

Acetylator Status Among Newly Diagnosed and Recurrent Tuberculosis Patients from Kupang, Eastern Part of Indonesia

Edhyana Sahiratmadja¹
Ika Agus Rini²
Simeon Penggoam³
Afandi Charles²
Ani Melani Maskoen^{1,2}
Ida Parwati⁴

¹Division of Biochemistry and Molecular Biology, Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia; ²Health Research Unit, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia; ³Laboratorium Mikrobiologi, RSUD Prof. Dr. WZ Johannes, Kupang, Indonesia; ⁴Department of Clinical Pathology, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia

Purpose: N-acetyltransferase-2 enzyme in the liver, encoded by *NAT2* gene, plays a central role in metabolizing tuberculosis (TB) drug isoniazid (INH). Low compliance of patients toward six-month TB therapy and internal host factors, ie comorbid diseases, immune status, and genetic profiles, are factors leading to treatment failure and recurrence of pulmonary TB infection. This study aimed to explore the *NAT2* acetylator status among newly diagnosed and recurrent pulmonary TB patients in eastern part of Indonesia.

Patients and Methods: Archived DNA of TB patients (n=124) and healthy controls (n=124) were sequenced, and *NAT2* acetylator status was determined, then categorized as fast, intermediate, or slow acetylators. Pulmonary TB patients who had no previous TB treatment history were designated as newly diagnosed pulmonary TB, whereas patients with a history of TB treatment were designated as recurrent pulmonary TB. The demographic, clinical, and microbiological data between pulmonary TB groups were compared, and acetylator status was described among groups.

Results: Male was more significantly prevalent in the recurrent pulmonary TB group (p=0.025), and anemia was more prevalent in new pulmonary TB (p=0.003). The acetylator status in pulmonary TB patients compared to healthy controls were rapid (33.9% vs 48.1%), intermediate (57.8% vs 33.0%), and slow acetylators (8.3% vs 18.9%), respectively. Interestingly, the rapid and intermediate acetylator were significantly more prevalent in pulmonary TB patients than in healthy controls (p=0.023, OR=2.58 (1.12–5.97)). Furthermore, no differences were found in acetylator status between new and recurrent pulmonary (p=0.776).

Conclusion: Rapid and intermediate acetylators status predominated the pulmonary TB patients in Kupang, eastern part of Indonesia, postulating different genetic makeup in this area. As the pulmonary TB patients in Kupang exhibit more rapid acetylator phenotype, the acetylator status might be relevant to be checked before TB therapy for adjusting treatment dose to prevent drug resistances.

Keywords: acetylator status, *NAT2*, Indonesia, tuberculosis, recurrence

Correspondence: Edhyana Sahiratmadja
Division of Biochemistry and Molecular Biology, Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Jl. Raya Bandung – Sumedang KM 21, Jatinangor, Sumedang, Jawa Barat, 45363, Indonesia
Tel +62 22 779 5594
Fax +62 22 779 5595
Email e.sahiratmadja@unpad.ac.id

Introduction

Pulmonary tuberculosis (TB) has been known for centuries as one of the deadliest diseases due to a single infection agent, *Mycobacterium tuberculosis*. Around 10 million people have been infected with TB and there were 1.2 million deaths related to TB globally in 2018, of which most cases occur in developing countries, including Indonesia. The estimated incidence rate per year in Indonesia is placed third globally.¹ Four-drugs regimen, including rifampicin, isoniazid,

pyrazinamide, and ethambutol, has been used for around 40 years to treat pulmonary TB, yet recurrence and death numbers due to this disease are still burdening.² Low compliance of patients toward six-month chemotherapy and internal host factors, ie comorbid diseases, immune status, and genetic profiles, become factors leading to treatment failure and pulmonary TB recurrence.³ Based on pharmacogenetics variation, recently, host-genetic profiles have emerged as a promising intervention in determining the success of treatment for pulmonary TB patients.⁴

One of the genes that play essential roles in TB drug pharmacokinetics is *NAT2* gene, encoding N-acetyltransferase-2 enzyme in the liver.^{4,5} This enzyme metabolizes INH into less active forms by acetylating the drug molecules, thus determining how much active drug concentration in the plasma.^{4,5} Previous studies showed three phenotypic host acetylator statuses were observed due to various polymorphisms in the *NAT2* gene: rapid, intermediate, and slow acetylators.⁴ Slow acetylator phenotype is associated with adverse drug responses such as nausea, drug-induced liver injury, peripheral neuropathy, and sideroblastic anemia which may lead to discontinuation of treatment.^{6,7} On the other hand, rapid acetylator phenotype could enhance drug metabolism in the liver, leading to the lower concentration of INH in the plasma below the requirement to kill the bacteria, thus causing treatment failure and the emergence of resistance.⁸ Therefore, pharmacogenetic profiling of *NAT2* gene becomes essential to achieve successful treatment.

As Indonesia accounts for many TB patients globally, the pharmacogenetic profiling of *NAT2* gene is of great interest. There is a high proportion of TB patients in this archipelagic country. Studies in Western and Central Indonesia have been reported recently, resulting in predominantly intermediate acetylators in this area.^{9,10} Interestingly, *NAT2* gene profile in other populations in the eastern part of Indonesia has not been explored. East Nusa Tenggara is a rural province in eastern Indonesia. This area has an estimated TB incidence of around 18,000 in 2019, with compliance percentage of treatment among patients just around 66%.¹¹ Socioeconomic factors may contribute to patient compliance in these populations. However, pharmacogenetic profiles may also affect anti-TB drug therapy's success and the rate of TB recurrence.¹² Therefore, this study aimed to explore *NAT2* acetylator

status gene among newly diagnosed and recurrent pulmonary TB patients in eastern part of Indonesia.

Materials and Methods

Study Design

This study was a descriptive and analytical study, with a retrospective design including pulmonary TB patients (newly diagnosed pulmonary TB patients and patients with recurrent TB) and healthy controls from the neighborhood. This study was part of a study exploring the susceptibility genes among TB patients from Kupang at the island of Timor, located in a remote area in the eastern part of Indonesia. Informed consent about the susceptibility genes study was obtained from all subjects, and DNA was stored for various genetic studies, including this study. Our study was conducted in accordance with Declaration of Helsinki and the study protocol was approved by the Ethical Committee of Faculty of Medicine Universitas Padjadjaran Bandung under no. 136/UN6.C2.1.2/KEPK/PN/2012.

In brief, archived DNA of TB patients (n=124) recruited in 2011 at Prof W. Z. Johannes General Hospital, Kupang, were used. Healthy controls (n=124) were also recruited to explore the *NAT2* acetylator status in the general population. Pulmonary TB patients were diagnosed according to standard hospital procedure as directed in WHO guideline, ie history taking of signs and symptoms of TB infection, physical examinations, chest radiology examinations, and positive result of three-time points of sputum examination. Mycobacterial culture was examined in a part of patients, of which drug susceptibility test and *Mycobacterium tuberculosis* spoligotyping test were performed as described previously.¹³

Pulmonary TB patients who had no previous TB treatment history were designated as newly diagnosed pulmonary TB, whereas patients with a history of TB treatment were designated as recurrent pulmonary TB. Patients with HIV seropositive or fasting glucose serum of >200 mg/dL were excluded from this study.

DNA Extraction and *NAT2* Gene Sequencing

Venous blood from pulmonary TB patients and healthy controls were collected in 3 mL EDTA tubes, then stored in 4°C before sending to Bandung, West Java, about 4 hours flight from Kupang. In brief, DNA was isolated from blood according to the manufacturer's protocol (QIAamp

DNA Blood Mini Kit, Cat no. 51104, Qiagen). The amplification of *NAT2* gene and the sequencing process was performed based on our study protocol published previously.¹⁴

Acetylator Status Assessment

The determination of acetylator status was conducted using seven single nucleotide polymorphisms in *NAT2* gene, ie rs1801279 for 191G>A, rs1041983 for 282C>T, rs1801280 for 341T>C, rs1799929 for 481C>T, rs1799930 for 590G>A, rs1208 for 803A>G, and rs1799931 for 857G>A. Sequence data analyses were performed using Bioedit software (version 7.2.5.0) and nucleotide BLAST (Genebank ref. KR231610). The *NAT2* genotype identification was performed by analyzing 7 SNPs of *NAT2* according to the Arylamine N-acetyltransferase Gene Nomenclature website (http://nat.mbg.duth.gr/Human%20NAT2%20alleles_2013.htm). The nucleotide changes at rs1801280 (341T>C), rs1799930 (590G>A), and rs1799931 (857G>A) were designated as NAT2*5, NAT2*6, NAT2*7, respectively. The subjects were then categorized as fast, intermediate, or slow acetylator phenotype.

Statistical Analyses

Data statistics were performed in Microsoft Excel 365 (Microsoft Corp., Redmond, WA, USA). The demographic, clinical, and microbiological data were compared between newly diagnosed and recurrent TB groups, then analysis was performed using Mann-U Whitney tests (for numerical data) and chi-square test (for categorical data), and p-value <0.05 was designated as significant. To determine whether the SNPs genotypes in *NAT2* were in Hardy-Weinberg equilibrium (HWE), Chi-square tests were conducted and p value ≥ 0.05 indicated that the SNPs genotype were in equilibrium. The *NAT2* haplotype status were presented in numbers (n). The acetylator status frequencies in different groups (newly diagnosed TB, recurrent TB, and healthy controls) were compared using Pearson Chi-square or Fischer's exact test as appropriate, then odds ratio (OR) with 95% confidence intervals (95% CI) were also calculated.

Results

Demographic, Clinical, and Microbiological Profile of Pulmonary TB Patients

Our study in Kupang recruited initially 124 participants pulmonary TB, consisting of 99 (79.8%) new and 25

(20.2%) recurrent pulmonary TB patients. However, patients were excluded (n=9) due to HIV seropositivity (n=1), high serum glucose suspected for Diabetes Mellitus (n=6; 4.8%) and incomplete data (n=2). The characteristic data of both group pulmonary TB patients is shown in Table 1.

When comparing new and recurrent pulmonary TB patients, there was a significant difference in gender. Male was more prevalent in the recurrent pulmonary TB group (p = 0.025). Furthermore, no significant differences in age, erythrocyte sediments rate, and body mass index between both groups. Interestingly, hemoglobin concentration in newly diagnosed pulmonary TB was significantly lower compared to recurrent TB (median 10.9 mg/dL vs 12.1 mg/dL; p = 0.018), of which anemia was more prevalent in new pulmonary TB (p = 0.003).

In a subset of the patients, drug sensitivity tests were performed, showing that the number of samples resistant to all drugs seemed to be higher in group of recurrent TB. Specifically, INH resistance cases were documented higher in recurrent TB than newly-diagnosed TB patients. Furthermore, spoligotyping detection showed that non-Beijing strain of *M.tuberculosis* predominated infection in this population, both in newly diagnosed and recurrent TB groups. Since the test was only performed in a limited number of patients, no statistical analyses were conducted, and data is only presented in frequency (Table 1).

NAT2 Polymorphisms and Acetylator Status of Pulmonary Tuberculosis Patients

The distribution of SNPs in seven rs contributed to *NAT2* gene variations among pulmonary TB patients from Kupang, Nusa Tenggara Timur, Indonesia is described in Table 2. Four polymorphisms that change the amino acid sequences (rs1801280, p = 0.616; rs1799930, p = 0.286; rs1208, p = 0.653; and rs1799931, p = 0.332) were in Hardy-Weinberg equilibrium. Based on those polymorphisms, there were 18 genotypes found in this population, and classified based on their inferred phenotype as rapid (n=37; 33.9%), intermediate (n=63; 57.8%), and slow acetylators (n=9; 8.3%). For comparison, the frequencies of acetylator status in healthy controls were 48.1%, 33.0%, and 18.9% for rapid, intermediate, and slow phenotype, respectively. The most common alleles found in this study were NAT2*4 (wild type; rapid phenotype), NAT2*6A, NAT2*6B, and NAT2*7A. There was "unknown" variation detected in this study, which had not been documented in *NAT2* gene bank; that was

Table 1 Clinical Characteristic of Patients with Newly Diagnosed and Recurrent Pulmonary Tuberculosis from Kupang, Nusa Tenggara Timur, Eastern Part of Indonesia

	Newly Diagnosed Pulmonary TB (n=91)		Recurrent Pulmonary TB (n=24)		p-value
	Median	(Min-Max)	Median	(Min-Max)	
Age; years	33	(15–74)	35	(15–64)	0.364
BMI ^a ; kg/m ²	14.6	(10.1–20.5)	15.6	(11.7–20.8)	0.201
Hb; g/dL	10.9	(7.3–15.5)	12.1	(7–14.8)	0.018*
ESR; mm/h	70	(1–140)	73.5	(4–119)	0.663
	N	(%)	N	(%)	p-value
Gender					
Male	41	(45.1)	17	(70.8)	0.025**
Female	50		7		
Nutritional status ^{c a †}					0.287
Normal	10		4		
Underweight	56	(84.8)	11	(73.3)	
Anemia status ††					0.003**
Anemia	78	(85.7)	14	(58.3)	
No Anemia	13		10		
ESR group					0.820
Normal	17		4		
High (20–25 mm/h)	8		3		
Very High (>50 mm/h)	66	(72.5)	17	(70.8)	
Drug Sensitivity Test ^b					n.d.
Sensitive	-		3	(25)	
Resistant to:					
Rif, INH, Str, Eth	1		6	(50)	
Rif, INH _____	1		2		
Rif, INH, Str _____	1		-		
Rif, _____ Str, Eth	1		-		
Rif, _____ Str, _____	1		-		
Str, _____	3		1		
Mtb genotype ^c					n.d.
Beijing	3		4		
Non-Beijing	13	(81.2)	7	(63.6)	

Notes: Data retrieved from ^a81 patients; ^b20 patients; ^c27 patients. † Nutritional Status according to BMI for normal 18–22 and underweight <18. †† Anemia designated as WHO guideline for men <13 mm/dL and women <12 mm/dL. Statistical analyses p<0.05 designated as significant for *non-parametric compared median and **chi-square
Abbreviations: BMI, body mass index; Hb, hemoglobin; ESR, erythrocyte sediment rate; TB, tuberculosis; Rif, Rifampicin; INH, isoniazid; Str, Streptomycin; Eth, Ethambutol; n.d, not determined; Mtb, *Mycobacterium tuberculosis*.

a combination of 282 C>T (rs1041983), 590 G>A (rs1799930), 625 C>T that inferred as slow phenotype due to homozygous recessive state. A new polymorphism 625 C>T had not been described elsewhere in *NAT2* gene database. However, since it was a non-sense mutation and did not change the amino acid sequence, this polymorphism might not change the function of NAT2 enzyme.

Interestingly, the rapid and intermediate acetylators were significantly more prevalent in pulmonary TB cases than in healthy controls (p = 0.023, OR = 2.58 (1.12–5.97)). However, comparing the frequency of acetylators status among newly diagnosed and recurrent pulmonary TB resulted in no significant difference between both groups (p = 0.776), as shown in Table 3.

Table 2 Distribution of Single Nucleotide Polymorphisms Contributed to NAT2 Gene Variations Among Pulmonary Tuberculosis Patients from Kupang, Nusa Tenggara Timur, Eastern Part of Indonesia

			rs1801279	rs1041983	rs1801280	rs1799929	rs1799930		rs1208	rs1799931
Genotypes	Inferred	n	191 G>A	282 C>T	341 T>C	481 C>T	590 G>A	625 C>T	803 A>G	857 G>A
	Phenotypes		R64Q	Y94Y	II14T	L161L	R197Q	L209L	K268R	G286E
					NAT2*5		NAT2*6		NAT2*12	NAT2*7
NAT2*4/4	Rapid (WT)	35	GG	CC	TT	CC	GG		AA	GG
NAT2*4/12A	Rapid	1							AG	
NAT2*4/12M	Rapid	1		CT		CT			AG	
NAT2*4/5B	Intermediate	4			TC	CT			AG	
NAT2*4/5C	Intermediate	1			TC	CT			AG	
NAT2*4/5E	Intermediate	1			TC		GA		AG	
NAT2*4/5Q	Intermediate	1			TC		GA			
NAT2*4/5S	Intermediate	1			TC					GA
NAT2*4/5U	Intermediate	1		CT	TC	CT	GA		AG	
NAT2*4/6A	Intermediate	4		CT			GA			
NAT2*4/6B	Intermediate	15					GA			
NAT2*4/6J	Intermediate	13		CT/TT			GA			GA
NAT2*4/7A	Intermediate	18								GA
NAT2*4/7B	Intermediate	3		TT						GA
NAT2*6A/6A	Slow	6		TT			AA			AA
NAT2*7B/7B	Slow	2		TT						AA
Unknown †	Intermediate	1			TC	CT	GA			
Unknown †	Slow	1		TT			AA	CT		

Notes: † Unknown is designated as variations that are not registered according to the data base of Arylamine N-acetyltransferase (NATs) for Human NAT2 Alleles (haplotypes) http://nat.mbg.duth.gr/Human%20NAT2%20alleles_2013.htm. Intermediate acetylator when the alleles are heterozygous; slow acetylators when the alleles are in homozygote recessive variant.

Abbreviations: NAT2, N-acetyltransferase 2; WT, wild type.

Table 3 Distribution of Acetylator Status in Newly Diagnosed and Recurrent Pulmonary Tuberculosis Compared to Healthy Controls from Kupang, Nusa Tenggara Timur, Eastern Part of Indonesia

Group	Acetylator Status, n (%)			P value*	OR (95% CI)
	Rapid	Intermediate	Slow		
Healthy Controls	51 (48.1)	35 (33.0)	20 (18.9)	0.023 ^a	2.58 (1.12–5.97)
Pulmonary TB patients	37 (33.9)	63 (57.8)	9 (8.3)		
Newly diagnosed	28 (32.2)	52 (59.8)	7 (8.0)	0.776 ^b	n.a.
Recurrent	9 (40.9)	11 (50.0)	2 (9.1)		

Notes: *rapid and intermediate vs slow acetylator status (chi-square test), ^a Control vs pulmonary TB patients, ^b Newly diagnosed vs recurrent pulmonary TB patients.

Abbreviations: TB, tuberculosis; OR, odds ratio; CI, confidence interval; n.a., not available.

Discussion

Recurrent pulmonary TB infection worldwide is reported in around 13% of all TB cases. Our study has detected that around 20% of cases are recurrent pulmonary TB, higher than estimated global data in 2017.¹⁵ The recurrent TB proportion varies significantly in different areas; for example, in Iran, Zambia, and South Africa, the recurrent TB proportions are 8.3%, 15.3%, and 26%, respectively.^{16–18} Recurrent pulmonary TB could be caused by either reactivation of *M.tuberculosis* from previous infection or re-

infected from exogenous sources.¹⁹ In this study, recurrent pulmonary TB is significantly higher in male patients, confirming that males become one of the risk factors contributing to recurrent cases.^{20,21} Other risk factors associated with recurrent TB cases include smoking, poor treatment adherence, uncontrolled HIV-comorbid infection, and specific clinical pictures such as residual cavitation, more significant area of involved lung tissue, and positive sputum culture at two months of first treatment.³ Previous studies have shown associations between TB

recurrence and drug resistance.^{22,23} Our study has revealed more MDR cases, as defined by simultaneous rifampicin and isoniazid resistance, documented in recurrent TB cases despite a small number of tests. These findings suggest the importance of using rapid methods to detect drug-resistance *M.tuberculosis* to determine which regimens should be used in recurrent pulmonary TB cases.

Genotype diversity of *NAT2* gene has been known to affect TB therapy response. Different genotypes can influence how rapidly *NAT2* enzyme metabolizes INH drugs in the liver. Different ethnicities have shown different proportions of rapid, intermediate, and slow phenotypes. The frequency of slow acetylators varies in different countries, for example, in Latvia (51.8%), Senegal (49%), and Brazil (41%),^{24–26} which is much higher than in our study population. Interestingly, several Asian countries reported slow acetylator phenotypes in lower frequencies, for example, China (15.2%) and South Korea (14.4%).^{27,28} Moreover, studies in western and central Indonesia have shown the frequencies of slow acetylators are around 36% in pulmonary TB patients with Javanese-Sundanese ethnicity. Of note, healthy Malay-Indonesian accounts for 38%, similar to healthy Buginese (42%).^{9,10,29} Interestingly, our study in Kupang, East Nusa Tenggara revealed that only around 8% of pulmonary TB patients were slow acetylator phenotypes. Predominance of rapid-intermediate acetylator in TB patients in Kupang, suggesting that different genetic background of this population may affect the *NAT2* gene polymorphism diversity. Based on historical perspective, Kupang people residing in Timor Island, East Nusa Tenggara, have mixed origin from Austronesian and Polynesian ancestry.^{30,31} This hypothesis should be explored in other parts of East Nusa Tenggara and other areas in eastern Indonesia such as Maluku and West Papua.

Both slow and rapid acetylation could influence the drug response in TB patients regarding of the INH metabolism speed in the liver. Slow acetylators are associated with increased INH levels in plasma and hepatotoxicity observed in TB patients, confirming the study conducted in Javanese and Sundanese TB patients.⁹ The *NAT2*6A* allele is reported as an ultra-slow acetylator that can be used to determine the effects of *NAT2*'s genetic polymorphism on metabolism and drug toxicity.³² On the other hand, a substantial percentage of rapid acetylators in this population should raise other awareness since a meta-analysis study showed that rapid acetylators

contribute to microbiological failure, acquired drug resistance, and TB relapse.⁸ The rapid acetylator individuals could exhibit lower INH levels as presented in bloodstream. This is due to INH molecules that is rapidly acetylated, thus decreasing INH exposure to MTB in the affected tissues.³³ Molecular docking study of mutated MT-K268R model (polymorphisms at 803 A>G in *NAT2*4*, *NAT2*12* and *NAT2*13*) has shown less binding affinity toward INH and increase acetyl-INH score, leading to more INH acetylation.³⁴ As we noted that INH drug resistance was found in this population (especially in people with recurrent pulmonary TB), further study with larger participants might be needed to confirm the association of rapid acetylator phenotypes and recurrent TB cases and drug resistance in Kupang population.

Our study has several limitations among others that the study was not designed to detect adverse effects such as hepatotoxicity and treatment failure, especially due to the nature of the study site which is in a remote area and laboratory examinations are scarce. Further study needs to be designed considering the role of pharmacogenetic background of individuals living in area with possible different genetic makeup across the islands of Indonesia. The study on population genetic profile is of great interest. Furthermore, the sample size is not calculated accordingly as we had included total respondents who were willing to participate in the study period. Moreover, some of the clinical information and other data are sometimes lacking. Therefore, it is imperative to register the complete information in the medical record or registry that would strengthen the results of the TB treatment with first-line drugs in Indonesia.

Conclusion

The slow acetylator status in pulmonary TB in Kupang population in our study was 8.3%, much lower than previous study conducted in other parts of Indonesia, suggesting different genetic background in eastern part of Indonesia. There was no difference in acetylator status between newly and recurrent pulmonary TB. As majority of pulmonary TB patients in Kupang are rapid and intermediate acetylator status, these might influence the emergence of drug resistance and recurrence. The acetylator status might be relevant as the rationalization in recurrent TB patients to be checked prior to INH therapy to adjust treatment dose to prevent further antimicrobial resistance for successful TB treatment.

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Disclosure

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