT2*6B/NAT2*12A	2	2	- 0.74% ; 4.74%
	NAT2*12A/NAT2*12A	2	2	- 0.74% ; 4.74%
	NAT2*14D/NAT2*12A	1	1	- 0.95% ; 2.95%
	NAT2*12A/NAT2*14A	1	1	- 0.95% ; 2.95%
	NAT2*14A/NAT2*14A	1	1	- 0.95% ; 2.95%
	NAT2*5C/NAT2*14D	1	1	- 0.95% ; 2.95%
	I114T, R197Q, K268R/I114T, R197Q, K268R	1	1	- 0.95% ; 2.95%
	Total	100	100	

Each one of the other genotypes *6C/*4, *14A/*4, *5C/*5C, *7A/*5C, *5C/*5E, *5E/*5E, *5E/*6B, *6C/*6B, *5E/*12A, *14D/*12A, *12A/*14A, *14A/*14A, *5C/*14D, I114T, R197Q, K268R/*5C, I114T, R197Q, K268R/5E, I114T, R197Q, K268R/I114T, R197Q, K268R was noted in one case.

Distribution of phenotype within each genotype shows that subjects with two substituted alleles have a higher mean of acetylation index than heterozygous subjects (1.29 *vs* 0.68); a lower mean of acetylation index was found in individuals with two wild type alleles than in heterozygous (0.68 *vs* 0.54).

Concordance value was 79%. The κ statistics indicated moderate agreement between genotype and phenotype with K = 0.55 (Fig. 1).

Distribution analysis of phenotype within each genotype was made in three groups of patients selected according to their genotypes (A: patients having two substituted alleles, B: heterozygous patients having the wild type and a substituted allele, C: patients were homozygous for the wild type (NAT2*4) allele). These results show that the group A consisted of 56 patients genotypically slow acetylators. Only one was phenotypically rapid acetylator (I3 = 0.58). The group B consisted of 39 heterozygotes among these 20 (51.28%) were phenotypically rapid and 48.72% were slow acetylators. Finally group C consisted of 5 patients having no transition and only one was phenotypically rapid.

The results also show a good sensibility (98.2%) and a high negative predictive value (96%) for the acetylation test for the detection of slow acetylators.

Genotypic study on NAT2 and correlation between acetylation phenotype and genotype was achieved in a large sample of the world population (Singh *et al*., 2009; Loktionov *et al*., 2002; Gross *et al*., 1999; Krause *et al*., 1998; Meisel *et al*., 1997; Cascorbi *et al*., 1995; Bell *et al*., 1993). The present study tries to evaluate this correlation in Tunisian population.

Determination of acetylation index after isoniazid administration is used for determining the acetylation phenotype. This method was chosen because the population under study was composed of tuberculosis patients treated by isoniazid. This method was described by Vivien *et al*. (1973) and had numerous interests: easy to perform, allows identification of phenotype as well as calculation of the therapeutic range and determination of the recommended dose.

Phenotypic results revealed a bimodal distribution of acetylation phenotype with 75% slow acetylators (SA) and 25% rapid acetylators (RA). Mehiri *et al*. (2005) determined

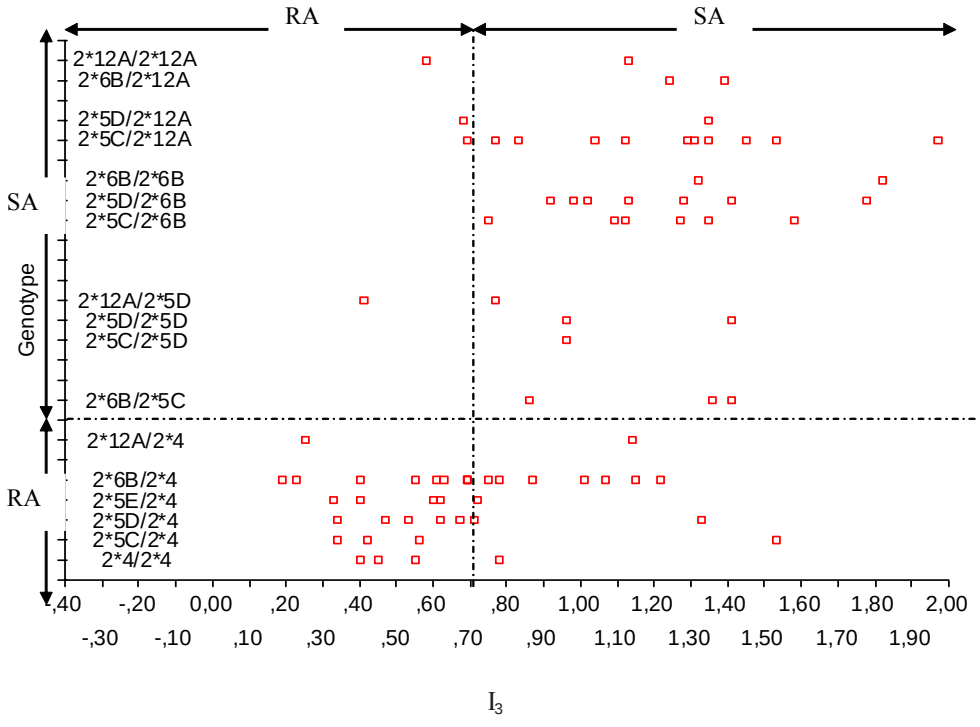

Fig. 1. Distribution of NAT2 phenotypes *vs* NAT2 genotype.

acetylator phenotype in 620 Tunisian tuberculosis patient, with 63.1% SA and 36.9% RA.

The nucleotide substitutions of NAT2 occur at several positions; all individuals were tested by PCR/RFLP for the five most frequent mutations described in literature (substitution at position: 191, 341, 590, 857 and 803). I114T, K268R and R197Q mutations were the predominant ones with an allelic frequency of the order of 37.5%, 34% and 31.5%, respectively. Whereas, R64Q and G286E mutations were not common, being observed in 3% and 1.5% of cases, respectively.

These results are in agreement with those of Cascorbi *et al*. (1995), who found that I114T mutation was the most frequent one (46.5%) and G286E mutation was rare (1.3%). Gross *et al*. (1999) did not find any transition at the nt 191 (R64Q). These results suggest that I114T mutation is common in Caucasians and that R64Q is rarely represented.

Distribution of NAT2*4 and NAT2*5C alleles in the data are close to those reported in Caucasian population (American, Polish and Turkish population) (Gross *et al*., 1999; Ayanacioglu *et al*., 1997; Morzikiewicz *et al*., 1996) they were, respectively, present in 24.5% of (IC 95%: 18.54 - 30.46) and in 16% of (IC 95%: 10.92 - 21.08) of the total.

NAT2*6B and NAT2*12A were also frequent, they accounted for 19% and 13.5%, respectively. These two alleles are not frequently represented in other populations. Gross *et al*. (1999) demonstrated that NAT2*6B is present only in 1.8% of American population and Kukongviriyapan *et al*. (2003) demonstrated that this allele accounted for 4.7% in Thai population.

In the present study, evidence of two new alleles was found; the first contained substitutions at nt 341, 803 and 857. The second contained substitutions at nt 341, 803 and 590.

The major genotypes found in the study were NAT2*6B/NAT2*4 which accounted for 15% (IC 95%: 801 - 21.99%). The next most frequent genotype was NAT2*5C/NAT2*12A which accounted for 12%.

Lee *et al*. (2002) found that in Korean population, NAT2*6B/NAT2*4 was present only in 1.1% of cases and difference in distribution of alleles and genotypes may be explained by genetic polymorphism of NAT2 gene.

Analysis of concordance between NAT2 genotype and acetylation phenotype showed a moderate agreement between genotype and phenotype (K = 0.55, concordance value was 79%) for the bimodal model. Results also show a good sensibility (98.2%) and a high negative predictive value (96%) of the acetylation test for the detection of slow acetylators.

Other studies indicate good correlation between phenotype and acetylation genotype; the rate of concordance was about 93.3% (Cascorbi *et al*., 1995), 90.2% (Lee *et al*., 2002), 90.9% (Gross *et al*., 1999), 97.5% (Graf *et al*., 1992), and 100% (Ilett *et al*., 1993).

Multiple hypothesis are possible to explain this disconcordance such as:

- Biological variability (inter and intra-individual) in phenotypic expression of certain genotype may explain such cases.
- Variability in procedure of acetylation test may also explain cases of discrepancy in which acetylation index are around 0.65. This may explain the case observed in the present study belonging to group A having a slow genotype with acetylation index of about 0.58.

Phenotypic determination by measurement of acetylation index has some limitations. Generally, a single phenotypic measurement is made and this one may be affected by hepatic or renal failure, by medications having acetylating metabolism that may interact with the substance test in the enzymatic site (Gross *et al*., 1999).

Cascorbi *et al*. (1995) found that discrepant cases tended to be more frequent among rapid genotypes of subjects older than the median age of 60 years and explain this due to slight reduction in hepatic or renal function in elderly patients.

The genotyping methods used in the present study analyzed five single nucleotide mutations within the coding region, which are the most described in literature (Bell *et al*., 1993; Vatsis *et al*., 1991; Deguchi *et al*., 1990).

The discrepancy observed in group B and C of the population may be explained by the presence of other nucleotide substitutions that are probably more frequent in Tunisian population than in Caucasian one.

Comparison of acetylation index between group A, B and C revealed that the group A, which was composed of patients having two substituted alleles, had the highest acetylation index (1.23). The group B had an acetylation index about 0.68 higher than the acetylation index found in group C (0.54).

These results are in concordance with those of Gross *et al*. (1999) and illustrate the fact that acetylation metabolism is lower in subjects having two substituted alleles and that the absence of nucleotide substitution allows a rapid acetylation metabolism.

Conclusion

In conclusion genotyping is an accurate method for determining acetylation status; phenotyping also allows to well

predict acetylation status, especially in slow acetylators who are more exposed to an overdose and consequently to a drug intoxication.

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