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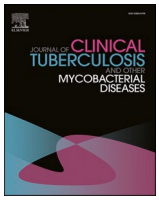

Variants in the *N*-acetyltranferase 2 gene, acetylator phenotypes and their association with tuberculosis: Findings in Peruvian patients

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ABSTRACT

Background: Tuberculosis (TB) is a highly prevalent chronic infectious disease in developing countries, with Peru being one of the most affected countries in the world. The variants of the *N*-acetyltransferase 2 (*NAT2*) gene are related to xenobiotic metabolism and have potential usefulness in TB studies.
Aim: To determine whether *NAT2* gene variants and acetylator phenotypes are associated with active TB in Peruvian patients.
Methods: This study included cases (patients with TB) and controls (population-based data). First, DNA isolation and the rs1799929, rs1799930, and rs1799931 variants of the *NAT2* gene were identified using sequencing methods. Subsequently, the acetylator phenotypes, namely slow (SA), intermediate (IA), and rapid acetylation (RA), were also analyzed.
Results: The comparison of the frequencies of the rs1799931 variant in the cases and controls revealed significant differences. Risk factors were found for both the A allele ($p = 0.00$; odds ratio [OR] = 3.04, 95 % confidence interval [CI]: 1.88–4.9) and AG genotype ($p = 0.00$; OR = 5.94, 95 % CI: 3.17–11.09). In addition, the non-rapid acetylator phenotype (SA + IA) was also found to be a risk factor ($p = 0.016$; OR = 3.16, 95 % CI: 1.29–7.72).
Conclusion: The A allele, GA heterozygous genotype of the rs1799931 variant of the NAT2 gene, and SA + IA acetylator phenotype showed an association with increased risk for the development of TB. In addition to xenobiotic metabolism, other metabolic and immunological functions of *NAT2* have also been postulated to confer susceptibility to TB in the Peruvian population owing to its characteristic high Native American component.

1. Introduction

Tuberculosis (TB) is a respiratory disease caused by *Mycobacterium tuberculosis* (*Mtb*) that primarily affects the lungs and other parts of the body, such as the lymph nodes, kidneys, bones, and joints. It is characterized by the formation of granulomas in the lungs, which are a collection of inflammatory cells that can lead to localized chronic inflammation, lung tissue necrosis, and even death [1]. TB greatly affects the Peruvian population. According to the latest report of the World Health Organization, Peru has the highest number of diagnosed TB cases in the entire Americas (119 cases per 100,000 inhabitants). This highlights a serious public health problem in the country that requires new policies and eradication strategies for the disease [2].

The TB-causing bacillus becomes active in some individuals but

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remains latent in others. The basis of this still needs to be elucidated, and the interaction between *Mtb* and the host is complex and not yet fully understood. Various studies have indicated a significant genetic component, and both conventional genetic and genomic studies (genome-wide association studies) have reported varied genes, among which are xenobiotic metabolism genes as candidate genes, including the *CYP450*, gluthatione S-transferases family genes and *NAT2* [3]. Other candidate genes are involved in the immune response, encoding recognition receptors such as Toll-like receptor (TLR) or CD4, C-type lectins, or vitamin D receptors [3,4,5].

In this study, to identify genetic factors of susceptibility to the development of TB, we tested *NAT2*, which has been previously associated with TB [3]. The downregulation of xenobiotics can inhibit apoptosis in macrophages, thereby reducing the neutralization of virulent mycobacteria. Specifically, with a defective *NAT2* gene, imbalance in biotransformation can result in the accumulation of metabolites and thus the development of respiratory diseases such as TB, which promotes incomplete phagocytosis of *Mtb* in individuals with impaired immunity and differential genetic profiles [6–8]. Thus, we hypothesized that NAT2 pathways impact the predisposition to TB development. In this work, to identify possible variants that could be risk factors for the development of TB in Peruvian populations, we tested three variants of the *NAT2* gene in a group of patients recruited from a reference hospital for TB in Lima, Peru: rs1799929, rs1799930, and rs1799931.

2. Methodology

2.1. Patients and controls

We evaluated 109 patients diagnosed with active TB based on the standard criteria (bacilloscopic and/or clinical criteria, supported with images) who attended the Guillermo Almenara Irigoyen Hospital-Essalud, Lima, Peru, a national reference center for TB. The control group consisted of 85 apparently healthy individuals (Peruvians of Lima [PEL]) whose data on genetic variants and other information were obtained from the international 1000 Genomes Project [9].

2.2. Blood samples and DNA extraction

With informed consent from the patients with TB, 3 ml of peripheral blood sample was collected into K3-EDTA tubes. DNA was extracted using the salting-out method, with some modifications [10], in the laboratory of the Centro de Investigacion de Genetica y Biologia Molecular (CIGBM), Facultad de Medicina Humana, Universidad de San Martín de Porres Lima, Peru. The DNA quality and concentration were tested using a NanoDrop Lite equipment.

2.3. NAT2 gene analysis

DNA samples were used to study *NAT2* gene genotypes and alleles, as well as to establish acetylator phenotypes, through next-generation sequencing (NGS) using panels and/or exome sequencing (Illumina Technology). The gene libraries and variant annotations generated were analyzed using an in-house bioinformatics pipeline at the CIGBM. The relevant variants (rs1799929, rs1799930, and rs1799931) were analyzed using polymerase chain reaction with specific primers [11,12] and Sanger sequencing with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

2.4. Statistical analysis

Genotype and allele frequencies were calculated for each polymorphism, and the haplotypes and acetylator phenotype were inferred for each individual on the basis of the combination of the three variants [12]. We verified whether they were under Hardy-Weinberg equilibrium. The chi-square test was used for comparison between the patient group and other population groups, and the odds ratio (OR) with the 95 % confidence interval was calculated for risk analysis. The statistical packages “dplyr” and “epiR” in the R software (https://www.r-project.org/) were used for the analysis.

2.5. Ethical aspects

Approval of the research protocol and informed consent for participation in the research were issued by the ethics committee of Universidad de San Martin de Porres IRB (IRB00003251-FWA0015320), Lima, Peru, on July 21, 2015, with legal No. 982-2015-CIEI-USMP-CCM.

3. Results

3.1. General characteristics

The study included 109 patients with TB, of whom 41.2 % were female and 58.8 % were male, with ages ranging from 18 to 84 years. The patients and controls (population-based) characteristics are summarized in Table 1.

3.2. NAT2 alleles

We specifically analyzed three variants of the *NAT2* gene: rs1799929, rs1799930, and rs1799931. The allelic frequencies in the patients and controls are reported in Table 2. We calculated the OR for each variant for comparison between the patients and controls and obtained ORs of 0.82 (95 % confidence interval [CI]: 0.52–1.31, p = 0.470) and 0.88 (95 % CI: 0.42–1.83, p = 0.440) for the rs1799929 and rs1799930 variants, respectively. However, for the rs1799931 variant, we obtained an OR of 3.04 (95 % CI: 1.88–4.90, p = 0.000), showing a significant association with the A allele as a risk factor for the development of TB. The A allele of the rs1799931 variant was present in 39.4 % of the patients and in 17.6 % of the controls. In the Peruvian patients with active TB, the prevalence of the A allele was almost double that in the controls (Table 2).

3.3. NAT2 genotypes

The rs1799929 and rs1799930 genotypes, when grouped into two categories (wild-type homozygous genotype and mutant homozygous + heterozygous genotype), showed no relationship with the development of TB. The ORs were 1.24 (95 % CI: 0.70–2.18) and 0.88 (95 % CI: 0.40–1.90) for the rs1799929 and rs1799930 variants, respectively (Table 3). For the rs1799931 variant, the OR was 5.94 (95 % CI: 3.17–11.09, p = 0.000), which indicates a strong association between the AG + AA genotypes (with the presence of the A allele) and TB. The AG genotype was more prevalent in the patients than in the controls (58.7 % vs. 18.8 %; Table 3).

3.4. NAT2 acetylator phenotypes

The acetylator phenotypes were analyzed and compared between the

Table 1
General characteristics of Peruvian patients with TB and the controls.

	Patients with TB	Controls*
Male, n	64	41
Female, n	45	44
Age in years	18–84	–
Diagnosed	Yes	No
Tuberculosis status	Active	–
Treatment	Yes	–
Type of sample	Blood	Blood
Sampling location	Lima	Lima

*PEL = Lima Peruvians (1000 Genomes Project).

Table 2
Three *NAT2* allelic frequency polymorphisms in the Peruvian patients with TB and the controls.

Variant (effect)	Reference allele/Variant allele	Position/protein change	Alleles	Patients with TB n (%)	Controls n (%)	p*	OR [95 % CI]
rs1799929 (synonimous)	C/T	p.L161L	C	168 (77.0)	125 (73.0)	0.470	0.82 [0.52–1.31]
			T	50 (22.9)	45 (26.4)		
rs1799930 (missense)	G/A	p.R197Q	G	202 (92.6)	156 (91.7)	0.440	0.88 [0.42–1.83]
			A	16 (7.3)	14 (8.0)		
rs1799931 (missense)	**G/A**	**p.G286E**	**G**	**132 (60.0)**	**140 (82.0)**	**0.000**	**3.04 [1.88–4.90]**
			A	**86 (39.4)**	**30 (17.6)**		

*Chi-square test, $p < 0.05$ indicates significant differences.

Table 3
Three *NAT2* genotypic frequency polymorphisms in the Peruvian patients with TB and the controls.

Variant (effect)	Reference allele/variant allele	Position/protein change	Genotype	Patients with TB, n (%)	Controls n (%)	p*	OR [95 % CI]
rs1799929 (synonymous)	C/T	p.L161L	CC	61 (56.0)	43 (50.5)	0.470	1.24 [0.70–2.18]
			CT	46 (42.0)	39 (45.9)		
			TT	2 (1.8)	3 (3.5)		
rs1799930 (missense)	G/A	p.R197Q	GG	93 (85.0)	71 (83.5)	0.840	0.88 [0.40–1.90]
			AG	16(14.6)	14 (16.5)		
			AA	0 (0.0)	0 (0.0)		
rs1799931 ** **(missense)**	**G/A**	**p.G286E**	**GG**	**34 (31.0)**	**62 (73.0)**	**0.000**	**5.94 [3.17–11.09]**
			AG	**64 (58.7)**	**16 (18.8)**		
			AA	**11 (10.0)**	**7 (8.0)**		

*Chi-square test, $p < 0.05$ indicates significant differences. Two categories in each variant were considered: CC vs CT + TT (rs1799929), GG vs AG + AA (rs1799930), and GG vs AG + AA (rs1799931). **The genotypic frequencies in both groups are in Hardy-Weinberg disequilibrium.

patient and control groups. The acetylator phenotype of each patient was determined on the basis of the combination of genotypes of the 03 polymorphisms rs1799929, rs1799930, and rs1799931. The acetylator phenotypes are summarized in Table 4.

The acetylator phenotypes of slow and intermediate acetylation (SA + IA) were grouped and compared with the rapid phenotype. An OR of 3.16 (95 % CI: 1.29–7.72, $p = 0.016$) was obtained, which indicates that the non-rapid acetylator phenotypes are a risk factor for the development of TB. Of the patients, 92.6 % exhibited a slow or intermediate acetylator phenotype and 7.4 % showed rapid acetylator compared with 20 % of the control group (Table 4).

3.5. Frequencies of NAT2 rs1799931 worldwide

The allele and genotype frequencies for the rs1799931 variant in the patients with TB were found to be significantly different from those of other populations in other continents, as shown in Table 5. The heterozygous and homozygous genotypes for the rs1799931 variant in the patients with TB (58.7 % for genotype GA and 10 % for genotype AA) were significantly different from those of European, American, and African populations.

4. Discussion

To our knowledge, this is the first study conducted in Peruvians, to investigate the association between the aforementioned polymorphisms in the *NAT2* gene and acetylator phenotypes and the development of TB.

Table 4
Frequencies of the acetylator phenotypes in the Peruvian patients with TB compared with those in the controls.

Acetylator phenotype	Patients with TB n (%)	Controls n (%)	p*	OR [95 % CI]
SA + IA	101 (92.6)	68 (80.0)	**0.016**	**3.16**
RA	8 (7.4)	17 (20.0)		**[1.29–7.72]**

*Chi-square test, $p < 0.05$ indicates significant differences. RA: Rapid acetylator, IA: Intermediate acetylator, SA: Slow acetylator.

We have proven that the current inhabitants of Lima have approximately 70 % native (pre-Columbian) genetic background from the Pacific Coast, Andes, and Amazon [13]. The patients with TB who were included in the study received the first-line treatment in the Peruvian health regimen, which includes isoniazid, rifampicin, pyrazinamide, and/or ethambutol. Although *NAT2* genetic polymorphisms are associated with anti-TB drug-induced liver injury [14], their relationship with susceptibility to TB is intriguing, as demonstrated in the results of the present study.

In general, different gene candidates for susceptibility to TB have been investigated in both conventional genetic and genomic studies, most of which were from the innate and adaptive immune systems, but some research groups have also studied xenobiotic metabolism genes [3,15]. However, more specific studies have evaluated the *NAT2* gene and multidrug-resistant TB to search for genetic variants associated with acetylation phenotypes and response to anti-TB treatments [16,17].

An important aspect is the condition of latent and/or active TB, for which personalized individual treatment may be more beneficial [18] and to which susceptibility genes from the immune system have been associated [19,20]. In Latin America, only few studies have reported antigen-processing genes associated with latent TB [21]. Previous studies in Peru have indicated that early progression to active TB is highly heritable, indicating gene variants in specific regions of chromosome 3 [22]. The results from our evaluation of the *NAT2* gene on chromosome 8 indicate the relevance of certain variants of this gene.

The results of this study show that 39.4 % of the patients had the mutant A allele of the rs1799931 variant of the *NAT2* gene, which is important in this context, with a frequency close to the 35.6 % reported in another group of Peruvian patients [23]. Genotypes presenting the rs1799931 variant (heterozygotes or homozygotes mutants) were found to be associated with the development of TB. This mutation is a missense variant positioned in exon 2 of the *NAT2* gene, where the amino acid glycine is replaced by a glutamic acid at position 286. Glycine at position 286 is adjacent to the active site of the enzyme. Replacing it with a glutamic acid can significantly alter the opening of the active site owing to steric clashes with nearby residues and the loss of a highly flexible residue such as glycine; thus, this could affect the functionality of the NAT2 enzyme [24]. The results also indicate that the non-rapid

Table 5
Allelic and genotypic frequencies of the rs1799931 variant of the NAT2 gene in the Peruvian patients with TB according to continent.

Continent*	n	Alleles n (%)		p**	Genotypes*** n (%)			p**
		G	A		GG	GA	AA	
Patients	506	132 (60.0)	86 (40.0)	–	34 (31.0)	64 (58.7)	11 (10.0)	–
Europe	287	983 (98.0)	23 (2.0)	0.000	480 (95.4)	26 (4.6)	–	0.000
America	671	616 (89.0)	78 (11.0)	0.000	278 (80)	60 (17.3)	9 (2.7)	0.000
Africa	109	1284 (97.0)	38 (3.0)	0.000	623 (94.3)	38 (5.7)	–	0.000

*Data from the 1000 Genomes Project. **Chi-square test, $p < 0.05$ indicates significant differences between the patients and the populations in other continents. ***All reported frequencies are in Hardy-Weinberg equilibrium except for those in the patient group.

acetylator phenotypes (SA + IA) are a risk factor in Peruvian patients. A study among native Russians [8] reached a similar conclusion, identifying the slow acetylator phenotype as a factor of increased risk of TB development but in interaction with the variant null GSTT1. The same study revealed no statistically significant differences in the genotype and allele frequencies of the *NAT2* gene between patients with TB and healthy individuals.

The explanation for the increased risk of TB development in Peruvian patients is based on cellular and immune mechanisms linked to the function of *NAT2*. In that sense, studies have found that cells involved in the innate immune response, such as monocytes and natural killer (NK) cells, express high levels of the NAT2 enzyme when combating the TB bacillus [25]. NK cells have been identified surrounding granulomas in lung tissue biopsy samples from patients with TB [26], and numerous studies have demonstrated the importance of NK cells in controlling the growth of the TB bacillus [27,28]. In this context, it is particularly interesting how impaired *NAT2* enzyme production may result in dysfunctional immune cells and consequently become a risk factor for the development of TB.

A genetic study focusing on the *NAT2* gene in different populations may yield contradictory results [29,30]. In the Peruvian population, allele and genotype frequencies are significantly different from those in other populations, as shown in Table 5. Genotypic frequencies for the rs1799931 variant, both in the patient and control groups, do not adhere to the Hardy-Weinberg equilibrium, which can be explained by the TB bacillus acting as a selective pressure [31]. The analyses demonstrated a degree of heritability for susceptibility to TB, indicating an important host genetic influence on the disease. However, studies have failed to replicate the genetic variants previously associated with TB. Therefore, there is a need to evaluate ancestries [32] experiencing high prevalence rates and differential pressures of *Mtb*, such as Peruvians.

Having coexisted with this disease for more than 70,000 years, it is likely that humans have developed some immunity to *Mtb*; hence, most infection cases remain as inactive TB. This immunity is likely to vary for each ethnic group. Genetic predisposition studies regarding TB have focused on analyzing genes related to the immune system, and only in recent years have studies began to consider the *NAT2* gene [25,33,34].

In developing countries, where TB is prevalent, environmental factors, lifestyles, and nutrition complement and interact with the genetic and pharmacogenetic components of population groups [19,35]. As *NAT2* is a critical gene in the metabolism of various xenobiotics, it is likely susceptible to specific selective pressures [30,36]. In this regard, the Peruvian population, particularly in the city of Lima, where the samples were taken, has approximately 70 % Native American genetic component [13]. The high frequency of slow acetylator phenotypes of *NAT2* has also been proposed in other populations as a partial explanation for the high TB incidence due to reduced isoniazid efficacy, failure to cure, or adverse side effects [37]. However, an important but unknown acetylation step in the *Mtb* infectious process is still possible. Other aspects that should be considered as possible explanations of the results and in future research are gene expression and epigenetic mechanisms (DNA methylation, microRNAs) in *NAT2* and other genes [38,39,40].

The main limitations of the study include the lack of complementary clinical, demographic and/or pharmacological information about the patients and specifically controls, where the lack of age data can be confounder and affect it as a representative group, moreover, the unknown type of *Mtb* strain that affected each patient and perhaps there are some other co-morbidities that are associated with the variant of the NAT2 gene that increase the risk of TB, and this requires further studies to confirm our findings.

5. Conclusion

The A allele and GA heterozygous genotype of the rs1799931 variant of the NAT2 gene show an association with the development of TB in Peruvian population. Moreover, the non-rapid acetylator phenotypes (SA + IA) may be risk factors for the development of TB disease. This study contributes to the current understanding of the *NAT2* gene, whose function extends beyond xenobiotic metabolism and may be related to TB infection and which is a highly prevalent disease in Peru. In addition, it sheds light on the influence of Native American ancestry on the Peruvian population. Further research is required to establish a relationship between The A allele and GA heterozygous genotype of the rs1799931 variant of the NAT2 gene and risk for TB disease.

Ethical statement

Approval of the study protocol and informed consent for participation in the study was issued by the ethics committee of Universidad de San Martin de Porres IRB (IRB00003251-FWA0015320), Lima, Peru, on July 21, 2015, with legal No. 982-2015-CIEI-USMP-CCM.

CRediT authorship contribution statement

Rodrigo Sánchez: Writing – original draft, Methodology, Investigation. **Oscar Acosta:** Writing – review & editing, Validation, Conceptualization. **Lina Laymito:** Methodology, Investigation. **Teodoro Oscanoa:** Writing – review & editing, Resources. **María Guevara:** Writing – review & editing, Methodology, Investigation. **Saul Moscol:** Writing – review & editing, Resources. **Daisy Obispo:** Writing – review & editing, Software, Formal analysis. **Doris Huerta:** Writing – review & editing. **Ricardo Fujita:** Writing – review & editing, Supervision, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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