

Original Article

**Influence of TLR-8 Gene Polymorphisms (rs3764880 and rs3788935)
Associated to Pulmonary Tuberculosis in Kupang, Indonesia**

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ABSTRACT

Toll-like receptor 8 (TLR-8) is known as part of intracellular signaling transduction for bacterial phagocytosis. *Mycobacterium tuberculosis* (Mtb) is intracellular pathogenic bacteria that is recognized by this receptor, and genetic variation of TLR-8 might alter susceptibility of the host towards pulmonary tuberculosis (PTB). This study aimed to determine whether TLR-8 gene polymorphisms were associated to PTB in Kupang, Indonesia. This case-control study compared demographic and clinical data between 115 PTB patients and 115 controls, then two TLR-8 single nucleotide polymorphisms (rs3764880 and rs3788935) were explored using the GoldenGate® Genotyping for VeraCode® / BeadXpress Illumina®. There is no significant difference between sex distribution of patient vs control groups. The polymorphisms (rs3764880 and rs3788935) are in Hardy-Weinberg Equilibrium in this population ($p > 0.05$). The distribution of major vs minor genotypes and alleles of TLR-8 polymorphisms in PTB patients were as followed: rs3764880 (GG vs GA vs AA, 50.0% vs 21.4% vs 28.6% ; G vs A, 60.9% vs 39.1%) and rs3788935 (GG vs GA vs AA, 53.0% vs 21.7% vs 25.3%; G vs A, 62.9% vs 37.1%). Neither genotypes nor alleles were associated with PTB in this population ($P > 0.05$). Besides, when the analyses were stratified by gender, none of the alleles of polymorphism in both genders were associated with PTB cases. None of the TLR-8 polymorphisms have associated the risk of developing PTB in Kupang, East Nusa Tenggara population (as opposed to other studies in different ethnic groups). These might reflect the diversity of genetic polymorphisms in eastern Indonesia populations, suggesting different genetic backgrounds with western part of Indonesia.

Keywords: Eastern Indonesia; Genetic polymorphisms; Pulmonary Tuberculosis; Toll-Like Receptor 8

ABSTRAK

Toll-like receptor 8 (TLR-8) dikenal sebagai reseptor patogen intraseluler terkait pensinyalan transduksi intraseluler setelah bakteri difagositosis. *Mycobacterium tuberculosis* (Mtb) adalah bakteri patogen intraseluler yang dikenali oleh reseptor ini. Variasi genetik pada gen TLR-8 memiliki kemungkinan asosiasi terhadap kerentanan tuberkulosis paru pada manusia. Penelitian ini bertujuan untuk mengetahui apakah polimorfisme gen TLR-8 berhubungan dengan kasus tuberkulosis paru di Kupang, Indonesia. Studi kasus-kontrol ini membandingkan data demografis dan klinis pada 115 pasien PTB dan 115 kontrol. Selanjutnya, dua polimorfisme nukleotida tunggal TLR-8 (rs3764880 dan rs3788935) dieksplorasi pada dua kelompok tersebut menggunakan GoldenGate® Genotyping for VeraCode® / BeadXpress Illumina®. Tidak ada perbedaan yang signifikan antara distribusi jenis kelamin pada kelompok pasien dan kelompok kontrol. Polimorfisme (rs3764880 dan rs3788935) berada dalam Ekuilibrium Hardy-Weinberg dalam populasi ini ($p > 0,05$). Distribusi genotipe dan alel mayor vs minor polimorfisme TLR-8 pada pasien PTB adalah sebagai berikut: rs3764880 (GG vs GA vs AA, 50,0% vs 21,4% vs 28,6% ; G vs A, 60,9% vs 39,1%) dan rs3788935 (GG vs GA vs AA, 53,0% vs 21,7% vs 25,3%; G vs A, 62,9% vs 37,1%). Baik genotipe maupun alel di atas tidak berasosiasi dengan tuberkulosis dalam populasi ini ($P > 0,05$). Selain itu, ketika analisis dikelompokkan berdasarkan jenis kelamin, tidak

Ditemukan adanya hubungan antara genotipe maupun alel polimorfisme pada kedua jenis kelamin terhadap kasus tuberkulosis paru.

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Pada penelitian ini tidak satupun dari polimorfisme TLR-8 terkait dengan risiko kasus PTB di populasi Kupang, Nusa Tenggara Timur (dibandingkan dengan studi lain di kelompok suku yang berbeda yang menunjukkan adanya keterkaitan polimorfisme TLR-8 terhadap kasus PTB). Hal ini mungkin mencerminkan keragaman polimorfisme genetik pada populasi Indonesia bagian timur, menunjukkan latar belakang genetik yang berbeda dengan Indonesia bagian barat.

Kata kunci: *Enterobacteriaceae; ESBL; flora usus; sapi perah; peternak; rural*

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INTRODUCTION

Tuberculosis still becomes a leading cause of morbidity and mortality among infectious diseases group, especially in low- and middle-income countries.¹ In 2015, the tuberculosis incident reached 10.2 million cases, and the mortality was 1.3 million cases globally.¹ Indonesia is ranked the third most prevalent countries in the world and about 845.000 new active tuberculosis cases emerged in 2018.²

Although *M. tuberculosis* (Mtb) is highly infectious, only 5-10 per cent of people infected with the bacilli had clinical manifestations; others remained to maintain latent infection status.³ This might reflect different gene expression of innate and adaptive immune system controlling the disease expression. Recently, genetic polymorphisms in various loci had been investigated, showing some relation with complex susceptibility and disease expression of pulmonary tuberculosis (PTB).⁴ Some of them were genes expressing toll-like receptors (TLRs), which have essential roles in recognizing pathogen by innate immunity frontline including Mtb.⁵ One of these receptors, TLR-8, becomes one of the interesting genes to be explored in relation to PTB. Recent investigations showed that connection of TLR-8 polymorphisms in PTB susceptibility differed among race, gender, and age.⁵⁻⁷

Previous study has shown that TLR-8 has association with the PTB in Jakarta (western part of Indonesia), and other studies in Pakistan, Russia, South Africa, and Turkey described similar results.⁸⁻¹⁰ However, some papers have also reported that these polymorphisms do not have significant

Kupang, the capital city of East Nusa Tenggara province in Indonesia, has high PTB cases with positive acid-fast bacilli sputum examination (incidence rate 210 cases per 100,000 population in 2013).¹³ Despite environmental and socioeconomic risk factors play essential role of PTB prevalence,¹⁴⁻¹⁶ genetic predisposition might also affect this disease becomes highly close contact with dairy cows. prevalent in this area. Since the genetic background of people in East Nusa Tenggara (as more closely related to Melanesian and Polynesian people) is different with Java as western part of Indonesia (which has high relationship with Austronesian ancestry), we were intrigued to investigate how TLR-8 association might be differed between these groups. Therefore, this study was aimed to explore the association of TLR-8 gene polymorphisms towards PTB in Kupang, Indonesia.

MATERIALS AND METHODS

Patient recruitment and sample collection

This retrospective, case-control study was part of a bigger research conducted in Prof. Dr. W. Z. Johannes General District Hospital, Kupang, East Nusa Tenggara, Indonesia between January to September 2012. Case group comprised of adult (more than 15 years old) diagnosed as post-primary pulmonary tuberculosis based on clinical manifestation and radiographic thorax evaluation, then confirmed by positive sputum smear using Indonesian guideline for tuberculosis diagnosis and treatment.¹⁷

On the other hand, control groups comprised of healthy individuals with no clinical symptoms of PTB and proven negative sputum smear for acid-fast bacilli. Subjects with severe comorbidities, i.e. diabetes mellitus, asthma, cardiovascular disease, cancer, and autoimmune disease like systemic lupus erythematosus, were excluded. Before Genotypic examination was held in Institute of Tropical Diseases, Universitas Airlangga, Surabaya. enrolment in this study, subjects were explained about the study procedure and written informed consents were obtained. This study was reviewed and approved by the Ethical Committee Board from Faculty of Medicine, Universitas Padjadjaran (no. 136/UN6.C2.1.2/KEPK/PN/2012).

The subjects answered questionnaires regarding their demography and pulmonary TB data. Their venous blood then was collected in EDTA-tubes and stored in 4°C before transported to Bandung, West Java, Indonesia. Routine blood profile tests were done using Hematology Analyzer Sysmex® XT-2000i (Illinois, USA), Tokyo Boeky TRX 7010 (Tokyo, Japan), and ABX Pentra 400 (Horiba Medical, Kyoto, Japan). The samples were also examined for the human immunodeficiency virus (HIV) reactivity using Alere Determine™ dipsticks (Alere Scarborough Inc, Maine, USA). Subjects with reactive-HIV tests and random blood sugar level ≥ 200 mg/dL were also excluded.

Genotyping of TLR-8 gene polymorphisms

Genomic DNA in each sample was extracted using QIAamp DNA Blood Minikit, Qiagen according to its protocol. Several single nucleotides from TLR-8 gene were analyzed using the GoldenGate® Genotyping Assay VeraCode® / BeadXpress (Illumina, San Diego, CA, USA) according to the manufacturer's protocol.¹⁸ The concentrations of DNA extracted from the samples were measured using spectrophotometer at absorbance 260 nm / 280 nm, and only DNA with concentration higher than 250ng/ml was included for further process according to the protocol (Illumina®).

In brief, the first step is DNA activation which enables DNA genomic samples to bind to paramagnetic samples, then hybridization was followed. Then, the BeadXpress® Reader identifies microbead code and fluorescent signal. A laser beam goes through each of VeraCode® microbeads to produce a unique code image during scanning. Illumina's GenomeStudio® software (San Diego, CA, USA) analyzed the data generated in this process, then allele and genotype frequencies as well as distribution in each SNPs were counted and compared.

Statistical Analysis

Data statistic was performed in Microsoft Excel 365 (Microsoft Corp., Redmond, WA, USA). Demographic and clinical data in this study was examined using Kolmogorov-Smirnov test to see whether they are normally distributed. The comparison between numeric and categoric variables within groups were analyzed using Student t-test or Pearson chi-square, respectively. In female subjects, genotype and allele frequencies were counted based on values predicted by Hardy-Weinberg equilibrium (HWE) using the Haldane exact test. Two SNPs were excluded due to deviation from HWE, and the remaining SNPs (rs3764880 and rs3788935) underwent further analysis. Analyses of genotype and allele frequencies of the SNPs among cases and control groups were compared using Pearson Chi's Square or Fischer's exact tests as appropriate, then odd ratios (OR) with 95% confidence intervals (95% CI) were also calculated.

RESULTS

A total of 230 subjects were enrolled in this study, comprised of 115 cases and 115 controls. The median of ages and gender distribution did not differ significantly between cases and control groups. Body-mass index and hemoglobin concentration were significantly lower, whereas leukocyte and thrombocyte counts were higher in PTB patients (Table 1). Among PTB patients, 79.1% percent of them

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Table 1. Demographic and clinical characteristic of population study

| Parameter | TB Patients n=115 | Control n=115 | P value |
|--|----------------------|------------------|---------|
| Clinical characteristic | | | |
| Age, median (IQR) | 34 (23) | 35 (18) | 0.543 |
| Male, n (%) | 58 (50.4%) | 49 (42.6%) | 0.234 |
| BMI in kg/m ² , mean ± SD | 15.38 ± 2.4 | 20.57 ± 2.6 | <0.001* |
| Haemoglobin in gr/dL, mean ± SD | 11.06 ± 1.9 | 13.24 ± 1.9 | <0.001* |
| Leucocyte count in x10 ³ cells/mm ³ , median (IQR) | 10.46 (4.95) | 8.25 (3.34) | <0.001* |
| Thrombocyte count, x10 ³ cells/mm ³ median (IQR) | 387 (197) | 260 (94) | <0.001* |
| Anemia, n (%) | 92 | 31 | <0.001* |
| Registration group | | | |
| Newly Diagnosed † | 91 | n/a | n/a |
| Relapsed / Defaulted ‡ | 22 | n/a | n/a |
| Failure § | 2 | n/a | n/a |

Notes: Test conducted in independent T-test or Mann U-Whitney test as appropriate. *P values were significant if < 0.05. † Newly diagnosed cases have never had treatment for TB, or have taken anti-TB drugs for less than 1 month.

‡ Relapsed is defined as the cases had been treated completely and cured, but now it is relapsed; Defaulted is defined as patients had been declared loss to follow up before now it is treated again. § Failure is defined as those who have previously been treated for TB but the treatment failed at the end of their most recent course of treatment.

Abbreviations: BMI, Body Mass Index; IQR, Interquartile Range.

The frequencies of genotypes in one plate of 96 individuals were described for each polymorphism as shown using Genome Studio® (Figure 1). Analysis of the genotype distribution of these polymorphisms of TLR-8 in this population showed no significant association with the risk of PTB ($P > 0.005$) (Table 2). Since the TLR-8 gene is located in chromosome X and might exhibit gender-inequality pattern distribution, sex-stratified analyses were also performed both in genotype and allele frequencies. Again, no association was observed between these polymorphisms with the presence of PTB in the male and female subgroup ($P > 0.005$) (data not shown).

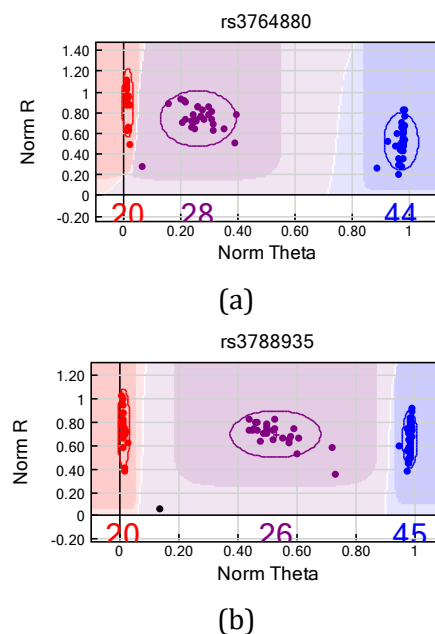


Figure 1. Distribution of SNP TLR-8 gene in Kupang population. As shown using Illumina's Genome Studio®, (a) rs3764880 and (b) rs3788935. Every single dot represents one individual genotype in one plate for 96 individuals. Pink areas represent minor allele genotypes, purple area as heterozygote genotype, whereas blue areas represent major allele (wild type) genotype, respectively.

Table 2. Genotype and allele distributions of TLR-8 polymorphisms in Kupang population

| Gene Polymorphisms | Cases, n (%) | Controls, n (%) | HWE P-value* | Chi-square P-value† |
|--------------------|--------------|-----------------|--------------|---------------------|
| rs3764880 | | | | |
| <i>Genotype</i> | | | | |
| GG or G- | 42 (50.0) | 30 (50.8) | 0.066 | 0.264 |
| AG | 18 (21.4) | 17 (28.8) | | |
| AA or A- | 24 (28.6) | 12 (20.3) | | |
| <i>Allele</i> | | | | |
| G | 81 (60.9) | 62 (62.0) | 0.865 | |
| A | 52 (39.1) | 38 (38.0) | | |
| rs3788935 | | | | |
| <i>Genotype</i> | | | | |
| GG or G- | 44 (53.0) | 27 (45.8) | 0.164 | 0.653 |
| AG | 18 (21.7) | 19 (32.3) | | |
| AA or A- | 21 (25.3) | 13 (22.0) | | |
| <i>Allele</i> | | | | |
| G | 83 (62.9) | 58 (58.6) | 0.508 | |
| A | 49 (37.1) | 41 (41.4) | | |

Notes: * *P* value was calculated in female population, *P* values > 0.05 indicate no deviation of the genotype from equilibrium. † Genotype chi-square calculation between dominant and intermediate vs recessive variants. *P* values were significant if < 0.05

Abbreviations: HWE: Hardy-Weinberg Equilibrium, CI: Confidence Interval

DISCUSSION

The linkage of PTB susceptibility and genetic polymorphisms had been studied intensively in recent decades.^{5,19,20} TLR-8 gene was included in these interests due to its important part in macrophage endosomal pathogen sensing.^{21,22} TLR-8 was firstly known as its function in response toward viral nucleic acids.¹⁹ However, its role in recognizing various bacteria in macrophage endosome and induction of IFN- β had been uncovered lately.^{19,21,22} Ex-vivo experiments showed that monocytes activated with living mycobacteria could induce follicular helper T-cell via TLR-8. Further real-time Polymerase Chain Reaction (PCR) analysis found that TLR-8 expressions were up-regulated in TB patients during their acute phase.²¹ Whereas the exact mechanisms have still to be elucidated, several studies depicted an intricate and complex association between TLR-8 polymorphisms and tuberculosis

susceptibility in different gender distribution.⁶ Davila et al in 2008 showed that these two TLR-8 SNPs (rs3764880 and rs3788935) were in perfect linkage disequilibrium.⁸ This study further revealed that minor A-allele in rs3764880, which is located in Exon 1 and could change start codon in the transcription, as well as rs3788935 located in the regulatory region had a profound influence for increasing PTB for adult male in both Jakarta and Russia population.⁸ Salie et al found that A-allele in rs3764880 was attributed to increased odds of developing tuberculosis in South African males.⁹ A study conducted with Dalgic et al also identified a strong association of A-allele of rs3764880 with PTB susceptibility in male pediatrics patients.⁷ On the other hand, Kobayashi et al found no significant association between any of TLR-8 polymorphisms and PTB susceptibility in several ethnicities of South East Asia (Javanese, Sundanese, and Vietnamese).¹¹ Hasheme-Shari et al in 2014 also revealed that rs3764880 was not risk factor for tuberculosis susceptibility in the Iranian population.¹² Interestingly, Bukhari et al found out that G-allele, rather than A-allele of rs3764880, was attributed to increased risk of PTB incidence and bacterial load in male Pakistani patients.¹⁰ Most of these studies showed no similar association found in female population subsets, excepts in the South African population for three TLR-8 polymorphisms (rs3761624, rs37647879, and rs3764880).⁹

This study, according to our best knowledge, was the first case-controlled report regarding the TLR-8 genetic variation study concerning PTB susceptibility in East Nusa Tenggara, eastern part of Indonesia. None of these TLR-8's polymorphisms associated with PTB, which was in accordance with Kobayashi et al results but differed with Davila et al study.^{8,11} Whereas Davila et al study were conducted in Jakarta (a metropolitan city which has heterogeneous ethnicities)⁸, this study population was located

in Kupang, East Nusa Tenggara which could be assumed to have relatively homogenous ethnicities and genetic pools rather than Jakarta. Kupang city is located in Timor Island in East Indonesia, and the Timor Island inhabitants were postulated to be ‘melting-pot’ ancestor genes coming from the Melanesian and Polynesian population (via Sahul continental shelf) and Austronesia (via Sundaland continental shelf).^{23,24} This unique ‘melting pot’ genetic background is further supported by Tumonggor et al study, who found that the maternal loci of West Timor population are dominated by Asian origin while paternal loci are dominated from Melanesian (Papuan) origin.²⁵ Therefore, this study findings might be caused by distinct genetic characteristics compared with western Indonesia studies.

CONCLUSION

There is no associated risk of having TLR-8 polymorphism in pulmonary TB occurrence among the population in Kupang, Nusa Tenggara Timur (as opposed to previous studies conducted in other different ethnic populations). Further studies in genetic polymorphisms should be explored to elucidate the susceptibility of people in these population to Mtb infection to understand more about the interaction of this microorganism and genetic host variability.

CONFLICT OF INTEREST

The authors declare no conflicts of interest in this work.

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