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NAT2 Gene Polymorphisms and Plasma Isoniazid Concentration in Vietnamese Tuberculosis Patients

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Abstract

Isoniazid (INH) is one of the most common drugs for tuberculosis (TB) treatment and INH acetylation catalyzed by non-inducible hepatic enzyme arylamine N-acetyltransferase type 2 (*NAT2*). The isoniazid acetylation rates, which depend on *NAT2* genotypes, is constant in an individual but can changes between patients. Phenotypic groups can be classified based on the genotype: slow, intermediate, and rapid acetylators. This study was performed to identify the relation between *NAT2* gene polymorphisms and plasma INH concentrations among the different genotypes of Vietnamese tuberculosis patients. Blood samples of 136 adult TB patients treated with INH were collected and genotyped for *NAT2* gene polymorphisms using Sanger sequencing. Two-hour post-dosing INH plasma concentrations were determined by high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). Among the 136 patients genotyped, there were 43 (31.62 %), 58 (42.65 %), and 35 (25.74 %) of slow, intermediate, and rapid acetylation phenotypes, with two-hour post dosing INH plasma concentrations of 3.4, 2.7, and 2.2 μg/ml, respectively. The differences in INH concentrations among the three genotypes were significant ($P < 0.05$). Genotyping of TB patients from Vietnam for *NAT2* gene polymorphism revealed that 48 percent of the study population comprised slow acetylators. Two-hour INH levels were significantly different among CC and TT homozygous genotypes of *NAT2*(C282T), as 2.7 μg/ml and 3.9 μg/ml, respectively. This suggests that *NAT2*(C282T) could play a role in INH metabolism in TB patients.

Keywords: N-acetyltransferase 2 enzyme, *NAT2* polymorphisms, Isoniazid, Tuberculosis

Introduction

Worldwide, tuberculosis (TB) is an infectious disease with the highest mortality; millions of people continue to fall sick with the disease each year. Vietnam ranks 16th among 30 high TB burden countries, with an estimated incidence of 124 new cases per 100,000 people in 2017 [1]. Isoniazid (INH) is a key agent and is widely used in TB treatment and prevention. The antimicrobial activity of INH is selective for *Mycobacterium tuberculosis*. The major pathway for metabolizing INH involves acetylation to acetyl INH, catalyzed by hepatic and intestinal enzyme N-acetyltransferase 2 (*NAT2*). This metabolism can create many hepatotoxic products, such as hydrazine and acetyldiazen. Despite the rather successful therapeutic effects with INH, there are still treatment failures and uncontrolled adverse effects (most commonly, liver injury and, occasionally, mortality) [1].

During the last ten years, personalized medicine has received lot of attention from scientists with the aims of giving each individual patient a precise medical treatment. Using inexpensive gene testing, such

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as direct PCR, many gene variants have been identified to support the treatment of non-infectious diseases, like psoriatic arthritis, or infectious disease, such as tuberculosis [2]. In tuberculosis treatment, the INH acetylation rate varies between patients, and is divided into three phenotypic groups: slow, intermediate, and rapid acetylators [3]. Several clinical reports have shown that slow acetylators have a higher risk of INH-induced liver injury in different populations [4]. In contrast, treatment failure usually occurs in rapid acetylators [5]. *NAT2* activity status is controlled by haplotypes composed of several single nucleotide polymorphisms (SNPs) in the *NAT2* gene [6,7]. The *NAT2* gene is located on chromosome 8p22, with a single open reading frame of 870 bp. Generally, single nucleotide substitutions in *NAT2* usually lead to low N-acetylation activity, decreased NAT2 expression, or enzyme instability. The wild type *NAT2*4* allele, which has no single nucleotide substitutions in *NAT2*, is associated with the rapid acetylator phenotype. The acetylation phenotype can be predicted through the genotype of some SNPs, including missense SNPs (T341C, G590A, A803G and G857GA) and silent SNPs (C282T and C481CT) [4,8].

Studies of the effect of *NAT2* genotypes on INH metabolism can improve the effectiveness of treatment, while the adverse effects associated with this drug can be minimized [9]. Previous studies in other populations have reported an association of *NAT2* genotypes with INH acetylation; however, this association has never been reported in Vietnam. INH acetylation and *NAT2* genotypes data can be used to improve the quality of TB management and to decrease the risk of INH toxicity in TB treatment. In this present study, we investigated the various *NAT2* genotypes found among Vietnamese tuberculosis patients and compared plasma INH concentrations among these different genotypes.

Materials and methods

Patients

TB patients were recruited from the National Lung Hospital, Hanoi Lung Hospital, and 74 military hospitals during March 2014 to September 2018. Patients were eligible to take part in this study if they met the following criteria: (i) aged 16 years or above; (ii) initial diagnosis: new and retreatment TB patients; (iii) indications for Isoniazid, and (iv) inpatient treatment at the hospital. This study was approved by the ethics committee of the School of Medicine and Pharmacy, Vietnam National University Hanoi, coding number IRB-VN01016. Our study was composed of 136 TB patients. Venous blood samples were collected in tubes containing ethylenediamine tetraacetic acid (EDTA) for *NAT2* genotyping, and in heparinized tubes after an oral administration of 5 mg of isoniazid per kg of body weight (Isoniazid Tablets BP100 mg, Macleods Pharmaceuticals Ltd.) to determine the INH concentration.

NAT2 genotyping

Genomic DNA was extracted from blood samples by using the E.Z.N.A blood DNA Mini Kit (Omega-Biotek Inc., USA) according to the manufacturer's recommended procedure. The extracted DNA products were analyzed on a 1 % agarose gel and measured at OD_{280} and OD_{260} on a NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., USA). The fragment 1093 bp of the *NAT2* gene was amplified by PCR with a pair of specific primers (5'-GGA ACA AAT TGG ACT TGG-3' and 5'-TCT AGC ATG AAT CAC TCT GC-3'). PCR mixture was composed of 20 ng/µl DNA template, 0.5 µM of each primer (Phusa Biochem Inc., Vietnam), and Kapa 2G™ Robust HotStart ReadyMix 2x (Kapa Biosystems Inc., USA). PCR program settings included preheating at 95 °C for 3 min, 35 cycle of 95 °C for 10 s, 57 °C for 15 s, 72 °C for 60 s, and then extension at 72 °C for 10 min. PCR product quality was tested on 1.5 % agarose gels electrophoresis. Next, the PCR products were purified by PCR E.Z.N.A® Cycle-Pure Kit (Omega-Biotek Inc., USA) and sent for sequencing (1st Base Laboratories Sdn. Bhd., Malaysia). Sequence analysis was performed by a BLAST search in the GenBank database (http://www.ncbi.nlm.nih.gov) and BioEdit version 7.1.9 software, thereby identifying the patient's genotype.

Plasma isoniazid (INH) estimation

This analysis was carried out within a week of sample collection, by high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) using a validated method [10]. A mobile phase containing methanol and ammonium acetate 5 mM, pH 3.5 and a Gemini C18 column were used for HPLC. Precision of this measurement was < 15 %, estimated by the coefficient of variation. The linear range of the calibration curve was 0.1 - 10 µg/ml, and the lower limit of quantification was 0.1 µg/mL.

Data analysis

The slow, intermediate, and rapid *NAT2* acetylator phenotypes were determined using the NAT2PRED web server (Kuznetsov *et al.* [8]). This software allows the use of the six SNP (T341C, G590A, A803G, G857A, C282T, and C481T) in *NAT2* to eventually determine the acetylator phenotype. The haplotypes and their frequencies were carried out by SNP Analyzer version 2.0 software (ISTECH, South Korea) (Yoo *et al.* [11]). Statistical analysis was performed using SPSS version 22.0 software (SPSS Inc., USA). Data were expressed as median and inter-quartile range (IQR). One-way ANOVA with Tukey post hoc test were performed to compare differences in two-hour INH concentrations among the different genotypes. Chi-square test was utilized for comparison of categorical variables among groups. The analysis was statistically significant if p-value < 0.05.

Table 1 Comparison of minor allele frequencies of SNPs located on *NAT2* gene in different populations of the world.

	Research subject populations in the world						
	Present study (n = 136)	**Kinh in HCM city, Vietnam (n =198)**	**East Asian (n =504)**	**South Asian (n = 489)**	**African (n = 661)**	**European (n = 503)**	**American (n = 347)**
C282T (rs1041983)							
Allele T frequency	0.47	0.54	0.44	0.43	0.47	0.31	0.29
T341C (rs1801280)							
Allele C frequency	0.06	0.06	0.04	0.35	0.29	0.45	0.36
C481T (rs1799929)							
Allele T frequency	0.06	0.05	0.04	0.32	0.24	0.44	0.34
G590A (rs1799930)							
Allele A frequency	0.32	0.35	0.26	0.36	0.24	0.28	0.17
A803G (rs1208)							
Allele G frequency	0.06	0.06	0.04	0.36	0.39	0.44	0.37
G857A (rs1799931)							
Allele A frequency	0.16	0.19	0.18	0.07	0.03	0.02	0.11

Results and discussion

The results of the minor allele frequency of SNPs located on the *NAT2* gene are presented in **Table 1**. In the analyzed loci, the frequencies of genotypes followed Hardy-Weinberg expectations. This means that the genetic compositions of these alleles were quite balanced, at least in 136 individuals of this study. Compared to the genome project in Phase 3, conducted on 2,504 people in 26 countries worldwide, including Kinh people living in Ho Chi Minh City (Vietnam) (1,000 Genomes Project Phase 3 Data, 2018), our results are similar to those of the research on Kinh people and East Asian (p > 0.05). Kinh

people, also known as "Vietnamese people", account for nearly 90 % of the population of Vietnam, and allelic frequencies of *NAT2* gene obtained from this group could represent normal Vietnamese people. The similarity in the *NAT2* allele frequencies in the normal group and the TB patient group is evidence that *NAT2* is not a tuberculosis susceptibility gene. Compared with the South Asian population, the frequency of the minor alleles of A803G in the present study and in the East Asian population was lower, with statistically significant difference ($p < 0.05$); however, the minor alleles of G857A is highest compared to other populations, and two times higher than the South Asian population.

Table 2 Observed frequencies of *NAT2* haplotypes in Vietnamese tuberculosis patients (n = 136).

***NAT2* haplotype**	**SNP**	**Observed frequency (%)**
***NAT2**4**	None	**46.22**
*NAT2**5A	T341C, C481T	0.37
*NAT2**5B	T341C, C481T, A803G	5.52
*NAT2**5E	T341C, C481T, G590A	0.37
Total *NAT25**		**6.26**
*NAT2**6A	C282T, G590A	30.20
*NAT2**6J	C282T, G590 A, G857A	1.02
Total *NAT26**		**31.22**
*NAT2**7B	C282T, G857A	14.50
*NAT2**7C	C282T, A803G, G857A	2.22
Total *NAT27**	C481T	**16.72**
***NAT2**12A**	A803G	**0.46**
***NAT2**13A**	C282T	**1.12**

A haplotype is a particular pattern of alleles at sequential loci on a single chromosome. In order to reconstruct haplotypes from the unphased diploid genotype data, we used NAT2PRED web server with EM-based algorithm [11]. Ten different haplotypes found among Vietnamese tuberculosis patients are presented in **Table 2**. The major alleles found were *NAT2**4, *6A, and *7B, followed by *NAT2**5B, *6J, *7C, and *13A. Additionally, some alleles were observed at frequencies below 1 % (*NAT2**5A, *5E, and *12A). The distribution of the *NAT2* genotype frequencies and predicted phenotypes are summarized in **Table 3**. Among genotypes, *NAT2**4/*4 was the most frequent genotype (25.74 %). The most frequent slow and intermediate acetylator genotypes observed were *NAT2**6/*6 (13.24 %) and *NAT2**4/*6 (23.53 %), respectively. The results of this study are consistent with the general trend of Asian haplotype distribution. Asian populations displayed lower haplotype diversity than either European or African populations. The mean haplotype diversities were estimated to be 0.59 ± 0.11, 0.69 ± 0.04 and 0.79 ± 0.06 in East Asians, Europeans, and Africans, respectively. Twenty-six haplotypes of *NAT2* genes were determined; only a few major haplotypes were found in Asian populations- *NAT2**4, *NAT2**6A and *NAT2**7B [12].

T341C, G590A, and G857A are the main SNPs which lead to reduced *NAT2* activity; many studies have only analyzed these 3 SNPs to predict acetylation phenotype. In particular, phenotypic groups can be classified based on the number of mutant alleles: rapid acetylators with no SNPs (homozygous for wild type *NAT2**4 allele), intermediate acetylators with heterozygous for *NAT2**4 and a mutant allele, and slow acetylators with a combination of mutant alleles [3]. 134/136 patients had the same predicted

phenotype results, based on genotypes of 3 SNP and 6 SNP, using NAT2PRED web server. The remaining two patients, who had only C282T mutation, were predicted to be rapid acetylator using 3 SNP, and intermediate acetylator using 6SNP. If all six SNPs are used, it is reported that the predictor can achieve an accuracy of 99.9 %, with sensitivities and specificities from 99.6 to 100. If data on two synonymous C282T and C481T are removed, the accuracy of the prediction drops from 99.9 to 93.2 % [8]. In fact, the number of slow acetylators who are at increased risk of adverse effects is almost larger than that of rapid acetylators (1,000 Genomes Project Phase 3 Data, 2018). So, at-risk individuals can be missed when the accuracy of the prediction decreases. Therefore, we conclude that six SNPs used in the present study are required to faithfully identify the acetylator phenotype.

Table 3 Distribution of *NAT2* genotypes and predicted acetylator phenotype (n = 136).

Genotype	Number of subjects	Observed frequency (%)	Predicted phenotype
*NAT2**5/*5	1	0.74	Slow
*NAT2**5/*6	4	2.94	Slow
*NAT2**5/*7	2	1.47	Slow
*NAT2**6/*6	18	13.24	Slow
*NAT2**6/*7	12	8.82	Slow
*NAT2****7**/***7**	6	4.41	Slow
Total	**43**	**31.62**	**Slow**
*NAT2**4*5	9	6.62	Intermediate
*NAT2**4*6	32	23.53	Intermediate
*NAT2**4***7**	13	9.56	Intermediate
*NAT2**4*13	2	1.47	Intermediate
*NAT2**6*12	1	0.74	Intermediate
*NAT2**7*13	**1**	0.74	Intermediate
Total	**58**	**42.65**	**Intermediate**
*NAT2**4/*4	**35**	**25.74**	**Rapid**

The median two-hour INH concentrations in slow, intermediate, and rapid acetylators were 3.4, 2.7, and 2.2 µg/ml, respectively. The differences in INH concentrations among the three genotypes were significant ($p < 0.05$). There existed a significant trend in the INH concentrations among the genotypes; the slow acetylators had the highest concentration, followed by the intermediate acetylators, and rapid acetylators had the lowest INH concentration **(Figure 1A**). Among 6SNP, the C282T genotype significantly affected INH concentration (**Figure 1B**). Patients with homozygous CC of C282T had a two-hour INH concentration median of 2.7 (1.4 - 3.6) µg/ml, which was significantly lower than patients with homozygous TT, which was 3.9 (2.6 - 4.4) µg/ml.

The metabolism of INH could be affected by variations in the *NAT2* gene among different populations. Specifically, several studies have reported the influence of *NAT2* genotypes on plasma INH concentrations, as well as INH-induced hepatotoxicity [13,14,15,16]. In general, significant variations in INH concentrations among different genotypes is an important subject, since INH is a first line TB drug and is widely used in Vietnam. Slower acetylators with higher concentrations of INH have a higher risk of drug-induced hepatotoxicity. A meta-analysis from 37 studies, comprising 1527 cases and 7184 controls, showed a significant association between *NAT2* slow acetylators and risk of anti-TB drug-induced liver toxicity [7]. On the other hand, fast acetylators are likely to benefit less from a prescribed drug dose. INH doses 1.5 times the currently recommended doses have been suggested for rapid acetylators [17]. A comparison of responses to TB treatment between slow and rapid acetylators of INH suggested an association between treatment response and rate of inactivation of INH. The difference in the rate of conversion to bacteriological negativity between slow and rapid acetylators was described [18].

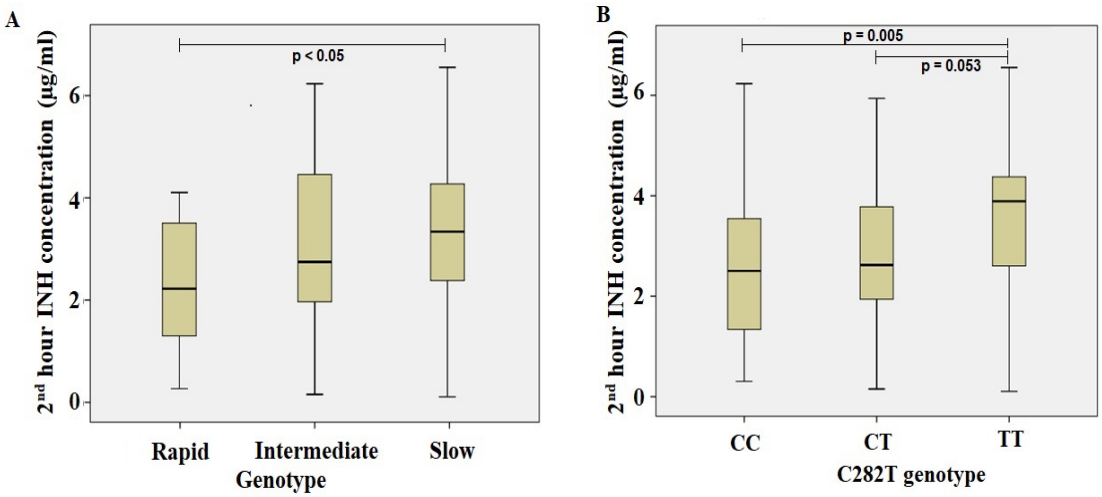

Figure 1 Median two-hour isoniazid concentrations in different genotypes. The vertical bars denote inter-quartile range. A: genotype of all SNP presented by acetylator capacity of rapid, intermediate, and slow. B: genotype of *NAT2*(C282T).

In this study, we carried out plasma INH concentrations at two hours after an oral drug administration. Because of logistical and financial difficulties during blood sample collection, the number of time points was limited. When only one sample can be taken, the two-hour post-dosing INH concentrations are the most common used, and INH concentration peaked at two hours in more than 95 per cent of patients in studies [19]. In the literature, the target peak concentration of INH is in the range of 3 - 6 µg/ml after oral administration of 5 mg/kg body weight [19]. The drug concentrations observed in our study were lower than those reported in several earlier studies, which could be because of differences in the INH dose used in these studies. Some studies used INH doses of up to 600 mg [20], two times higher than the dose used in this study. We chose the INH dose based on the recommendation of WHO Guidelines for the programmatic management of drug-resistant tuberculosis [21]. Interestingly, a pilot study applied a new INH dosing regimen which takes into consideration *NAT2* genotype and body weight and demonstrated potential for subsequent large-scale studies [22].

Conclusions

Genotyping of TB patients for *NAT2* gene polymorphism identified 31.62, 42.65, and 25.74 % patients as slow, intermediate, and rapid acetylators. Two-hour INH levels were significantly different among slow and rapid acetylators. The data from the present study and other published reports suggest that the determination of the *NAT2* genotype prior to INH administration is clinically relevant for the prediction of pharmacokinetic variability and the possible adjustment of INH dosing regimens in practice.

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