

Association Between Arg753Gln and Arg677Trp Polymorphisms of TLR2 Gene with Active Pulmonary Tuberculosis in an Indonesian Population

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ABSTRAK

Latar belakang: Toll-like receptor (TLR) adalah reseptor pengenalan pola yang mengenali pola molekul terkait patogen dalam mikroorganisme. Makrofag mengenali adanya mikobakteri melalui Toll-Like Receptor 2 (TLR2) dan penanda lanjutan pada produksi sitokin, proinflammatory TNF- α , IL-1 β , IL-6, IL-12, IL-15, IL-18 dan IFN- γ , serta anti-inflamasi IL4, IL-10 dan TGF- β . Polimorfisme gen TLR2 sangat ditentukan oleh etnisitas dan geografi, karenanya diperlukan analisis hubungan polimorfisme Arg753Gln dan Arg677Trp gen TLR2 dengan kerentanan menderita TB paru aktif dan peranan beberapa sitokin pada populasi Indonesia di Bandung Jawa Barat. **Metode:** penelitian observasional analitik dengan rancangan potong lintang dilaksanakan pada periode April 2011–Mei 2012 di RS Dr. Hasan Sadikin Bandung. Populasi penelitian terdiri dari penderita TB paru aktif dengan BTA positif dan sebagai pembanding adalah TB laten. Adanya polimorfisme gen Arg753Gln dan Arg677Trp diperiksa dengan metode polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Kadar sitokin IFN- γ , TNF- α , IL-10, dan IL-12 juga dibandingkan antara kelompok TB aktif dan TB laten. **Hasil:** polimorfisme heterozigot Arg753Gln gen TLR2 ditemukan pada 9 dari 86 (10.5%) penderita TB paru aktif dan tidak ditemukan pada TB laten. Pada kedua kelompok tidak ditemukan polimorfisme Arg677Trp. Odds Ratio untuk polimorfisme Arg753Gln adalah 28,07 ($p=0,022$). Tidak ditemukan perbedaan bermakna kadar sitokin IFN- γ , TNF- α , IL-10, dan IL-12 antara penderita TB paru dengan polimorfisme Arg753Gln dan penderita TB paru tanpa polimorfisme tersebut. **Kesimpulan:** polimorfisme Arg753Gln gen TLR2 merupakan faktor risiko menderita TB paru pada populasi orang Indonesia di Bandung, sedangkan polimorfisme Arg677Trp gen TLR2 bukan. Peningkatan risiko ini tidak terkait dengan perubahan kadar IFN- γ , TNF- α , IL-10 maupun IL-12.

Kata kunci: Toll-like receptor 2 (TLR2), Arg753Gln, Arg677Trp, sitokin, *M. tuberculosis*.

ABSTRACT

Background: Toll-like receptor is a pattern recognition receptor (PRR) that recognize pathogen-associated molecular pattern (PAMP) in a microorganism. Macrophages recognize the presence of mycobacteria through Toll-Like Receptor 2 (TLR2) and signaling further lead to the production of cytokines, both proinflammatory TNF- α , IL-1 β , IL-6, IL-12, IL-15, IL-18 and IFN- γ , as well as anti-inflammatory IL4, IL-10 and TGF- β . TLR2 gene polymorphism is strongly determined by ethnicity and geography. Therefore it is necessary to uncover the existence and association between Arg753Gln and Arg677Trp TLR2 gene polymorphism with TB susceptibility and its underlying mechanisms in Indonesian population in Bandung West Java. **Methods:** analytical observational

study with cross-sectional design was conducted in Hasan Sadikin General Hospital Bandung from April 2011 to May 2012. Study population consisted of active pulmonary TB patient with positive AFB smear and Latent TB to ascertain previous MTb exposure. Polymorphism of gen Arg753Gln and Arg677Trp gene was determined with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. Plasma levels of IFN- γ , TNF- α , IL-10 and IL-12 were also compared between active and latent TB group. **Results:** heterozygote Arg753Gln TLR2 gene polymorphism was found in 9 of 86 pulmonary TB subjects (10.5%) but none in the latent TB group. The Arg677Trp polymorphism was not found in both groups. The odds ratio for Arg753Gln existence was 28.07 ($p=0.022$). No differences in the levels of IFN- γ , TNF- α , IL-10 and IL-12 between active pulmonary TB and latent TB subjects with and without Arg753Gln TLR2 gene polymorphism. **Conclusion:** Arg753Gln polymorphism of TLR2 gene is a risk factor for active pulmonary TB while Arg677Trp polymorphism is not. The Increased risk is not mediated by the difference in IFN- γ , TNF- α , IL-10 and IL-12 serum levels.

Keywords: Toll-like receptor 2 (TLR2), Arg753Gln, Arg677Trp, cytokines, *M. tuberculosis*.

INTRODUCTION

Toll-like receptor is a pattern recognition receptor (PRR) that recognizes pathogen-associated molecular pattern (PAMP) in a microorganism. Macrophages recognize the presence of mycobacteria through Toll-Like Receptor 2 (TLR2) and signaling further leading to the production of cytokines, both proinflammatory TNF- α , IL-1 β , IL-6, IL-12, IL-15, IL-18 and IFN- γ , as well as anti-inflammatory IL4, IL-10 and TGF- β . Macrophage activation occurs, followed by the killing of Mycobacterium tuberculosis (MTb) in the macrophage and activation of the adaptive immune system specific to MTb in the form of cell mediated immunity and delayed-type hypersensitivity.¹⁻⁵

Toll Like Receptor 2 gene polymorphisms Arg753Gln decreases macrophage response against bacterial peptide thereby reducing the immune response of the host.⁶ Research in Turkey found the TLR2 gene polymorphism Arg753Gln in 17.9% of patients with tuberculosis (TB) and 7.7% in control and increased the risk of developing tuberculosis by 6.04 times in homozygous mutant and 1.60 times in heterozygous mutants.⁷ Jin et al.⁸ in China and Mishra et al.⁹ in India also found role of Arg753Gln heterozygous polymorphisms on susceptibility to tuberculosis.

Other TLR2 gene polymorphism Arg677Trp increase the vulnerability of leprosy and decreases production of IL-12.¹⁰ This polymorphism inhibit immune response to *M. Leprae* and MTb infection.¹¹ Research in Tunisia in 2004 by

Ben-Ali et al.¹² found heterozygous form of this polymorphism in 94% of patients with TB and 31% of controls.

TLR2 gene polymorphism is strongly determined by ethnicity and geography.¹³⁻¹⁶ Therefore it is necessary to uncovered role of both TLR2 gene polymorphism above in TB in Indonesian population and subsequent cytokine levels alteration.

METHODS

An observational analytic study with cross-sectional design was conducted at Hasan Sadikin General Hospital Bandung Indonesia. Ethical Clearance No 97/ FKUP-RSHS/KEPK/ Kep./EC/2011 was obtained from Health Research Ethics Commttee Faculty of Medicine Universitas Padjadjaran – dr. Hasan Sadikin Hospital, Bandung. Study was done between April 2011 to May 2012 in Outpatient and Inpatients Departement, Hasan Sadikin General Hospital. Study population were enrolled according to consecutive admission and after informed consent. Study population consist of active tuberculosis patients over 18 years old and latent TB with similar age and sex were choosen as control to ensure exposure to Mycobacterium tuberculosis in both groups. Active pulmonary TB was defined as TB patient with smear positive, while latent TB was defined as positive tuberculin test, with normal chest x-ray and no clinical symptom. Patients were excluded if having risk factors for TB such as HIV infections, diabetes mellitus, end stage renal disease,

silicosis, gastrectomy and jejunioileostomy, malignant diseases, post organ transplantation, pregnancy, sarcoidosis, acute infectious diseases, and current use of drugs that interfere immune system such as corticosteroids, cytostatic and malnutrition.

For DNA isolation, 300 μ L of blood is fed into Eppendorf tube, 900 μ L RBC lysis solution was added and incubated for 10 minutes at room temperature, centrifuged at 13,000-16,000 rpm for 20 seconds to obtain leukocyte pellet, and the supernatant was then discarded. Add 300 μ L cell lysis on a tube containing leucocyte pellets. 1.5 μ L RNase was added and incubated for 5 min at 37° C. Protein precipitation solution of 100 μ L was added, vortex 15 seconds later, centrifuged for 3 minutes and the supernatant transferred into new Eppendorf tube. Then, 600 μ L of isopropanol to supernatant was added to precipitate the DNA, mixed by turning the tube about 10 times, then centrifuged for one minute to get the DNA pellet and the supernatant was removed. The DNA pellets were washed using ethanol by adding 600 μ L of cold ethanol. It was centrifuged to precipitate the DNA pellet, discard ethanol and dry by turning the tube over the tissue until ethanol evaporates. The pellet was then dissolved with 50 μ L of TE buffer. The preparations were stored in a freezer (-20° C).

Arg677Trp, Arg753Gln polymorphisms of TLR2 gene were determined from leukocyte nucleus using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods at the Molecular Biology Lab Medical Research Unit Faculty of Medicine, Universitas Padjadjaran. After DNA was isolated, the next step was to determine the existence of Arg753Gln and Arg677Trp polymorphism of gene TLR2 by restriction fragment length polymorphism (RFLP) method. The restriction process was performed twice for each Arg753Gln polymorphism and Arg677Trp TLR2 gene.

For detection of Arg677Trp (CGG >TGG), restriction enzyme used was MwoI (GCNNNNNNNGC). Primers used were:

- Primer 677F: 5'CCCCTTCAAGTTGTGGCTTCATAAG 3'
- Primer 677R: 5'AGTCCAGTTCATACTTGCACCAC 3'

For detection of Arg753Gln (CGG >CAG), restriction enzyme used was MspI (CCGG) and Primers used were:

- Primer 753F: 5'CATTCCCCAGCGCTTCTGCAAGCTCC3'
- Primer 753R: 5'GGAACCTAGGACTTTATCGCAGCTC3'

Cytokines was measured at Research and Esoteric Division of Clinical Lab Prodia Jakarta.

IFN- γ examination using Human IFN- γ high sensitivity ELISA kit BMS228HS (eBioscience). TNF- α Examination were done using Quantikine HS ELISA catalog number HSTA00D for the quantitative determination of human tumor necrosis factor alpha (TNF- α) concentration in serum and plasma, IL-10 examination were done using Quantikine catalog number HS100C for the quantitative determination of Human interleukine 10 IL-10) concentration in serum and plasma. IL-12 examination were done using Quantikine HS human IL-12 immunoassays catalog number HS120 for concentrations in cell culture supernatants, serum and plasma.

Sample size was determined based on formula for unpaired categorical comparative analysis technique. From this formula, a minimum of 127 subjects in each group were needed for Arg753Gln polymorphism TLR2 gene study, and for polymorphism Arg677Trp TLR2 gene 42 subjects were needed. Samples were collected from consecutive admission at Outpatient and Inpatient Department of dr. Hasan Sadikin General Hospital, Bandung, Indonesia.

Statistical Analysis

Analysis of differences in Arg753Gln and Arg677Trp polymorphism of TLR2 gene was conducted using Fisher's exact test or Chi-square. Analysis of differences in levels of cytokines with unpaired t-test or Mann-Whitney. Limit of statistical significance was set at p value of <0.05.

RESULTS

A total of 200 subjects consisted of 86 active pulmonary TB smear-positive and 114 subjects with latent TB were eligible and enrolled between April 2011 to May 2012. Characteristics

of study population are described in **Table 1**. No symptoms were reported in latent TB group, because by definition TB latent is a person with no sign and symptom of TB and also with no radiological appearance of TB but with positive tuberculin skin test.

Table 1. Characteristics of study population

Characteristic	Active Pulmonary TB	Latent TB
Age (years), median (min-max)	32 (18–70)	31 (18–70)
Gender, n (%)		
- Male	46 (53.5)	58 (50.9)
- Female	40 (46.5)	56 (49.1)
BMI (kg/m ²), mean (SD)	17.22 (2.83)	24.64 (4.147)
Complain, n (%)		
- Cough	79 (91.9)	0
- Hemoptisis	18 (20.9)	0
- Sputum Production	77 (89.5)	0
- Shortness of Breath	55 (64.0)	0
- Fever	62 (72.1)	0
- Night Sweat	47 (54.7)	0
- Wight Loss	72 (83.7)	0
TB history, n (%)		
- History of previous TB disease	27 (31.4)	8 (7.0)
- TB contact		
- Yes	15 (17.558)	40 (35.1)
- No	26 (30.2)	14 (12.3)
- Not Sure	45 (52.3)	60 (52.6)

Distribution of Arg753Gln and Arg677Trp polymorphism TLR2 Gene

Heterozygote Arg753Gln Polymorphisms of gene TLR2 was found in 9 (10.5%) cases consisted of 5 (5.8%) male and 4 (4.7%) female, but none in the latent TB group and no homozygote polymorphism was found. There

was no polymorphism Arg677Trp found in both groups (**Table 2**).

Fisher's exact test shows that there were significant differences in TLR2 gene polymorphism Arg753Gln groups between patients with active pulmonary TB and latent TB group ($p < 0.001$) (**Table 3**).

Table 3. Comparison of polymorphism Arg753Gln group of pulmonary TB active and latent TB

Genotype	Active Pulmonary TB	Latent TB	Total
Mutants	9	0	9
Wild Type	77	114	191

Note: exact Fisher test, $p < 0.00$. RO: 28.07, CI (95%): 1.61 to 489.5, $p = 0.022$; Mutants: genotype Arg/Gln, Gln/Gln; Wild Type: Arg/Arg

Odds ratio for heterozygous mutant polymorphism Arg753Gln TLR2 gene for active pulmonary TB was 28.07 with 95% CI from 1.61 to 489.5 and $p = 0.022$. This wide range of confidence interval is probably due to small number of Arg753Gln mutant found. Odds ratios was calculated by adding 0.5 to each cell because the cell (b) was zero.¹⁷

Comparison of cytokine levels between patients with active pulmonary TB with and without Arg753Gln gene polymorphism and latent TB were done.

Active pulmonary tuberculosis patients with and without polymorphism Arg753Gln gene TLR2, and latent TB shows significantly different levels in IFN- γ , TNF- α and IL-10 serum level, but not IL-12 level (**Table 4**).

Further analysis showed that there were no significant differences in levels of IFN- γ , TNF- α , IL-10 and IL-12 serum between active pulmonary tuberculosis with polymorphism Arg753Gln and active pulmonary tuberculosis patients without this polymorphism (**Table 5**).

Table 2. Distribution of polymorphism Arg753Gln and Arg677Trp in active pulmonary TB and latent TB

	Active Pulmonary TB (n = 86)				Latent TB (n = 114)			
	753Gln	753Arg	677Trp	677Arg	753Gln	753Arg	677Trp	677Arg
Man	5	41	0	46	0	58	0	58
Woman	4	36	0	40	0	56	0	56
Total	9	77	0	86	0	114	0	114

Table 4. Differences in cytokines level active TB wild type and mutant alleles with latent TB

Variables	Active Pulmonary TB		Latent TB	X ² KW	P value
	allele 753Arg pg/mL	allele 753Gln pg/mL	allele 753Arg pg/mL		
IFN- γ					
- X (SD)	12.33 (7.99)	16.02 (10.28)	1.04 (1.57)	53.361	<0.001
- median (range)	13.2 (1.21 to 28.0)	11.6 (2.56 to 31.52)	0.53 (0.06 to 8.37)		
TNF- α					
- X (SD)	6.72 (6.05)	5.33 (2.97)	1.87 (0.76)	52.215	<0.001
- median (range)	5.6 (1.97 to 34.84)	4.44 (2.35 to 11.81)	1.65 (0.95 to 5.52)		
IL-10					
- X (SD)	0.98 (2.29)	0.51 (0.43)	0.21 (0.09)	24.418	<0.001
- median (range)	0.30 (0.13 to 12.73)	0.34 (0.13 to 1.48)	0.02 (0.09 to 0.55)		
IL-12					
- X (SD)	0.41 (0.12)	0.42 (0.08)	0.41 (0.11)	0.556	0.757
- median (range)	0.38 (0.24 to 0.71)	0.46 (0.28 to 0.50)	0.39 (0.25 to 0.76)		

X²KW= Kruskal Wallis**Table 5.** Differences in cytokine levels between patients with active pulmonary TB Arg753Gln Gene Polymorphism of TLR2 and pulmonary TB patients without Polymorphism

Variables	Active Pulmonary TB		Z _{MW}	P value
	allele 753Arg pg/mL	allele 753Gln pg/mL		
IFN- γ				
- X (SD)	12.33 (7.99)	16.02 (10.28)	0.891	0.388
- median (range)	13.2 (1.21 to 28.0)	11.6 (2.56 to 31.52)		
TNF- α				
- X (SD)	6.72 (6.05)	5.33 (2.97)	0.437	0.679
- median (range)	5.6 (1.97 to 34.84)	4.44 (2.35 to 11.81)		
IL-10				
- X (SD)	0.98 (2.29)	0.51 (0.43)	0.130	0.899
- median (range)	0.30 (0.13 to 12.73)	0.34 (0.13 to 1.48)		
IL-12				
- X (SD)	0.41 (0.12)	0.42 (0.08)	0.746	0.463
- median (range)	0.38 (0.24 to 0.71)	0.46 (0.28 to 0.50)		

Z_{MW}= Mann-Whitney

DISCUSSION

This was the first study in Indonesia about the existence and role of Arg677Trp and Arg753Gln polymorphism in toll-like receptor (TLR) 2 gene in pulmonary TB. Polymorphisms of genes varies and strongly depends on the ethnicity and geography.^{7,12,16,18} Kang¹⁹ showed close relationship between polymorphisms Arg677Trp TLR2 gene and diseases caused by mycobacteria. A study in Korea showed that polymorphism

was found in 22% of patients with lepromatous leprosy but not found in lepromatous tuberculoid and healthy subjects. Ryu²⁰ in Korea studied non-tuberculosis mycobacteria (NTM) and healthy controls and did not find Arg753Gln and Arg677Trp polymorphisms. Yoon et al.²¹ in Korea also did not find these TLR2 gene polymorphism, both in 154 patients with bacteremia and in 179 controls.

In Tunisia, Arg677Trp polymorphism

was found to be associated with TB disease. This polymorphism was found in 31 of 33 (94%) of TB patients compared with 10 of 33 (34%) healthy controls.¹² In native Germany, Arg677Trp polymorphism was not found at all, but Arg753Gln polymorphism was found 9.4%.²² In healthy Turkish people, Arg753Gln polymorphism was found in 10.34% and 12.3%²³ while Arg677Trp was not found. Lorenz et al.⁶ found Arg753Gln polymorphism in 3% of the Caucasian population.

Association between TB and Arg753Gln polymorphism clearly demonstrated by Oğus et al.⁷ in Turkey. This study involved 151 newly diagnosed TB patients and 116 healthy control. Arg753Gln polymorphism was found in 17.9% of patients with TB and 7.7% in controls with an increased risk for tuberculosis by 6.35 times for homozygous polymorphism Arg753Gln and 1.60 times for subjects with heterozygous. In meta-analysis of 7 studies which also include the above study, Wu²⁴ in 2015 provide evidence that TLR2 gene Arg753Gln polymorphism is a risk factor of TB (OR 3.17, 95% CI: 2.31 – 4.35).

Biswas et al.¹⁸ in India did not find Arg753Gln and Arg677Trp polymorphisms at 100 TB cases and 100 healthy controls. In contrast, Mishra et al⁹ also in India, found the prevalence of homozygous mutant Arg753Gln TLR2 gene polymorphism was 5%. This finding emphasized the importance of geographical factor because their ethnicity were the same.

At 170 TB patients and 199 controls Zhejiang Han Chinese population, heterozygous polymorphisms Arg753Gln TLR2 gene was found in 41.77% of cases and in 15.8% of healthy controls, all in heterozygous form.⁸ Xue et al²⁵ in Chinese population failed to find a relationship Arg753Gln and Arg677Trp gene polymorphisms TLR2 with tuberculosis. A study in Iran also did not found association between Arg677Trp polymorphism and tuberculosis.²⁶ A study in Sudan also did not found association between Arg753Gln with susceptibility to MTb.²⁷

Our study, the first in Indonesia, found the prevalence of polymorphisms of TLR2 gene Arg753Gln in 10.5% of patients with active pulmonary tuberculosis but did not found Arg677Trp polymorphism TLR2 gene. This

study showed association between Arg753Gln polymorphism of TLR2 gene and active pulmonary TB disease ($p < 0.001$) with Odds Ratio of 28.07 times in person with Arg753Gln polymorphism of TLR2 gene. By using the formula $p = RO / (1 + RO)$, then the probability of a person with a polymorphism in the gene TLR Arg753Gln to have pulmonary tuberculosis is 96.6%. The difference with other similar studies is that in this study, we use latent TB as control to ensure exposure to MTb in both study groups. Although our study population for Arg677Trp Gene TLR2 polymorphism is sufficient but we did not found this polymorphism. This reflects that Arg677Trp TLR2 gene was not a risk factor for active pulmonary tuberculosis in Indonesia. The weakness of this study was the amount of study population in Arg753Gln Polymorphism TLR2 gene which didn't reach minimal sample calculated, although we still found Arg753Gln polymorphism TLR2 gene in this study.

No difference was found in serum cytokines among active pulmonary tuberculosis patients with and without polymorphisms of genes Arg753Gln TLR2 suggesting that increased susceptibility to TB in the TLR2 gene polymorphism Arg753Gln did not caused by changes in cytokine levels. There are several possible explanations. Firstly, Arg753Gln polymorphisms in TLR2 gene disrupt the process of phagocytosis by macrophages and intramacrophage killing but do not alter cytokines production. Secondly, the production of cytokines (IFN- γ , TNF- α , IL-10 and IL-12) is not merely through TLR2 signaling pathway. Aravalli et al²⁸ showed that mortality did not increase in mice with TLR2 - / - when infected with HSV; and the chemokine CXCL10 production was not affected by TLR2 signaling. Beijing genotype was turned out to stimulate the production of cytokines IL-6, TNF- α , IL-10, and GRO- α lower than wild strain H37Rv MTb. This study does not rule out the possibility of MTb genotype role.²⁹ Other possible explanation of the susceptibility mechanisms is that polymorphism of TLR2 gene control the expansion of NK cells.³⁰

Therefore, it is necessary to do further studies to prove these allegations. These studies include association between TLR and

Vitamin D level, also with natural resistance-associated macrophage protein (Nramp) gene that important in activation of macrophages and intramacrophage killing. It is also necessary to uncovered the association between TLR2 with intramacrophage effectors such as reactive oxygen intermediate and reactive nitrogen intermediate.^{5,31}

CONCLUSION

Arg753Gln TLR2 gene polymorphism is a risk factor for active pulmonary TB in Indonesian population, in Bandung but not Arg677Trp polymorphism. This increased risk of active pulmonary TB disease in subjects with TLR2 gene Arg753Gln polymorphism is not associated with changes in the levels of IFN- γ , TNF- α , IL-10 and IL-12 serum.

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