Genetic characterization of *N*-acetyltransferase 2 variants in acquired multidrug-resistant tuberculosis in Indonesia

Rika Yuliwulandari^{*1,2} , Kinasih Prayuni², Intan Razari³, Retno W Susilowati^{2,4}, Yenni Zulhamidah⁵, Soedarsono Soedarsono^{6,7}, Abdul Salam M Sofro^{8,9} & Katsushi Tokunaga¹⁰

¹Department of Pharmacology, Faculty of Medicine, YARSI University, Jakarta, Indonesia

²Genetic Research Center, YARSI Research Institute, YARSI University, Jakarta, Indonesia

³YARSI Research Institute, YARSI University, Jakarta, Indonesia

⁴Department of Histology, Faculty of Medicine, YARSI University, Jakarta, Indonesia

⁵Department of Anatomy, Faculty of Medicine, YARSI University, Jakarta, Indonesia

⁶Department of Pulmonology & Respiratory Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

⁷Dr. Soetomo General Hospital, Surabaya, Indonesia

⁸Graduate School, YARSI University, Jakarta, Indonesia

⁹Department of Biochemistry, Faculty of Medicine, YARSI University, Jakarta, Indonesia

¹⁰Genome Medical Science Project (Toyama), National Center for Global Health & Medicine, Tokyo, Japan

*Author for correspondence: Tel.: +62 21 420 6674; rika.yuliwulandari@yarsi.ac.id

Background: Owing to the high resistance rate of tuberculosis (TB) to isoniazid, which is metabolized by *N*-acetyltransferase 2 (NAT2), we investigated the associations between NAT2 variants and multidrug-resistant (MDR)-TB. **Materials & methods:** The acetylator status based on NAT2 haplotypes of 128 patients with MDR-TB in Indonesia were compared with our published data from patients with anti-TB drug-induced liver injury (AT-DILI), TB and the general population. **Results:** NAT2*4 was more frequent in the MDR-TB group than in the AT-DILI group, TB controls and general controls. NAT2*4/*4 was significantly more frequent in patients with MDR-TB than in those with AT-DILI. NAT2*5B/7B, *6A/6A and *7B/*7B were detected at lower frequencies in patients with AT-DILI. Rapid acetylators were significantly more frequent in patients with MDR-TB than in those with AT-DILI. **Conclusion:** These results provide an initial data for optimizing TB treatment in the Indonesian population, and suggest that NAT2 genotyping may help to select appropriate treatment by predicting TB-treatment effect.

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Keywords: anti-TB drug-induced liver injury • multidrug-resistant-TB • NAT2 • rapid acetylator • tuberculosis

Tuberculosis (TB) is a major infectious disease worldwide. The increase in multidrug-resistant (MDR)-TB, primarily caused by bacteria that do not respond to antibiotics, is a potential threat to TB control programs. These antibiotics include isoniazid (INH) and rifampicin, which are currently the two most powerful anti-TB treatment options [1,2]. According to the Global Tuberculosis Report of 2019, Indonesia is one of the top 30 countries with respect to the MDR-TB incidence, with an estimated 24,000 new patients eligible for MDR-TB treatment every year, including 2.4% new TB cases and 13% previously treated TB cases [1].

INH is an important first-line treatment regimen for TB, which has high bactericidal activity and a low risk of adverse events [3]. However, approximately 8% of patients with TB worldwide have INH-resistant TB. Patients with confirmed INH resistance have been reported to show worse outcomes such as higher rates of treatment failure, relapse and acquired drug resistance [4]. INH is primarily metabolized by *N*-acetyltransferase 2 (NAT2) encoded by the NAT2 gene, which can produce three phenotypes: rapid, intermediate and slow acetylators [5,6]. The NAT2 slow acetylator (SA) type is associated with the development of anti-TB drug-induced liver injury (AT-DILI) [7–9]. By contrast, the NAT2 rapid acetylator (RA) type has been associated with treatment failure due to suboptimal doses of INH [5,10]. An *in silico* study revealed that the NAT2 RA status can be considered a risk factor for the development of INH resistance [11]. However, the NAT2 mutational spectrum in patients with MDR-TB

has not been well characterized. Therefore, in this study, we investigated the associations between *NAT2* variants and MDR-TB to gain insights into possible strategies for optimizing TB treatment, particularly for preventing the development and spread of antibiotic resistance.

Materials & methods

Subjects

In this retrospective study, a total of 128 patients (68 males and 60 females) diagnosed with MDR-TB were recruited from Dr. Soetomo Hospital, Surabaya, Indonesia. The AT-DILI and the control samples data were obtained from our previous published studies [12,13]. All patients and controls ethnicities were native inhabitants of Java Island (Javanese, Madurese or Sundanese ethnicity). The samples were obtained from pulmonary MDR-TB patients who were confirmed to have received or were continuing to receive treatment with an MDR-based regimen. All of MDR-TB samples in this study were acquired resistance caused by inadequate treatment, either patients with sputum-smear positive for acid-fast bacilli that remain positive after 2 months TB treatment or patients with acid-fast bacilli that turn or remain positive after the whole TB treatment. Whole blood samples (3 ml) were collected from each patient in ethylenediaminetetraacetic acid (EDTA) tubes and stored in a -20°C freezer until further processing. All patients were ≥21 years old and provided written informed consent to participate in the study. Exclusion criteria were patients with pulmonary MDR-TB comorbidities (including diabetes, kidney failure, liver failure, bronchial asthma, chronic obstructive pulmonary disease and human immunodeficiency virus) and patients with MDR-TB who were being treated with corticosteroids. The study was approved by the Institutional Research Board of YARSI University (No. 127/KEP-UY/BIA/VIII/2019).

DNA extraction

Genomic DNA was extracted from venous blood samples (3 ml) using a Maxwell DNA Blood Kit (Promega, WI, USA). The extracted genomic DNA was quantified using an Infinite 200 Pro NanoQuant Plate Reader (TECAN, Mannedorf, Switzerland). DNA purity was determined by calculating the ratio of absorbance at 260 and 280 nm.

NAT2 genotyping

NAT2 genotyping in samples from the MDR-TB patients was performed by standard Sanger sequencing. PCR was performed using a T-100 thermocycler (Bio-Rad, CA, USA) in a 20-μl reaction mixture containing 2 μl of 20-ng genomic DNA, 2.5 μl of PCR Reaction Buffer, with 20 mM MgCl₂ 10x concentrated (Roche Applied Science, Penzberg, Germany), 2.5 μl of 10 mM PCR nucleotide mix (Promega, WI, USA), 0.5 μl of 100 μM primer (each forward and reverse; Macrogen, Inc., Seoul, South Korea), 0.2 μl of 1 U FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) and 11.8 μl of nuclease-free water (NFW; Promega, WI, USA). The PCR conditions were as follows: initial denaturation at 96°C for 4 min; 40 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s; and a final elongation step at 72°C for 5 min. The forward and reverse primers used for PCR and DNA sequencing were based on a previously published report targeting the following six SNPs: rs1041983 (282C4T), rs1801280 (341T4C), rs1799929 (481C4T), rs1799930 (590G4A), rs1208 (803A4G) and rs1799931 (857G4A) [12]. All amplified fragments were sequenced by Macrogen Sequencing service (Macrogen, Inc.).

Data analysis

All SNPs were detected using NovoSNPs [14]. Haplotypes were inferred from the six SNPs using PHASED v2.1.1 [15,16]. The *NAT2* acetylator status for each haplotype was inferred based on the annotation table published in the human *NAT2* allele database (http://nat.mbg.duth.gr/Human%20NAT2%20alleles_2013.htm). A comparative analysis of MDR-TB data was performed with our previously published *NAT2* genotype and phenotype data for AT-DILI cases and TB controls and the general population [12,13]. The χ^2 -test was used for comparisons between the groups. Odds ratios (ORs) and 95% CIs were calculated using conditional logistic regression. A two-tailed $p < 0.05$ was considered statistically significant.

Results

We identified eight *NAT2* alleles from six SNPs in MDR-TB samples. *NAT2**4 is the reference wild-type allele (Table 1). *NAT2**4, *12A and *13A are known as *NAT2* RA-type alleles, whereas *5A, *5B, *6A, *6B and *7B are known as *NAT2* SA-type alleles. *NAT2**4 was more frequent in MDR-TB cases (41.02%) than in AT-DILI

Table 1. Comparison of NAT2 alleles in multidrug-resistant tuberculosis patients, antituberculosis drug-induced liver injury patients, tuberculosis controls and general controls.

NAT2 allele	MDR-TB (2n = 256)	AT-DILI (2n = 200) [†]	TB control (2n = 420) [†]	General control (2n = 424) [‡]	MDR-TB vs TB controls		MDR-TB vs AT-DILI		MDR-TB vs general controls	
					p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)
*4	105	45	154	158	0.29	1.2 (0.87–1.65)	0.000035	2.4 (1.58–3.63)	0.37	1.17 (0.85–1.61)
*5A	2	1	0	0	0.14	NA	1	1.57 (0.14–17.4)	0.14	NA
*5B	18	24	49	38	0.06	0.57 (0.33–1)	0.07	0.55 (0.29–1.05)	0.39	0.77 (0.43–1.38)
*6A	89	97	133	156	0.44	1.15 (0.83–1.59)	0.0039	0.56 (0.39–0.83)	0.62	0.92 (0.66–1.27)
*7A	1	0	1	0	1	0.88 (0.05–13.81)	1	NA	0.37	NA
*7B	31	32	57	65	0.6	0.88 (0.55–1.4)	0.27	0.72 (0.42–1.23)	0.26	0.76 (0.48–1.20)
*12A	8	0	20	4	0.33	0.64 (0.28–1.49)	0.01	NA	0.06	3.38 (1–11.36)
*13A	2	1	6	3	0.5	0.54 (0.11–2.71)	1	1.57 (0.14–17.4)	1	1.1 (0.18–6.66)

[†] Previously published data for AT-DILI and TB controls [13].[‡] Previously published data for the Javanese and Sundanese populations [12].

Bold numbers indicate a significant p-value.

AT-DILI: Anti-TB drug-induced liver injury; MDR-TB: Multidrug-resistant TB; NA: Not available; OR: Odds ratio; TB: Tuberculosis.

Table 2. Comparison of NAT2 diplotypes in multidrug-resistant tuberculosis and antituberculosis drug-induced liver injury cases.

NAT2 diplotype	MDR-TB cases (n = 128)	AT-DILI cases [†] (n = 100)	p-value	OR (95% CI)
*4/*4	23	7	0.02	2.91 (1.19–7.09)
*4/*5A	1	1	0.86	0.78 (0.05–12.62)
*4/*5B	9	5	0.53	0.05 (0–0.89)
*4/*6A	34	20	0.25	1.45 (0.77–2.71)
*4/*7A	1	0	0.38	NA
*4/*7B	12	4	0.11	0.06 (0–1.19)
*4/*12A	1	0	0.38	NA
*4/*13A	1	1	0.86	0.78 (0.05–12.62)
*5A/*6A	1	0	0.38	1.57 (0.05–47.42)
*5B/*5B	0	2	0.11	0.11 (0.01–2.58)
*5B/*6A	7	8	0.44	0.66 (0.23–1.90)
*5B/*7B	1	7	0.02	0.1 (0–0.86)
*5B/*12A	1	0	0.38	NA
*6A/*6A	16	25	0.02	0.42 (0.21–0.86)
*6A/*7B	10	19	0.02	0.36 (0.15–0.81)
*6A/*12A	5	0	0.06	NA
*7B/*7B	3	1	0.44	0.24 (0.01–7.16)
*7B/*12A	1	0	0.38	NA
*7B/*13A	1	0	0.38	NA

[†] Previously published data for AT-DILI [13].

Bold numbers indicate a significant p-value.

AT-DILI: Anti-TB drug-induced liver injury; MDR-TB: Multidrug-resistant tuberculosis; NA: Not available; OR: Odds ratio.

cases (22.5%), TB controls (36.67%) and general controls (37.26%). However, allele frequencies did not differ significantly among MDR-TB cases, TB controls and general controls (Table 1). NAT2*4 and *12A frequencies were significantly higher in MDR-TB cases than in other groups. The frequency of *6A was significantly higher among AT-DILI cases than that of the other groups (OR: 0.56; 95% CI: 0.39–0.83; p = 0.0039; Table 1).

We further compared diplotypes between MDR-TB and AT-DILI cases. As shown in Table 2, the frequency of NAT2*4/*4 (OR: 2.9; 95% CI: 1.19–7.09; p = 0.02) was significantly higher in MDR-TB cases than in AT-DILI cases, whereas NAT2*5B/*7B, *6A/*6A and *7B/*7B diplotypes were less frequent in patients with AT-DILI (OR:

Table 3. Inferred *N*-acetyltransferase 2 phenotype in multidrug-resistant tuberculosis cases, antituberculosis drug-induced liver injury cases, tuberculosis controls and general controls.

NAT2 phenotype	MDR-TB cases (n = 128)	AT-DILI cases [†] (n = 100)	TB control [†] (n = 210)	General control (n = 212) [‡]
RA	25 (19.53%)	8 (8%)	35 (16.67%)	29 (13.68%)
IA	65 (50.78%)	30 (30%)	110 (52.38%)	107 (50.47%)
SA	38 (29.69%)	62 (62%)	65 (30.95%)	76 (35.85%)

[†]Previously published data for AT-DILI [13].[‡]Previously published data for the Javanese and Sundanese populations [12].AT-DILI: Anti-TB drug-induced liver injury; IA: Intermediate acetylator; MDR-TB: Multidrug-resistant TB; NAT2: *N*-acetyltransferase 2; RA: Rapid acetylator; SA: Slow acetylator; TB: Tuberculosis.**Table 4. Comparison of inferred *N*-acetyltransferase 2 phenotypes.**

Phenotypes	p-value	OR (95% CI)
MDR-TB vs TB control		
RA + IA vs SA	0.81	1.06 (0.66–1.71)
RA vs SA	0.61	1.22 (0.64–2.34)
IA vs SA	1	1.01 (0.61–1.67)
MDR-TB vs AT-DILI		
RA + IA vs SA	0.0000012	3.86 (2.22–6.72)
RA vs SA	0.00023	5.1 (2.09–12.44)
IA vs SA	0.000028	3.53 (1.95–6.39)
MDR-TB vs general control		
RA + IA vs SA	0.28	1.32 (0.83–2.12)
RA vs SA	0.12	1.72 (0.88–3.34)
IA vs SA	0.45	1.22 (0.74–1.99)

Bold numbers indicate a significant p-value.

AT-DILI: Anti-TB drug-induced liver injury; IA: Intermediate acetylator; MDR-TB: Multidrug-resistant TB; NAT2: *N*-acetyltransferase 2; OR: Odds ratio; RA: Rapid acetylator; SA: Slow acetylator; TB: Tuberculosis.

0.1; 95% CI: 0–0.86; $p = 0.02$, OR: 0.42; 95% CI: 0.21–0.86; $p = 0.02$, OR: 0.36; 95% CI: 0.15–0.81; $p = 0.02$, respectively).

The distribution of inferred NAT2 acetylator status is shown in Table 3. The RA type was more frequent in MDR-TB cases than in AT-DILI cases, TB controls and general controls. Comparative inferred phenotype analysis (summarized in Table 4) showed that the frequency of RA was significantly higher in MDR-TB cases than in AT-DILI cases (OR: 5.1; 95% CI: 2.09–12.44; $p = 0.00023$).

Discussion

NAT2 is an important enzyme involved in the metabolism of INH, a key drug used for the treatment of TB. *NAT2* polymorphisms cause variation in INH *N*-acetylation, producing the RA, intermediate acetylator and SA phenotypes [6,17]. Previous studies have revealed that *NAT2* genotypes correlated with the SA phenotype are associated with AT-DILI [7–9]. MDR-TB is another serious issue, affecting approximately 18% of patients globally in 2018 [1]. Approximately 8% of patients with TB worldwide are estimated to have INH-resistant TB [4]. However, little is known about the characterization of *NAT2* genotypes in MDR-TB.

Many studies have shown that the RA type is associated with treatment failure in patients receiving the standard regimen of TB drugs [6,17,18]. Treatment failure may be caused by insufficient exposure to INH [6,17,18]. In this study, we found that *NAT2**4/*4, a known RA genotype [6,19], is more frequent in MDR-TB cases than in AT-DILI cases and was significantly associated with MDR-TB. *NAT2**4/*4 is a potential marker for INH resistance in MDR-TB cases. In contrast, *NAT2**5B/7B, *6A/6A and *7B/*7B, which are classified as SA genotypes [6,19], were detected at lower frequencies in patients with AT-DILI. Several studies have revealed that these genotypes are associated with the development of AT-DILI [5,8,20]. Additionally, Selinski *et al.* [21] classified *NAT2**6A/6A and *7B/*7B as ultra-SA genotypes, in which very low NAT2 enzyme activity contributes to a higher risk of AT-DILI relative to the SA and RA types [22].

We found that the RA inferred phenotype was significantly more frequent in MDR-TB cases than in AT-DILI cases. This suggests that the RA phenotype may increase the risk of INH resistance; it is possible that lower serum concentrations result in reduced exposure of the bacteria to INH in affected tissues [11]. Sotsuka *et al.* showed that the INH concentration was the lowest in patients with an RA status and was the highest in patients with the SA phenotype [23]. Another pharmacokinetics study demonstrated that patients with an RA phenotype are at an elevated risk of treatment failure, whereas the SA phenotype emerged as a risk factor for AT-DILI [5,10,17]. Therefore, appropriate dose adjustment of INH based on the *NAT2* genotype before prescribing TB treatment is important to prevent treatment failure and unwanted side effects such as MDR-TB and AT-DILI.

This study had several limitations. We did not determine the pharmacokinetic profile of INH concentrations in MDR-TB patients according to *NAT2* genotypes. Additionally, we did not analyze other factors such as age, sex, body weight, BMI and liver function before the anti-TB treatment. Jung *et al.* revealed that body weight independently affects INH concentrations [10]. Therefore, further studies are needed to confirm our findings. Nevertheless, this study provides an initial data for the development of an optimal INH dosage in the treatment of TB for the Indonesian population. Additionally, our results suggest that *NAT2* genotyping prior to therapy would be useful to predict unfavorable outcomes such as MDR-TB and AT-DILI.

Conclusion

To the best of our knowledge, this is the first study to characterized *NAT2* variants in acquired MDR-TB. We detected a significant association between the inferred *NAT2* RA phenotype and MDR-TB. *NAT2**4/*4, an RA-associated genotype, was significantly more common in MDR-TB cases than in other groups, whereas the frequencies of *NAT2**5B/7B, *6A/6A and *7B/*7B, which are SA-associated genotypes, were relatively reduced in MDR-TB cases. This study provides an initial data for further pharmacokinetics analyses of MDR-TB based on *NAT2* variants.

Future perspective

TB treatment using a standard regimen of antimicrobial drugs can cause an unfavorable outcome of either MDR-TB or AT-DILI. INH, as the powerful drug for TB treatment that is metabolized by *NAT2*, may not be appropriate for some patients with certain *NAT2* genotypes due to failure to maintain the therapeutic concentrations of the drug owing to rapid metabolism. Our study makes an important contribution owing to the lack of studies investigating the characterization of *NAT2* genotype in acquired MDR-TB. Our study can provide an initial data for further investigations and pharmacokinetics analyses in MDR-TB patients. The determination of INH dosing according to *NAT2* genotype may help to maintain patients within the therapeutic range, thereby minimizing treatment failures and adverse events such as MDR-TB and AT-DILI.

Summary points

- Long duration and multi drugs treatment of tuberculosis may result in unfavourable outcome of tuberculosis (TB) treatment such as multidrug-resistant (MDR)-TB and anti-TB drug-induced liver injury (AT-DILI).
- *NAT2* plays an important role in the metabolism of isoniazid.
- The role of *NAT2* genotype and phenotype in MDR-TB is still unclear.
- Investigating the role of *NAT2* genotype and phenotype may help to avoid unfavourable treatment outcomes such as MDR-TB and AT-DILI.
- Eight *NAT2* alleles were identified in this study.
- *NAT2**4 and *12A frequencies were significantly higher in MDR-TB cases than in AT-DILI patients, TB patients or the general population.
- *NAT2**4/*4 diplotype was significantly more common in MDR-TB cases, whereas *NAT2**5B/7B, *6A/6A and *7B/*7B diplotypes were less frequent in MDR-TB than in AT-DILI cases.
- The inferred rapid acetylator phenotype was significantly more frequent in MDR-TB cases than that in other groups.

Author contributions

R Yuliwulandari is the principal investigator that was responsible for all aspects of this study. All authors were responsible for the study conception, study design, manuscript writing and manuscript revision. R Yuliwulandari, RW Susilowati, Y Zulhamidah and S Soedarsono were responsible for sample collection. K Prayuni and I Razari were responsible for data acquisition and data valida-

tion. R Yuliwulandari and K Prayuni were responsible for data analysis and interpretation, and for writing the original manuscript draft. R Yuliwulandari, ASM Sofro and K Tokunaga validated the study results and supervised the study.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The study protocol was approved by the ethical review committee at YARSI University (No. 127/KEP-UY/BIA/VIII/2019) and the study followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. All participating patients provided their written informed consent for participation and inclusion of their data in the study.

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