

Natural resistance-associated macrophage protein 1 gene polymorphisms in thalassemia patients with tuberculosis infection

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Abstract

Background Thalassemia is a hereditary disorder of hemoglobin that needs regular blood transfusions leading to accumulation of iron in the cells. This iron overload level in macrophage might cause intracellular bacteria, particularly *Mycobacterium tuberculosis* (MTB) to multiply. Polymorphisms in natural resistance-associated macrophage protein 1 (NRAMP1), a metal transporter across the phagosome membrane, play important role in regulating iron, which is also needed by MTB. Increased iron in thalassemia patients may have an increased potential risk for TB.

Objective To compare natural resistance-associated macrophage protein 1 (NRAMP1) gene polymorphisms (INT4, D543N, and 3'UTR) in thalassemia patients with and without tuberculosis (TB) infection.

Methods A cross-sectional measurement of NRAMP1 genetic polymorphisms was performed in pediatric thalassemia patients with TB (n=40) and without TB (n=50). Iron status including serum iron, total iron-binding capacity (TIBC), and ferritin, was compared between the two groups. The NRAMP1 genetic polymorphisms were analysed using polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP). Allelic and genotypic distributions of each polymorphism were assessed for possible associations with TB infection.

Results Mean serum iron and TIBC in thalassemia patients with TB were higher compared to thalassemia patients without TB (mean serum: 166.26 vs. 134.92 $\mu\text{mol/L}$, respectively; $P=0.026$) and (mean TIBC: 236.78 vs. 195.84 $\mu\text{mol/L}$, respectively; $P=0.029$). In thalassemia patients with TB, we observed significantly higher frequency of the C allele in INT4 (10% vs. 2%, respectively; OR=5.44; 95%CI 1.1 to 26.4; $P=0.02$) and the TGTG deletion allele (78.8% vs. 51%, respectively; OR=3.56; 95%CI 1.83 to 6.9; $P=0.0002$) in 3'UTR polymorphisms than in thalassemia patients without TB. There were no significant

differences in distributions of the A allele between TB and non-TB groups (16.3% vs. 15%, respectively; $P=0.84$) or the GA genotype (32.5% vs. 30%, respectively; $P=0.79$) in D543N.

Conclusion The NRAMP1 polymorphisms are known to be associated with major gene susceptibility to TB, and in our thalassemia patients this association is even more pronounced. [Paediatr Indones. 2016;56:84-9].

Keywords: NRAMP1, iron, tuberculosis, and thalassemia

Thalassemia is a blood disorder which requires regular blood transfusion, leading to accumulation of iron in the cells.¹ Excessive iron in the cells may cause vulnerability to various infections, including salmonellosis and

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tuberculosis (TB).² Indonesia has a high TB prevalence rate, and was ranked as having the 5th largest morbidity rate and latent TB infection in the world.³ Moreover, the Indonesian archipelago is located in a geographic belt of genetic diseases such as thalassemia.⁴

The level of iron overload in thalassemia patients makes them more vulnerable to TB infection.⁵ With current, better quality therapy, thalassemia patients can live longer, hence, the probability of exposure to TB infection is increased.⁶ However, only 5-10% of people who are infected by *Mycobacterium tuberculosis* (MTB) will develop active TB disease.⁷ A previous study revealed that vulnerability to TB infection is not only due to MTB virulence factors, but also depends on the body's immunity in eliminating MTB.⁸ Iron overload level in macrophages may cause MTB to multiply and attack the human immune system.⁹ Natural resistance-associated macrophage protein 1 (NRAMP1) regulates intracellular iron transport, especially in the membrane of the phagosome where MTB reside. Therefore, this protein is very important in providing the iron source for MTB growth and proliferation.^{10,11} Nonetheless, iron regulation in the macrophage and susceptibility to infection remain subjects of debate, especially in thalassemia patients who receive recurrent transfusion therapy which leads to cellular iron accumulation.

The roles of iron overload and susceptibility to infection have been previously described.^{5,9} However, publications on the model for thalassemia have been limited. Most studies have used hemochromatosis as a disease model for iron overload, but this disease is largely found in Western countries.¹²⁻¹⁴ About 200,000 Indonesian children are believed to be thalassemia patients. We report here on three types of NRAMP1 variations (INT4, D543N, and 3'UTR) in pediatric thalassemia patients with and without TB infection.

Methods

This cross sectional study was performed in pediatric thalassemia patients who visited the Pediatric Thalassemia Clinic of the Dr. Hasan Sadikin Hospital/ Padjadjaran University Medical School, Bandung on March 17, 2011. Thalassemia patients with TB infection as the case group were compared to thalassemia patients without TB infection as the control

group. After written informed consent was obtained from the parents of all subjects, blood specimens were acquired by venipuncture for routine and complete blood examinations before blood transfusion. Blood profiles such as hemoglobin (Hb), hematocrit, leukocyte, platelets, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), as well as iron status including serum iron, ferritin, and TIBC were performed. Tuberculosis was diagnosed according to the TB diagnostic scoring system based on *National Guidelines for Tuberculosis in Children, Association of Pediatricians in Indonesia Pulmonology Working Group (Pedoman Nasional Tuberkulosis Anak, UKK Pulmonologi PP IDAI, 2005)*.¹⁵ This study was approved by the Health Research Ethics Committee of Padjadjaran University Medical School/Dr. Hasan Sadikin General Hospital, Bandung.

The NRAMP1 genomic polymorphisms were determined using PCR-RFLP analysis.¹⁶ The polymorphisms were named accordingly: a G/C single nucleotide change in intron 4 (469+14 G/C) was termed INT-4, a non-conservative single base substitution at codon 543 that changes aspartic acid (Asp) to asparagine (Asn) was termed D543N, and a TGTG deletion in the 3' un-translated region (1729+55del4) was termed 3'UTR. The PCR was performed in a total volume of 50 μ L of solution, containing 0.1 μ g of genomic DNA, 5 μ L Mg²⁺ free 10X PCR buffer (1st Base, Singapore), 200 μ M dNTPs, 1.5mM MgCl₂, 0.4 μ M of each primer, and 2.5 units of Taq DNA polymerase (Fermentas, USA). Thermal cycling was performed on a C1000 Thermal Cycler device (Bio-Rad, USA). For D543N and 3'UTR NRAMP1 polymorphisms, PCR was performed as follows: 3 minutes at 94°C, followed by denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C, extension for 1 minute at 72°C, repeated for 30 cycles, followed by 3 minutes at 72°C, then stored at 4°C. For INT4, annealing was done at 56°C for 1 minute, and all other procedures were identical to those described above. The INT4 fragment (623 bp) was amplified using 5'-CTC TGG CTG AAG GCT CTC C-3' and 5'-TGT GCT ATC AGT TGA GCC TC-3' primers. A region of 244 bp D543N and 3'UTR were amplified using 5'-GCA TCT CCCCCA TTC ATG G-3' and 5'-AAC TGT CCC ACT CTA TCC TG-3' primers. For RFLP analysis of INT4, *ApaI* (New England Biolabs, USA) was used to confirm the G→C mutant

type; two bands of 455bp and 169bp were verified. For RFLP analysis of D543N, *Ava*II (New England Biolabs, USA) was used to confirm a G → A mutation. Allele G (Asp) showed three bands of 126bp, 79bp and 39bp, and allele A (Asn) showed two bands of 201bp and 33bp. For the 3'UTR with allele TGTG+, *Fok*I (New England Biolabs, USA) was used, resulting in 211bp and 33bp fragments; the TGTG deletion showed a 240bp fragment.¹⁶ Digested products were electrophoresed on a 2% polyacrylamide gel, followed by staining with Gel Red (Biotium, Singapore).

Data from laboratory and genotype analyses were examined using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) software. Kolmogorov-Smirnov test was used to check the normality of data. Independent T-tests were used to compare means. Chi-square tests were used to compare contingency tables. All statistical analyses were two-sided and P values < 0.05 were considered to be statistically significant.

Results

There were 40 thalassemia patients with TB and 50 thalassemia patients without TB participated in this study. The clinical characteristics of patients are shown in Table 1. Thalassemia patients with TB tended to have lower Hb than thalassemia patients without TB, but the difference was not significant (P=0.588). Other hematological indices were also

not significantly different between groups. However, the serum iron level (P=0.026) and TIBC (P=0.029) were significantly higher in the case group compared to the control group. Nonetheless, ferritin level in the TB group tended to be lower than in the non-TB group, but this difference was not significant (P = 0.437).

The allele and genotype distribution of *NRAMP1* single nucleotide polymorphisms (SNPs) of INT4, D543N, and 3'UTR in thalassemia patients with and without TB are depicted in Table 2. For the INT4 locus, there was a significantly higher distribution of *NRAMP1* SNP associated with TB infection, as demonstrated by C allele distribution (10% in the case group vs. 2% in the control group; OR=5.44; 95%CI 1.1 to 26.4; P=0.02). For the same locus, higher distributions of genotypic variants, but without significant differences, were demonstrated in the heterozygous GC variant (10% vs. 4%, respectively) and the homozygous CC variant (5% vs. 0%, respectively; P=0.13). For the D543N locus, there were no significant differences in distributions of the A allele between the TB and non-TB groups (16.3% vs. 15%, respectively; P=0.84) or the GA genotype (32.5% vs. 30%, respectively; P=0.79). We found no AA variants in the D543N locus. For the 3'UTR polymorphism, there was a significantly higher distribution of the TGTG deletion allele (78.8% case group vs. 51% control group; OR=3.56; 95%CI 1.83 to 6.91; P=0.0002) and the homozygous TGTG deletion variant (75% TB group vs. 45% non-TB group;

Table 1. Clinical characteristics of thalassemia patients with and without TB

	Thalassemia with TB group (n=40)	Non-TB thalassemia group (n=50)	P value
Gender, n (%)			
Males	16 (40)	27 (54)	0.186
Females	24 (60)	23 (46)	
Hematological indicators			
Mean Hb (SD), g/dL	7.7 (1.3)	7.8 (1.51)	0.588
Mean hematocrit (SD), %	23.4 (3.8)	24.1 (4.5)	0.428
Mean leukocyte (SD), /mm ³	8,762.5 (6,132.4)	15,716 (33,219.1)	0.195
Mean platelets (SD), /mm ³	275,200 (114,005.4)	337,780 (446,486.8)	0.345
Mean MCV (SD), fl	76.1 (6.1)	73.8 (14.8)	0.327
Mean MCH (SD), pg/cell	25.5 (2.8)	27.4 (12.2)	0.294
Mean MCHC (SD), gr/dL	32.9 (1.7)	32.6 (1.3)	0.387
Iron status indicators			
Mean ferritin (SD), μmol/L	3,556.7 (1,906.5)	3994.5 (3,340.1)	0.437
Mean serum iron (SD), μmol/L	166.3 (79.9)	134.9 (51.1)	0.026
Mean TIBC (SD), μmol/L	236.8 (104.3)	195.8 (70.1)	0.029

Table 2. Association between polymorphisms of *NRAMP1* and tuberculosis

<i>NRAMP1</i> variant locus	Allele or genotype	Frequency in TB group		Frequency in non-TB group		P value	OR (95% CI)
		n	(%)	n	(%)		
INT4	G	72	(90)	98	(98)	0.02	5.44 (1.12 to 26.42)
	C	8	(10)	2	(2)		
	GG	34	(85)	48	(96)	0.13	4.23 (0.80 to 22.27)
	GC	4	(10)	2	(4)		
	CC	2	(5)	0	(0)		
D543N	G	67	(83.8)	85	(85)	0.84	1.1 (0.49 to 2.47)
	A	13	(16.3)	15	(15)		
	GG	27	(67.5)	35	(70)	0.79	0.89 (0.36 to 2.18)
	GA	13	(32.5)	15	(30)		
	AA	0	(0)	0	(0)		
3'UTR	TGTG+	17	(21.2)	49	(49)	0.0002	3.56 (1.83 to 6.91)
	TGTG-	63	(78.8)	51	(51)		
	+/+	7	(17.5)	19	(38)	0.004	2.89 (1.07 to 7.82)
	+/-	3	(7.5)	11	(22)		
	-/-	30	(75)	20	(40)		

OR=2.89; 95%CI 1.07 to 7.82; P=0.004) in thalassemia patients with TB infection than in those without TB infection.

Discussion

NRAMP1 plays an essential role in regulating iron transport within the cell, thereby influencing the replication rate of intracellular bacteria, including MTB.¹ Allelic variants of the human *NRAMP1* locus are associated with susceptibility to TB.^{16,23} Thalassemia patients who receive regular transfusions are burdened by iron overload. Excess intracellular iron may cause MTB to multiply, leading to an active MTB infection. We aimed to assess for *NRAMP1* polymorphisms and susceptibility to TB infection in thalassemia patients. We analyzed three types of *NRAMP1* SNPs, namely, D543N, INT4, and 3'UTR. The two latter types of SNPs (INT4 and 3'UTR) were found to be significantly higher in thalassemia patients with TB infection, and associated with TB infection.

The present investigation involving thalassemia patients with and without TB, showed, as expected, iron overload with higher level of serum iron and TIBC in TB patients. Iron overload in thalassemia patients is due to regular transfusion, causing a reduction to the detoxification tolerance limit and iron storage in the form of ferritin. At the same time, transferrin becomes

saturated.¹⁴ The final phase is the rising rate of iron in its free form that is very toxic to the cells and tissues, especially red blood cells. Free iron can catalyze the production of radical OH⁻ substances from peroxide molecules, known as the Fenton reaction.¹⁷ *Mycobacterium tuberculosis* (MTB) infect macrophages and become dormant in the phagosome. These MTB need iron to survive. The human body also needs iron for various processes.¹⁸ The MTB's ability to survive the phagolysosome may be due to NRAMP1 regulation of metal transport in the membrane.¹⁰ Macrophages obtain iron to support the function of the body's defense from several sources: the erythrocyte phagocytosis process, as well as bound or free iron on transferrin, heme, and hemoglobin. Yet, this essential metal can be toxic. Pathogens such as MTB may compete with host cells for iron in order to survive.¹⁹ Therefore, excess iron in thalassemia patients may weaken the host immune system so it is more susceptible to TB infection.^{9,19}

In contrast to the hypothesis that iron overload makes thalassemia patients prone to TB infection, we found that our thalassemia patients had lower ferritin levels, though not significantly so. This discrepant result may have been due to the success of iron chelation therapy in our population. Iron chelation therapy is used to remove excess iron in blood, and is generally required in thalassemia patients receiving regular transfusions. This therapy is fundamental in the strategy to prevent and overcome iron overload

and high tuberculosis prevalence, such as in Indonesia. Consequently, iron chelation therapy can reduce the replication and infection of MTB, thereby restoring the immune system.²⁰ A future study may be pursued to evaluate the effects asymptotic thalassemia minor patients who limit foods with high iron content.

The NRAMP1 metal transporter protein on the phagosome membrane has an essential function in the development of immunity to tuberculosis.²¹ It may be involved in the rapid initiation of innate or adaptive immune responses after infection with MTB. Therefore, NRAMP1 may help to develop early and effective granulomatous responses, which are critical for containing bacterial burdens or the spread of pulmonary MTB infection.^{22,23}

The functional NRAMP1 polymorphisms may explain the association with tuberculosis. Several studies on different populations showed that NRAMP1 gene polymorphisms were related to TB.^{16,21,24} Similarly, the results of our investigation also support the presumption that NRAMP1 variants in thalassemia patients correlate with increased susceptibility to TB. The NRAMP1 protein acts to transport iron in cells, although this mechanism in macrophages and their vulnerability to infection are unclear.²⁵ The NRAMP1 polymorphisms may lead to more hospitable phagolysosomal conditions that pathogens such as MTB tend to favor. In addition, these polymorphisms may affect the macrophage capability to present antigens, after they engulf bacilli, as part of the normal immune response to infection.^{21,23} Therefore, it is probable that macrophage malfunction affects the innate and adaptive immune responses to MTB infection.^{9,11} Further studies are needed to elucidate the role of NRAMP1 variants in the pathogenesis of tuberculosis in thalassemia patients, particularly with regards to the innate and adaptive immune responses to MTB.

In conclusion, there are significantly more NRAMP1 polymorphisms in thalassemia patients with TB than those without TB. Further study on the role of iron in immunity during TB infection in thalassemia patients is needed.

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Conflict of interest

None declared.

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