Allelic Frequencies of Single Nucleotide Polymorphisms (SNPs) in the *N-acetyltransferase 2* (*NAT2*) Gene of Filipinos

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ABSTRACT

Objectives. The present study aims to determine the frequency of occurrence of *NAT2*4*, *NAT2*5A, NAT2*6B, NAT2*7A* and *NAT2*14A* alleles by PCR-RFLP among Filipino volunteers. These alleles correspond to substitutions in the following sites: *C341T, G590A, G857A* and *G191A*, respectively, of the *NAT*2 gene. The presence of specific SNP combination was also used to deduce acetylation status and estimate genotype frequency and describe them in comparison with other populations based on literature.

Methods. Genomic DNA from peripheral blood lymphocytes from 129 healthy Filipino volunteers was used to amplify the *NAT2* gene segment. The RFLP analysis was done by restricting the expected PCR product with *Kpn1, Taq1, BamH1* and *Msp1/Al1*, respectively, to detect the 4 alleles: *NAT2*4*, *NAT2*5A, NAT2*6B, NAT2*7A* and *NAT2*14A*.

Results. The calculated allelic frequencies in Hardy-Weinberg equilibrium of *NAT2**5A (C481T), *NAT2**6B (G590A), *NAT2**7A (G857A) and *NAT2**14A (G191A) were 0.058, 0.097, 0.182 and 0.046, respectively. *NAT2**4 had an allele frequency of 0.617. Nine genotypes were determined: *NAT2**4/*4, *NAT2**4/*5A, NAT2*4/*6B, *NAT2**4/*7A, *NAT2**4/*14A, *NAT2**5A/*7A, *NAT2**6B/*7A, *NAT2**6B/*14A and *NAT2**7A/*14A. From these genotypes, acetylator phenotypes were deduced. A trimodal pattern of distribution was established: rapid, intermediate and slow acetylators with the following percentages, 47.3%, 41.1 % and 11.6%. Among the slow acetylator SNPs determined, *NAT2*7A* was found as the most frequent allele and *NAT2*14A* was found as the least frequent allele.

Conclusion. The study showed the mutation profile and observed genotypic similarities and differences of Filipinos with other Asian populations and Americans and other Caucasians based on literature. The results also suggest a trimodal pattern of distribution of acetylators and lesser number of slow acetylators among Filipino populations, a characteristic similar to other Asian populations but significantly different from Americans and other Caucasians. The occurrence of *NAT2*7A* and *NAT2*14A* can be further sequenced to verify the observed genotype.

Key Words: variation in NAT2 gene; single nucleotide polymorphism (SNP); Filipinos; acetylator status; trimodal; Asians

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Introduction

N-acetyltransferase 2 (NAT2) is a phase II drug-metabolizing enzyme involved in the transformation of several arylamine and hydrazine drugs.[1] Metabolic activity of the polymorphic NAT2 is determined by the mutation pattern of the *NAT*2 gene.[2] There has been sustained interest in studying the variability in N-acetylation status among groups of individuals. These differences may be used as biomarkers to evaluate the effectiveness of therapy and development of adverse reactions or toxicity during treatment.

Traditionally, acetylation capacity has been detected by metabolic phenotyping tests performed with a number of surrogate drugs, such as isoniazid. Early studies done on healthy volunteers and tuberculous patients showed large differences in the elimination rate of isoniazid. Based on these studies, a great majority of the patients could be identified as being either slow or rapid acetylators of the drug.[2,3] The distribution of isoniazid metabolism was characterized by a longer drug half-life among the slow acetylators and a shorter drug half-life among rapid acetylators. This divided the population into two distributions and was described as bimodal. Earlier studies among the Filipino population using dapsone[4] and isoniazid[5] showed that a greater number of Filipinos were rapid acetylators. On the other hand, other studies

established a trimodal distribution among the Americans and Africans as shown by phenotype–genotype concordance studies.[6,7]

In humans, *N-acetyltransferase* (*NAT*) has three loci: 2 expressed genes, *NAT1* and *NAT*2, and a pseudogene, *NATP*.[1] Both expressed genes are 870 base pair (bp) intronless coding regions[8] and are located on chromosome 8p21.3-23.1.[9] The isozymes differ in their substrate specificities: isoniazid, dapsone, caffeine and sulfamethazine are *NAT*2-specific substrates; p-aminobenzoic acid and p-aminosalicylic acid are *NAT1*-specific substrates. *NAT*2 is primarily expressed in the liver, whereas *NAT1* is expressed at other sites, including the colon.[1] Both *NAT1* and *NAT*2 genes are highly polymorphic. The polymorphisms are due to point mutations within the coding region of the *NAT* genes. The association between *NAT*2 polymorphism and acetylation phenotype have been studied extensively.[7,10,11] The phenotype-*NAT*2 genotype association studies have become the basis of deducing phenotypes from the genotypes. [6,7, 12,13]

There are over 60 *NAT*2 alleles that had been identified in human populations,[14] but there are only seven major human *NAT2* alleles (*G191A, C282T, T341C, C481T, G590A, A803G* and *G857A*).[12] Out of these seven alleles, only four alleles (*G191A, C341T*, *G590* and, *G857A)* are commonly found in human populations. These four alleles result in an amino acid substitution that leads to a significant decrease in acetylation capacity.[12,15] They are diagnostic for defective *NAT2* function, and hence for the slow acetylator phenotype.[12] Other single nucleotide polymorphisms (SNPs) did not affect acetylation capacity because they are either silent mutations (*C481T* and *C282T*) or non-synonymous substitutions that do not alter phenotype.[16] It was previously reported that *C481T* and *C341T* were observed to cosegregate and, that *C341T* rarely occurs without *C481T.* [1]

Current methods of molecular genotyping such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing have been used widely to identify mutations, like base substitutions, deletions and additions. PCR-RFLP makes use of specific oligonucleotide primers and appropriate restriction enzymes to locate specific SNPs.[11,17,18] This method was found to be an accurate and cost-effective means of determining acetylator status.[17]

The present study aims to determine the frequency of occurrence of *NAT2*4*, *NAT2*5A, NAT2*6B, NAT2*7A* and *NAT2*14A* alleles by PCR-RFLP. These alleles correspond to substitutions in the following sites: *C341T*, *G590A, G857A* and *G191A*, respectively, of the *NAT*2 gene. The presence of specific SNP combination was also used to deduce acetylation status and estimate genotype frequency.

Methods

Study Sample

DNA samples were obtained from 129 healthy Filipino volunteers residing in Metro Manila, Philippines for not less than 5 years. Their ages ranged from 15-75 years old. The study sample consisted of 73 females and 56 males. The research protocol of the present study had been approved by a local Independent Ethics Committee in accordance with the ethical standards with the principles enunciated in the Declaration of Helsinki of 1975 as revised in 2005. An informed consent was taken from the volunteers after orientation of the nature of the study.

Genotyping by PCR-RFLP

Genomic DNA was extracted from peripheral blood lymphocytes using the phenol-chloroform method. *NAT*2 genotyping was determined by PCR-RFLP according to Heuvel.[13] The expected 1109bp amplicon was obtained using the following primer sequences: forward primer, 5′-CCA TTg TgT TTT TAC gTA TT-3′ and reverse primer, 5′-gTA TTT gAT gTT TAg gAT TTT-3′. Amplification was carried out in an Minicycler™ (MJ Cycler, USA) under the following conditions: initial denaturation at 94^0C for 4 minutes, followed by 30 cycles performed at denaturation (94^0C, 30 seconds), annealing (50^0C, 30 seconds), and elongation (72^0C, 1 minute and 30 seconds). The final extension was done at 72^0C for 4 minutes.

RFLP analysis was done by restricting the PCR product with four different enzymes, namely: *Kpn1* at 65^oC for 16 hours to determine *NAT2*5A* allele, Taq*1* at 37^oC for 1 hour to determine *NAT2*6B* allele, *BamH1* at 65^oC for 16 hours to determine *NAT2*7A* allele and *Msp1*/*Alu1* at 65^oC for 16 hours to determine *NAT2*14A* allele. The identification of the alleles as wild-type allele (*NAT2*4*) and *NAT*2 SNPs (*NAT2*5A, NAT2*6B, NAT2*7A* and *NAT2*14A*) was based on the restriction and migration pattern of the PCR product digests as described in detail by Heuvel.[13]

The data interpretation and rules on *NAT*2 nomenclature and allele assignment were based on previous reports.[12-14, 18] *NAT*2*4 or wild type allele is associated to a rapid acetylator *NAT*2 phenotype while *NAT*2*5A, *NAT*2*6B, *NAT*2*7A and *NAT*2*14A are associated to a slow acetylator *NAT*2 phenotype. The combination of homozygous *NAT*2*4 alleles made up a rapid acetylator phenotype while a wild type and a mutant allele led to an intermediate phenotype. Combination of any two *NAT*2 SNPs (*NAT*2*5A, *NAT*2*6B, *NAT*2*7A and *NAT*2*14A) were associated to a slow acetylator phenotype. The assignment of genotype from allele combinations and deduced phenotype from genotype was based on previous reports.[12-15,19]

Statistical analysis

The genotype distribution of acetylator status was compared with the expected obtained from Hardy-Weinberg equilibrium test using a chi-square test. The difference in genotypic frequency distribution between the study population and published reference was also tested by chi-square.

Results

The expected 1109 bp amplicon was generated using *NAT*2 specific oligonucleotide forward and reverse primers. Restriction digestion of the 1109 bp PCR product with *Kpn*1, *Taq*1, *Bam*H1 and *Msp*1/*Alu*1 resulted in specific migration patterns as shown in Figure 1.

Restriction digestion of the PCR product with *Kpn1* showed two digestion products, 593bp and 516bp. *Taq1* digestion of the PCR amplicon produced four digestion products, 474bp, 238bp, 227bp and 170bp as shown in lanes 2-5 of Figure 1b. Likewise, restriction digestion with *BamH*1 produced two digests, 969bp and 140bp as shown in lanes 2, 4 and 5 of Figure 1c. In lane 3, three restriction digests upon *BamH1* restriction were produced, 1109 bp, 969bp and 140 bp. There were four restriction products, 618bp, 209bp, 189 bp and 94 bp shown in lanes 2-5 of Figure 1d upon treatment with *Msp*1/*Alu*1.

The distribution of individuals carrying homozygous wild type, heterozygous wild type/mutant and homozygous mutant alleles upon *Kpn1* digestion are as follows: 114, 15 and 0. On the other hand, *Taq1* digestion resulted into 104 individuals carrying homozygous wild type alleles and 25 individuals with heterozygous wild type allele. No individual was observed carrying homozygous mutant alleles. *BamH1* digestion yielded 82 individuals with homozygous wild type allele and 47 individuals with heterozygous wild type allele. Similarly, no individual was determined carrying the homozygous mutant alleles. Digestion of the PCR product with *Msp1*/*Alu1* identified 117 individuals with homozygous wild type allele, 12 individuals with heterozygous wild type allele and the absence of individuals with homozygous mutant alleles.

The summary of calculated allelic frequencies is shown in Table 1. *NAT*2*4 was found to be the most frequent allele followed by *NAT*2*7A allele. On the other hand, *NAT2*14A was observed as the least frequent allele.

Table 2 summarizes the number of individuals with their respective genotypes. The composite genotype of each subject was determined based on the published study of Heuvel (1998). The assignment of genotype nomenclature was based on the consensus rules of *NAT*2 nomenclature.[12,15] Out of the nine NAT2 genotypes determined, NA*T2*4/*4 genotype was the most frequent, followed by *NAT2*4/*7A,* while *NAT2*7A*/*14A was the least frequent genotype.

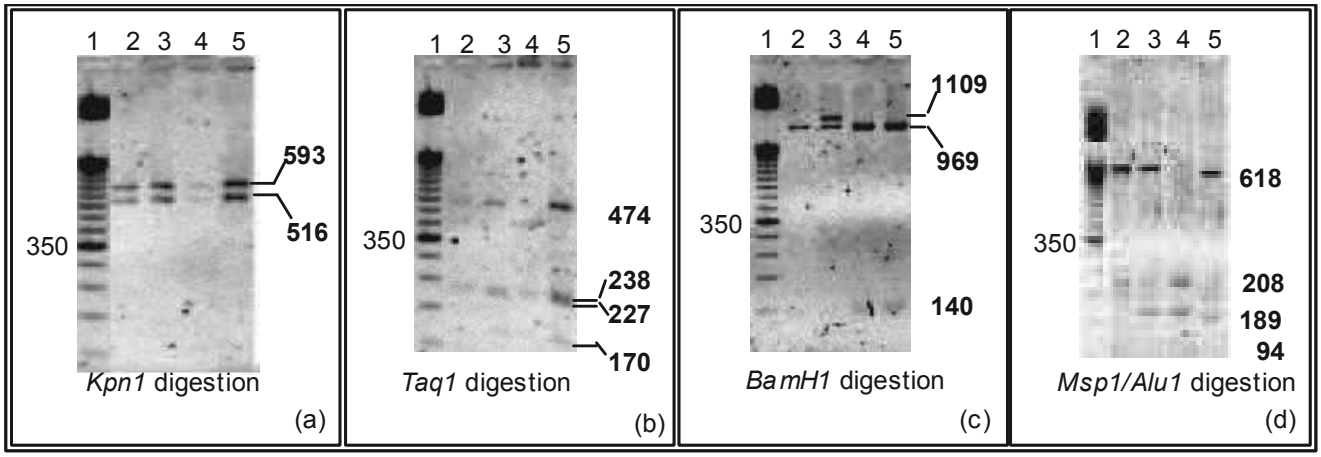

Figure 1. NAT2 genotyping using PCR-RFLP. **1a**. restriction digestion with *Kpn1* showing 2 digestion products with sizes 593bp and 516bp, indicating that samples in lanes 2 to 5 are *NAT2*4/NAT2*4* (lane 1 in all gels, 50-bp marker), **1b**. restriction digestion with *Taq1* with digestion products 474bp, 238bp, 227bp and 170bp, indicating that all samples in lanes 2 to 5 are designated as *NAT2*4/ NAT2*4*; **1c**. restriction digestion with *BamH*1 wherein samples in lanes 2, 4 and 5 have 2 restriction products: 969bp and 140 bp, designated as *NAT2*4/ NAT2*4* and sample in lane 3 with 3 restriction products: 1109 bp, 969bp and 140 bp, and designated as *NAT2*4/NAT2*7A*, and, **1d**. restriction digestion with *Msp*1/*Al*1 wherein samples in lanes 2 to 5 produced 4 restriction products: 618bp, 209bp, 189 bp and 94 bp, and designated as *NAT2*4/NAT2*4*. DNA samples in lanes 2, 4 and 5 have *NAT2*4/ NAT2*4* overall genotype and sample in lane 3 has a *NAT2*4/ NAT2*7A* overall genotype (based on Heuvel [11]).

Table 1. Allele frequencies of the *NAT2* alleles observed among 129 Filipino volunteers

NAT2 allele	Mutation Sites	Allele Frequency
*4	Wild type	0.617
*5A	*C481T*	0.058
*6B	*G590A*	0.097
*7A	*G857A*	0.182
*14A	*G191A*	0.046

Table 2. *NAT2* Genotype frequencies in 129 healthy Filipinos

Allele Combinations	Mutation	Number of Individuals (n)
*NAT2*4/*4*	wild-type	54
*NAT2*4/*5A*	C481T	7
*NAT2*4/*6B*	G590A	10
*NAT2*4/*7A*	G857A	33
*NAT2*4/*14A*	G191A	4
*NAT2*5A/*7A*	C481T;G857A	6
*NAT2*6B/*7A*	G590A;G857A	7
*NAT2*6B/*14A*	G590A;G191A	6
*NAT2*7A/*14A*	G857A;G191A	2

NAT2 acetylator phenotype was deduced from the *NAT2* genotype. Deduced phenotype was based on a consensus NAT2 phenotype identification[15] based from several phenotype-genotype association studies.[6,7] The phenotype-genotype association is summarized in Table 3.

Table 3. Genotype and Phenotype frequencies in 129 healthy Filipinos

Genotype	% Genotype Frequency	Deduced Phenotype	% Phenotype Frequency
*NAT2*4/*4*	41.9	rapid	41.9
*NAT2*4/*5A*	5.4		
*NAT2*4/*6B*	7.8		
*NAT2*4/*7A*	25.6	intermediate	41.9
*NAT2*4/*14A*	3.1		
*NAT2*5A/*7A*	4.6		
*NAT2*6B/*7A*	5.4		
*NAT2*6B/*14A*	4.6	slow	16.2
*NAT2*7A/*14A*	1.6		

Discussion

Arylamine N-acetyltransferase is one of the major phase II liver enzymes involved in the biotransformation of drugs. Three forms of *N-acetyltransferase* (*NAT1*, *NAT2* and *NATP*) exist in humans. Both *NAT1* and *NAT2* are highly polymorphic[2] and their difference lies in their substrate specificities.[1]

NAT2 is involved in the metabolism of aromatic amines and hydrazines, and catalyzes the transfer of an acetyl group from acetyl CoA to the terminal nitrogen of the substrate.[20] The N-acetylated form renders the drug inactive.[21] The common genetic variations in *N-acetyltransferase 2* (*NAT2)* could result in striking differences in the half-life and plasma concentrations of drugs metabolized by this enzyme. Such drugs included the antituberculosis agent isoniazid,[22] the antihypertensive agent hydralazine[23] and the antiarrhythmic drug procainamide.[24] Polymorphisms in drug-metabolizing enzymes also influence interindividual variability in their function.[2] The NAT2 phenotype could account for the increased risk of certain side effects in slow acetylators treated with isoniazid, although therapeutic efficacy seems to be independent of the acetylation status.[25]

With the advent of genotyping methods, it became clear that many drug-metabolizing enzymes like NAT2 are coded by genes bearing multiple mutations or allelic combinations.[2]

The current study demonstrates the presence of *NAT2*4, NAT2*5A, NAT2*6B, NAT2*7A* and *NAT2*14A* among the Filipino population. A combination of *NAT2* alleles resulted in variation in *NAT2* genotypes. From the genotype pattern, the distribution of acetylator phenotypes was deduced.[12,13]

*NAT2*4*, the wild type allele, was found as the most common allele. *NAT2*4* allele is associated with a rapid acetylator phenotype based on rules on consensus *NAT2* nomenclature.[12,15,19] This allele, as previously reported[13] retains the recognition sites of *Kpn1*, *Taq1*, *Bam H1* and *Msp1*/*Alu1* at the following polymorphic sites: *C481, G590, G857 and G191* of the *NAT2* gene.

*NAT*2 SNPs designated as *NAT*2*5A, *NAT*2*6B, *NAT*2*7A and *NAT*2*14A have been associated to a slow acetylator phenotype based on the rules on consensus *NAT*2 nomenclature.[12,15, 19]

Among the *NAT*2 SNPs, *NAT*2*6B allele was found as the most frequent allele (0.097). Mutation at this site, G590A, led to alteration of *Taq1* recognition site. This nucleotide substitution would lead to an arginine to glutamine change (R197Q) (Hein et al, 2003) with a decreased enzyme activity.[26]

On the other hand, *NAT2*14A*, also associated to slow acetylator phenotype, was found as the least frequent allele (0.046). The corresponding *G191A* substitution was associated to a change of amino acid from arginine to glutamine (R64Q).[19] This site was reported to be the highly conserved region of the active site of acetyl transfer.[27]

The nine observed genotypes were grouped into three phenotypes: rapid, intermediate and slow. The lone homozygous *NAT2*4/NAT2*4* genotype is associated with a

rapid acetylator phenotype. Four genotypes are associated with an intermediate acetylator phenotype. Likewise, four genotypes are associated with slow acetylator phenotype. Based on the results obtained from the current PCR-RFLP genotyping study done among the Filipino population, the data suggest that the acetylation metabolism shows a trimodal pattern of distribution.

The differences between Asian and Caucasian populations as shown in Table 4 rest on the frequency distribution of the following three *NAT2* alleles: *NAT2**4, *NAT2**5A and *NAT2**7A. In this study, the alleles *NAT2**4 and *NAT2**7A were found to be the most frequent among the Filipinos, similar to other Asian populations. The result obtained, however, is in contrast to the lesser occurrence of *NAT2**4 and *NAT2**7A among the Caucasian populations.

On the other hand, *NAT2**5A has been found as the most frequent allele among the Caucasian populations compared to its lesser occurrence among the Asian populations and Filipinos as seen in this study. Observed in this study is the occurrence of *NAT2**14A. This has been reported only in African-Americans.[29]

It was observed that rapid and intermediate phenotype acetylators were more common among the Filipino populations (Table 5). This observation is consistent with previous studies among the Koreans, Japanese, Chinese and Thai populations. The slow acetylator phenotypes, on the other hand, were more common among Americans, French and other Caucasian populations.

In conclusion, the present study using PCR-RFLP was able to determine the presence of the four most common *NAT2* SNPs among Filipino study population. Among the slow acetylator SNPs determined, *NAT2*7A* was found as the most frequent allele and *NAT2*14A* was found as the least frequent allele. The occurrence of the rare allele, *NAT2*7A* was also identified. The present molecular genotyping study showed a trimodal acetylation distribution. The results of the study also showed that there were a lesser number of slow acetylators among Filipino populations, a characteristic similar to other Asian populations.

Table 4. *NAT2* Allelic frequencies of the Filipinos compared with different races

POPULATION	N	SNP frequency					Reference
		NAT2*4	*NAT2*5A* C481T	*NAT2*6B* G590A	*NAT2*7A* G857A	*NAT2* 14A* G191A	
Filipino	129	0.617	0.058	0.097	0.182	0.046	Present study
Japanese	173	0.660	0.010	0.200	0.130	*	[28]
Korean	85	0.692	0.018	0.180	0.110	*	[28]
Taiwanese	100	0.515	0.025	0.310	0.150	*	[29]
HongKong, Chinese	70	0.553	0.057	0.230	0.160	*	[28]
Polynesians	25	0.600	0.040	0.340	0.020	*	[28]
Egyptian	199	0.215	0.497	0.260	0.028	*	[28]
Caucasian American	372	0.250	0.450	0.280	0.020	0	[29]
American	266	0.242	0.47	0.28	0.008	*	[28]
German	844	0.244	0.465	0.278	0.013	*	[28]
Portuguese	128	0.212	0.433	0.328	0.027	*	[28]
Danish	242	0.254	0.473	0.25	0.023	*	[28]
Scottish	96	0.203	0.49	0.271	0.036	*	[28]
Swedish	70	0.194	0.507	0.278	0.021	*	[28]
South Indians	166	0.16	0.22	0.37	0.25	0	[29]
Hispanic Americans	65	0.39	0.32	0.19	0.1	*	[28]
African -Americans	128	0.37	0.3	0.22	0.02	0.09	[29]

* Not determined

Table 5. Percentage occurrence of phenotype acetylators in various population groups

Population	N	Method	Number of acetylator			References
			rapid, n (%)	intermediate, n (%)	slow, n (%)	
Filipino	129	PCR-RFLP	54 (41.9)	54 (41.9)	21 (16.2)	Present study
Korean	65	phenotyping with INH	29 (44.6)	29 (44.6)	7 (10.8)	[10]
Japanese	1808	phenotyping with INH	798 (44.1)	803 (44.4)	207 (11.5)	[10]
Chinese	441	PCR-RFLP	132 (29.9)	197 (44.7)	112 (25.4)	[29]
Thai	108	phenotyping with INH	21 (19.4)	57 (52.8)	30 (27.8)	[10]
Egyptian	200	allele-specific RT-PCR	7 (5.6)	37 (68.5)	56 (28.0)	[28]
French	72	PCR-RFLP	9 (12.5)	35 (48.6)	28 (38.9)	[31]
American	222	phenotyping with caffeine	6 (2.7)	87 (39.2)	129 (58.1)	[17]
American	222	PCR-RFLP	13 (5.9)	77 (34.6)	132 (59.5)	[17]
Caucasian	18	PCR-RFLP	3 (16.7)	2 (11.1)	12 (72.2)	[30]

Recommendations

It is also recommended to sequence representative alleles to verify some of the RFLP data.

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