NRAMP1/SLC11A1 GENE POLYMORPHISMS AND HOST SUSCEPTIBILITY TO *MYCOBACTERIUM TUBERCULOSIS* AND *M. LEPRAE* IN SOUTH SULAWESI, INDONESIA

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Abstract. Genetic host factor may play an important role in controlling mycobacterial infections such as tuberculosis and leprosy. Natural resistance associated macrophage protein1 (Nramp1, alias Slc11a1) gene has been suggested to an associated gene of the host susceptibility to mycobacterium infection. To determine the association of Nramp1/Slc11a1 with tuberculosis and leprosy, we analyzed using polymerase chain reaction restriction fragment length polymorphisms three variants (D543N, 3'UTR and INT4) of Nramp1/Slc11a1 gene in 58 tuberculosis patients (mean age, 34.0±13.1), 42 leprosy patients (mean age, 35.0±14.3) and 198 healthy controls (mean age, 32.0±12.9) from South Sulawesi, Indonesia. We observed an association of INT4 polymorphism with paucibacillary type of leprosy (p=0.032, 1df, OR=2.975, CI=1.057-8.373), but not to multibacillary type (p=0.173, 1df, OR=2.248, CI=0.682-7.404). No significant association was found in the three variants with tuberculosis in this population.

Key words: gene polymorphisms, host susceptibility, *M. tuberculosis, M. leprae,* Indonesia, macrophage protein 1

INTRODUCTION

Mycobacterial diseases, including tuberculosis (TB) and leprosy, remain a major threat to human health in developing countries. The human pathogenic bacterium, *Mycobacterium tuberculosis*, infects 2 billion people, equal to one-third of the world's total population, resulting in about 2.0 million deaths every year. In 2005, it was estimated there were 8.8 million new TB cases, and the total number of cases is still rising every year in the world (WHO, 2007a). Leprosy is a chronic disease caused by bacillus *Mycobacterium leprae*, and the number of new cases detected is now stabilizing and there is a steady declining trend (WHO, 2007b). However, in some countries, including India, Brazil, and Indonesia, leprosy is still considered a public health problem (WHO, 2007b).

The outcome of infection is influenced by many factors, such as nutritional status, co-infections, exposure to environ-

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mental microbes, and previous vaccinations (Fine, 1988; Esther *et al*, 2004; Gisner *et al*, 2004; Neil, 2005; Elena *et al*, 2006). Genetic host factors also play an important part in controlling disease susceptibility to intracellular pathogens (Philippe *et al*, 1981; Silvia *et al*, 1995). Natural resistance associated macrophage protein1 (Nramp1/SLC11A1) gene is a candidate gene in regulating resistance and susceptibility to *Salmonella typhimurium*, *Leishmania donovani*, *Mycobacterium bovis* BCG, and it was isolated by positional cloning in mice (Ellen *et al*, 2001; Jenefer *et al*, 2003). A human homolog has been cloned, containing 16 exons and located on chromosome 2q35 (Cellier *et al*, 1994). Nramp1 protein localizes to late endosome/lysosome of macrophage and is now classified as solute carrier family 11 member 1 (slc11a1) (Gruenheid *et al*, 1997). Nramp1/SLC11A1 delivers divalent cations from phagosome depending on the pH gradient, which leads to decreased DNA replication and respiratory chain of microorganism, but the precise mechanism of Nramp1/SLC11A1 function remains unknown (Samantha and Philippe, 2000; Bryan and Matthias, 2004; Yaniv and Nathan, 2006; Courville *et al*, 2006). Studies in inbred mouse strains revealed that susceptibility was associated with a G169D mutation in the fourth transmembrane domain of the protein, but this substitution was not found in the human homologs (Danielle *et al*, 1994; Silvia *et al*, 1996). However, several reports showed an association of Nramp1/SLC11A1 polymorphism and host susceptibility to tuberculosis and leprosy (Liu *et al*, 1995; Richard *et al*, 1998; Gao *et al*, 2000; Ryu *et al*, 2000; Meisner *et al*, 2001; Abe *et al*, 2003; El Baghdadi *et al*, 2003; Fitness *et al*, 2004; Hoal *et al*, 2004; Malik *et al*, 2005; Sahyana *et al*, 2007; Soborg *et al*, 2007; Vejbaesya *et al*, 2007).

In the present study, the allele frequency of each of the 3 variants (D543N, 3'UTR and INT4) in Nramp1/Slc11a1 gene were determined in blood samples of 58 tuberculosis patients, 42 leprosy patients and 198 healthy controls in Indonesia. We observed an association of INT4 polymorphism with paucibacillary (PB) type of leprosy, but no significant association with tuberculosis was found in the 3 variants of Nramp1/Slc11a1 in this population.

MATERIAL AND METHODS

Study population

All patients and healthy controls enrolled in this study were Bugisness from South Sulawesi, Indonesia to avoid possible confounding gene-phenotype association due to differences in ethnic groups. Patient with leprosy and tuberculosis were recruited from several primary health care in South Sulawesi, Indonesia. All patients provided informed consent before enrolment in this study. The characteristics of the all patients and healthy controls are presented in Table 1. The number of patients with tuberculosis and leprosy was 58 and 42, respectively, and healthy controls were 198. Patients with tuberculosis consisted of 29 males and 29 females, and the patients with leprosy consisted of 27 males [including 14 paucibacillary (PB) and 13 multibacillary (MB) patients] and 15 females (including 9 PB and 6 MB patients). Healthy controls consisted of 96 males and 102 females. The mean age of TB patients, leprosy patients and healthy controls was 34.0±13.1, 35.0±14.3 and 32.0±12.9, respectively.

This study was approved by Medical Ethical Committee of Hasanuddin University, Makassar Indonesia.

Sample collection

Diagnosis and classification of leprosy

Table 1
Characteristics of the subjects.

	Tuberculosis	Leprosy (PB/MB)	Healthy
Total	58	42 (23/19)	198
Male	29	27 (14/13)	96
Female	29	15 (9/6)	102
Age (years)			
0-4	0	0 (0/0)	0
5-14	0	0 (0/0)	1
15-24	16	14 (9/5)	67
25-34	15	9 (5/4)	58
35-44	14	5 (2/3)	37
45-54	5	9 (6/3)	21
55-64	7	3 (1/2)	9
≥65	1	2 (0/2)	5
Mean age	34.0±13.1	35.0±14.3 (32.3/38.3)	32.0±12.9

were based on WHO criteria, namely, the appearance and distribution of skin lesions by clinically and acid-fast bacilli in slit-skin smear examination by microscopy. Diagnosis of tuberculosis was smear-positive or culture positive by acid-fast bacilli in sputum. Healthy controls samples were from blood donors in South Sulawesi Blood Transfusion Services, Indonesian Red Cross.

Polymerase chain reaction

DNA was extracted from venous blood samples using QIAamp DNA Mini Kit (QIAGEN, Tokyo, Japan). All PCR amplification was carried out in 50 μl reaction volume containing 15-60 ng of extracted genomic DNA, 0.40 μM specific primers, and AmpliTaq Gold PCR Master Mix (Applied Biosystems, California, USA) in a Program Temp Control System PC-701 (ASTEC, Fukuoka, Japan). The primers used are shown in Table 2. Parameters for thermocycling of D543N and 3'UTR were as follows: incubation for 5 minutes at 95ºC, followed by 35 cycles of 45 seconds at 94ºC, 45 seconds at 57ºC, 45 seconds at 72ºC, and a final extension step of 10 minutes at 72ºC. Thermocycling parameters for INT4 was as follows: incubation for 5 minutes at 95ºC, followed by 35 cycles of 30 seconds at 94ºC, 45 seconds at 59ºC, 1 minute at 72ºC, and a final step of 10 minutes at 72ºC. Amplicons were visualized by electrophoresis in 1.8% agarose gel stained with ethidium bromide. Amplicons were used for restriction fragment length polymorphism (RFLP) analysis and direct sequencing.

PCR-RFLP

Nramp1 polymorphisms were investigated by PCR-RFLP. Amplicon of D543N, 3'UTR and INT4 was digested with restriction enzyme, *AvaII*, *FokI*, and *ApaI*, respectively under conditions recommended by manufacturer (New England BioLabs, Tokyo, Japan). Restriction-enzyme digestion products were visualized by electrophoresis in 1.8-3.8% agarose gel stained with ethidium bromide.

Direct sequencing

The presence of polymorphisms was confirmed by direct sequencing of each

Table 2
PCR primers and restriction enzymes employed in PCR- RFLP study of Nramp1/SLC11A1.

Name	Location/base change	Primer sequence	Annealing temperature	Product length	Restriction enzyme
D543N (1627G/A)	G or A at nucleotide 1703; GAC(Asp) or AAC (Asn) at codon 543 in exon 15	5'-GCATCTCCCCAATTCATGGT-3' 5'-AACTGTCCCCACCTATCCTG-3'	57ºC	244 bp	*Ava*II
3'UTR (1929+55del4)	Deletion of TGTG in the 3'UTR (55 nt 3' to the last codon in exon15)	5'-GCATCTCCCCAATTCATGGT-3' 5'-AACTGTCCCCACCTATCCTG-3'	57ºC	240 or 244 bp	*Fok*II
INT4 (469+14G/C)	G or C at nucleotide +14 of intron 4	5'-TCTCTGGCTGAAGGCTCTCC-3' 5'-TGTGCTATCAGTTTGAGCCTC-3'	57ºC	624 bp	*Apa*I

band after RCR-RFLP analysis. PCR products were sequenced directly as follows: 5 μl of PCR product was incubated with 2 μl of ExoSAP-IT (USB, Cleveland, Ohio) at 37ºC 15 minutes, followed by 80ºC for 15 minutes and sequenced using the BigDye^R Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington UK) according to the manufacturer's instructions. In brief, sequencing reaction was carried out in 20 μl of final volume containing 7 μl of PCR product, 3.2 pmol of forward primer, 4 μl of Ready Reaction Mix and 2 μl of BigDye Sequencing Buffer. Parameters for sequencing reaction were as follows: incubation for 1 minute at 96ºC, followed by 25 cycles of 10 seconds at 96ºC, 5 seconds at 50ºC, 1 minute at 60ºC, and final step of 10 minutes at 72ºC in a Program Temp Control System PC-701 (ASTEC, Fukuoka, Japan). Unincorporated dyes were removed by DyeEx 2.0 Spin Kit (QIAGEN, Maryland, USA). Products were dissolved in Hi-Di Formamide (Applied Biosystems, Warrington, UK) and analyzed using an ABI PRISM^R 310 Genetic analyzer (Applied Biosystems, Forster City, California).

Statistical analysis

For each polymorphism, genotype frequency difference between patients and healthy controls was examined by chi-square test or Fisher's test depending on the number. Difference was considered significant when *p*-value was <0.05.

RESULTS

Analysis of INT4 polymorphisms in intron 4 of Nramp1/Slc11a1 gene showed increased frequency of G/C allele among leprosy patients compared with healthy controls [*p*=0.020, 1df, odds ratio (OR)= 2.634, confidence interval (CI)=1.135-6.113] (Table 3). As for leprosy type, there are

Table 3
Distribution of Nramp1/SLC11A1 genotype among leprosy patients.

		Healthy (*N*=198)	[a]Leprosy total (*N*=42)			PB Leprosy total (*N*=23)			MB Leprosy total (*N*=19)		
Name	Genotype	*n* (%)	*n* (%)	Odds ratio (95% CI)	*p*-value	*n* (%)	Odds ratio (95% CI)	*p*-value	*n* (%)	Odds ratio (95% CI)	*p*-value
D543N	G/G	115 (58)	23 (55)			14 (63)			9 (47)		
	G/A	77 (39)	16 (38)	1.039 (0.526-2.093)	0.915	7 (32)	0.747 (0.288-1.935)	0.547	9 (47)	1.494 (0.567-3.932)	0.414
	A/A	6 (3)	2 (5)	1.667 (0.316-8.781)	0.543	1 (4)	1.369 (0.288-12.214)	0.567	1 (5)	2.13 (0.231-19.668)	0.434
3' UTR	TGTG +/+	115 (58)	23 (55)			14 (63)			9 (47)		
	TGTG +/-	77 (39)	16 (38)	1.039 (0.526-2.093)	0.915	7 (32)	0.747 (0.288-1.935)	0.547	9 (47)	1.494 (0.567-3.932)	0.414
	TGTG -/-	6 (3)	2 (5)	1.667 (0.316-8.781)	0.543	1 (4)	1.369 (0.288-12.214)	0.567	1 (5)	2.13 (0.231-19.668)	0.434
INT4	G/G	177 (89)	32 (76)			17 (74)			15 (79)		
	G/C	21 (11)	10 (24)	2.634 (1.135-6.113)	0.02	6 (26)	2.975 (1.057-8.372)	0.032	4 (21)	2.248 (0.682-7.404)	0.173
	C/C	0 (0)	0 (0)			0 (0)			0 (0)		

Table 4
Distritubution of Nramp1/Slca1 genotype among tuberculosis patients.

		Healthy (*N*=198)	Tuberculosis (*N*=58)		
Name	Genotype	*n* (%)	*n* (%)	Odds ratio	*p*-value
D543N	G/G	115 (58)	33 (57)		
	G/A	77 (39)	20 (34)	0.905 (0.484-1.693)	0.755
	A/A	6 (3)	5 (9)	2.904 (0.833-10.119)	0.082
3' UTR	TGTG +/+	115 (58)	33 (57)		
	TGTG +/-	77 (39)	20 (34)	0.905 (0.484-1.693)	0.755
	TGTG -/-	6 (3)	5 (9)	2.904 (0.833-10.119)	0.082
INT4	G/G	177 (89)	52 (90)		
	G/C	21 (11)	6 (10)	0.973 (0.373-2.536)	0.955
	C/C	0 (0)	0 (0)		

increased G/C allele frequency of PB leprosy patient compared with healthy controls [(*p*=0.032, 1df, (OR)=2.975, (CI)=1.057-8.373]. On the other hand, there is no significant difference between MB leprosy patients and healthy controls [*p*=0.173, 1df, (OR)=2.248, (CI)=0.682-7.404]. Homozygotes of C/C in INT4 was absent in this population. For the D543N and 3'UTR polymorphisms, no significant differences could be observed between healthy controls and leprosy patients.

None of the polymorphisms in D543N, 3'UTR and INT4 investigated in Nramp1/Slc11a1 were associated with TB (Table 4). Furthermore, we found perfect linkage disequilibrium between D543N and 3'UTR in this population.

DISCUSSION

The human Nramp1/Slc11a1 gene has been studied as one of the host genetic factor for increased risk of TB and leprosy. For TB, heterozygotes for INT4, 3'UTR (TGTG) and D543N variants are at increased risk in Gambia (Richard *et al*, 1998). 3'UTR (TGTG) polymorphism influences host susceptibility to smear-positive TB in Korea (Ryu *et al*, 2000). Heterozygosity for D543N is observed in active TB cases in Japan (Gao *et al*, 2000), whereas INT4 polymorphism is not associated with smear-positive TB in Korea (Ryu *et al*, 2000). In Morocco, INT4, 3'UTR (TGTG) and D543N variants are not associated with pulmonary TB (El Baghdadi *et al*, 2003).

For leprosy, 3' UTR (TGTG) polymorphism is associated with leprosy type not with leprosy *per se* in West Africans (Meisner *et al*, 2001). In northern Malawi, a large-scale candidate gene study showed no association with Nramp1 and leprosy (Fitness *et al*, 2004). In the present study, polymorphisms of Nramp1/slc11a1 gene among patients with TB (*n*=58) and lep-

rosy (n=43) were investigated compared with 198 healthy controls. We observed an association of INT4 polymorphisms with PB type of leprosy (p=0.032), but not with MB type of leprosy (p=0.173). PB leprosy patients have strong cellular immune response mediated by TH1-type immune response (Yamamura *et al*, 1992). Increasing cellular immune response accompany the over-production of Th1 cytokines in skin and peripheral blood. In contrast, MB leprosy patients have strong humoral immune response mediated by TH2-type immune system, but they do not have a strong cellular immune response enabling killing of the microbes in macrophage (Yamamura *et al*, 1992; Straohl *et al*, 2001). An early study by Soo *et al* (1998) showed the association of Nramp1 and type of immune response: congenic mice carrying wild-type Nramp1 allele mount predominantly T-helper-1 response to vaccination, and mice carrying mutant Nramp1 allele mount T-helper-2 response to vaccination. The polymorphisms of Nramp1 could play an important role in the type of immune response in leprosy.

In the case of TB patients, no significant association was observed for any of the three polymorphisms in this population. Variation in the human Nramp1 is associated with susceptibility to TB in Gambia (Richard *et al*, 1998), but not in Indonesia (Sahyana *et al*, 2007). The difference between these reports might be attributed to the diversity of racial groups. The size of our study was sufficient, but relatively smaller than the earlier two studies.

Perfect linkage disequilibrium between D543N and 3'UTR was found in this population. This relationship is observed in West Africans (Richard *et al*, 1998), Asians (Gao *et al*, 2000; Ryu *et al*, 2000) and Europeans (Liu *et al*, 1995). In Nramp1/slc11a1, D543N and 3'UTR is located within and near exon 15 respectively.

In summary, Nramp1/slc11a1 polymorphism is associated with host susceptibility to PB leprosy but not to tuberculosis in this Indonesian population. Nramp1/slc11a1 polymorphism could be one of the important host genetic factors of the immune response to leprosy, but the susceptibility to mycobacterial disease chould be determined not only by Nramp1 but also by many other factors.

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