TNF α and NRAMP1 Polymorphisms in Leprosy

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Objective: *To determine the association of TNF α and NRAMP1 polymorphisms in leprosy.*
Material and Method: *The polymorphisms of TNF α at -238, -308, and NRAMP1 at INT4, D543N, and 3´UTR were examined in 37 patients with leprosy (24 multibacillary and 13 paucibacillary) and 140 healthy controls. PCR-SSP and PCR-SSO method were used to type TNF and NRAMP1 polymorphisms.*
Results: *The genotype frequency of TNF-308 G/A was significantly increased in all leprosy patients compared to the controls (p = 0.04, OR = 2.69). When leprosy types were divided, the allele frequency of TNF-308A was significantly increased in multibacillary leprosy compared to the normal controls (p = 0.04, OR = 2.93). There was no significant difference in the distribution of the genotypes and allele frequencies of TNF -238 and NRAMP1 polymorphisms between the patients and controls.*
Conclusion: *TNF-308A was associated with susceptibility to multibacillary leprosy.*

Keywords: *TNF, NRAMP1, Leprosy*

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Leprosy is a chronic infectious disease with a broad clinical spectrum, ranging from the paucibacillary tuberculoid form, in which a TH1-type response predominates, to the multibacillary lepromatous form, which is associated with TH-2 type immune response. Twin and family studies indicate that host genetic factors influence susceptibility to leprosy and possibly leprosy type[1,2]. Many studies reported the involvement of human leukocyte antigens (HLA) genes in susceptibility to leprosy types[3]. However, the effects of HLA genes are insufficient to explain the whole host genetic component to susceptibility. Non-HLA genes such as tumor necrosis factor (TNF), NRAMP1 (SLC11A1) were found to be important candidate genes. Tumor necrosis factor alpha (TNF α) gene is located within MHC class III region. It was shown that high serum TNF level was associated with lepromatous leprosy[4,5]. The association of TNF genes with leprosy were reported in only a few ethnic groups and the results are inconsistent[6,7]. Natural resistance associated macrophage protein-1 (Nramp1) was mycobacterial susceptibility genes identified in the mouse. The human homologue of the Nramp1 (NRAMP1) maps to chromosome 2q35. Associations of NRAMP1 have been widely reported in tuberculosis[8], however the information on the role in susceptibility to leprosy is limited. In the present study, polymorphisms of TNF α and NRAMP1 genes were analyzed in leprosy and healthy controls.

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Material and Method

Subjects

Thirty-seven patients with leprosy from the Department of Dermatology, Siriraj Hospital, Bangkok, Thailand were included in the present study. Diagnosis of leprosy was established according to World Health Organization (WHO) criteria. Patients were classified as multibacillary (MB) and paucibacillary (PB) by clinical, slit skin smear for acid-fast bacilli and histological criteria. The MB group had more than five skin lesions, and acid-fast bacilli were found from slit skin smear, whereas The PB group had less than five lesions and acid-fast bacilli were not found. Histopathological findings were classified according to Ridley and

Jopling classification[9], and interpreted in correlation with clinical findings. The control population consisted of 140 unrelated, healthy donors from the blood bank. Informed consent was given by all subjects and the present study was approved by the Ethics Committee, Faculty of Medicine Siriraj Hospital.

DNA extraction

Genomic DNA from peripheral blood cells was isolated using a modified guanidine hydrochloric acid extraction method.

TNF α genotyping

The TNF SNP -238,-308 were typed by PCR-SSP (polymerase chain reaction, sequence-specific priming). The primer sequence and primer mixture were as previously described[10]. Each reaction mixture consisted of 5 ul of primer mix and 8 ul of PCR reaction mixture. The final concentration of reaction components were as follows: 200 uM of each dNTP, primers, 2 mM $MgCl_2$, 67 mM Tris Base pH 9-8.8, 16.6 mM ammonium sulfate, 0.01% (v/v) Tween 20, 0.1 ug of DNA and 0.2 units of Taq polymerase. PCR amplification was carried out in Perkin Elmer 9600. The cycling parameters were 96°C for 1 min, followed by five cycles of 96°C for 25 s, 70°C for 45 s, and 72°C for 25 s; 21 cycles of 96°C for 25 s, 65°C for 50 s, 72°C for 30 s; and four cycles of 96°C for 30 s, 55°C for 60 s, and 72°C for 120 s. The PCR reaction plus 10 ul of loading dye was loaded into a 1% agarose gel. Electrophoresis was done for 20 min at 200 V/cm^2. The gels were photographed under ultraviolet light. The presence of an allele-specific band of the expected size, in conjunction with a control band was considered as positive.

NRAMP1 genotyping

The NRAMP1 polymorphisms were a single nucleotide change in intron 4 (469 + 14G/C) denoted as INT4, a non-conservative single-based substitution at codon 543 that change aspartic acid to asparagines (D543N) and a TGTG deletion in the 3′ untranslated region (1729 + 55del4), denoted as 3′ UTR[11]. NRAMP1 typing was performed using PCR-SSO technique (polymerase chain reaction using sequence-specific oligonucleotides). The primers and probes were as previously described[12,13]. For INT4, the PCR conditions were 94°C for 10 s, 58°C for 20 s and 72°C for 30 s (35 cycles) using 0.32 mM of each dNTP, 0.1 uM for each primer, 3 mM $MgCl_2$, 67 mM Tris Base pH 9-8.8, 16.6 mM ammonium sulfate, 0.01% (v/v) Tween 20, 0.1 ug of DNA and 1 units of Taq in a 25 ul reaction. For D543N and TGTG deletion, the PCR conditions were 94°C for 15 s, 58°C for 30 s and 72°C for 30 s (5 cycles) followed by 94°C for 15 s, 53°C for 30 s and 72°C for 30 s (30 cycles) using 0.32 mM of each dNTP, 0.1 uM for each primer, 3 mM $MgCl_2$, 67 mM Tris Base pH 9-8.8, 16.6 mM ammonium sulfate, 0.01%(v/v) Tween 20, 0.1 ug of DNA and 1 units of Taq in a 25 ul reaction. Amplified DNA was hybridized with non-radioactive (DIG-ddUTP)-labeled oligonucleotide probes in a dot blot-type assay. The reaction was detected using anti-digoxigenin alkaline phosphatase conjugate by the chemiluminescence method.

Statistical analysis

Comparison of genotype and allele frequencies between patients and controls was done by Chi-square test and Fisher's exact test when appropriate. Odds ratio (or) was presented as risk factor. A difference was considered significant when the p-value was < 0.05. Hardy-Weinberg equilibrium was tested for each single nucleotide polymorphisms (SNP).

Results

The genotype and allele frequencies of TNF polymorphisms at position -238, -308 in the patients and controls are shown in Table 1. For position TNF -238, the frequency of TNF-238A was higher in PB leprosy than in the controls and MB leprosy, although this was not significant. At position TNF-308, a higher frequency of TNF heterozygous TNF*2 (A) genotype or TNF-308 (G/A) was observed among all patients with leprosy than among control subjects ($p = 0.04$, OR = 2.69). When leprosy was divided into multibacillary and paucibacillary group, the frequency of individuals heterozygous for TNF*2 or TNF-308 G/A was significantly higher in multibacillary leprosy than in the controls ($p = 0.04$, OR = 3.26) and higher than in PB leprosy ($p > 0.05$). No individual homozygous for TNF*2 or (-308A) was identified in the patients and controls. The allele frequency of TNF-308A was also significantly higher in multibacillary leprosy than in the controls ($p = 0.04$, OR = 2.93).

For the NRAMP1 polymorphisms in intron 4, D543N, and 3′ UTR, comparison of the genotype and allele frequencies between all leprosy or leprosy type and controls showed no significant differences (Table 2). The 3′ UTR TGTG alleles was always associated with D543N G allele, indicating strong linkage disequilibrium between them. The control genotypes at each single nucleotide polymorohisms (SNP) were in Hardy-Weinberg equilibrium.

Table 1. TNF α polymorphisms in leprosy patients and controls

	Multibacillary (n = 24)	Paucibacillary (n = 13)	All leprosy (n = 37)	Controls (n = 140)
TNF-238				
G/G	23 (95.8)	11 (84.6)	34 (91.9.)	132 (94.3)
G/A	1 (4.2)	2 (15.3)	3 (8.1)	8 (5.7)
A/A	0 (0)	0 (0)	0 (0)	0 (0)
Allele G	47 (97.9)	24 (92.3)	71 (95.9)	272 (97.1)
Allele A	1 (2.1)	2 (7.7)	3 (4.1)	8 (2.9)
TNF-308				
G/G	18 (75)	11 (84.6)	29 (78.4)	127 (90.7)
G/A	6 (25)*A	2 (15.3)	8 (21.6)*C	13 (9.3)
A/A	0 (0)	0 (0)	0 (0)	0 (0)
Allele G	42 (87.5)	24 (92.3)	66 (89.2)	267 (95.4)
Allele A	6 (12.5)*B	2 (7.7)	8 (10.8)	13 (4.6)

* A, Multibacillary vs controls p = 0.04, OR = 3.26
* B, Multibacillary vs controls, p = 0.04, OR = 2.93
* C, All leprosy vs controls, p = 0.04, OR = 2.69

Table 2. NRAMP1 polymorphisms in leprosy patients and controls

	Multibacillary (n = 24)	Paucibacillary (n = 13)	All leprosy (n = 37)	Controls (n = 140)
INT 4				
G/G	22 (91.7)	13 (100)	35 (94.6)	125 (89.3)
G/C	2 (8.3)	0 (0)	2 (5.4)	15 (10.7)
C/C	0 (0)	0 (0)	0 (0)	0 (0)
Allele G	46 (95.8)	26 (100)	72 (97.3)	265 (94.6)
Allele C	2 (4.2)	0 (0)	2 (2.7)	15 (5.4)
D543N				
G/G	17 (70.8)	7 (53.8)	24 (64.9)	103 (73.6)
G/A	7 (29.2)	6 (46.2)	13 (35.1)	32 (22.9)
A/A	0 (0)	0 (0)	0 (0)	5 (3.6)
Allele G	41 (85.4)	20 (76.9)	61 (82.4)	238 (85)
Allele A	7 (14.6)	6 (23.1)	13 (17.6)	42 (15)
3′ UTR				
TGTG++	17 (70.8)	7 (53.8)	24 (64.9)	103 (73.6)
TGTG+/del	7 (29.2)	6 (46.2)	13 (35.1)	32 (22.9)
TGTGdel/del	0 (0)	0 (0)	0 (0)	5 (3.6)
Allele TGTG+	41 (85.4)	20 (76.9)	61 (82.4)	238 (85)
Allele TGTG-	7 (14.6)	6 (23.1)	13 (17.6)	42 (15)

Discussion

In the present study, a significant association was seen between TNF-308*2 or -308A allele and MB leprosy. The authors confirmed a previous study in an Indian population that found association of TNF-308A with lepromatous leprosy[6], although a sample size in the present study was small. This was in contrast with a previous study in a Brazilian population, which found that TNF-308*2 was protective against MB leprosy[7]. This divergent finding might be from ethnic specific difference of TNF polymorphism. The frequency of TNF-308 *2 carriers in Brazilian control population was

higher than in Asians[6,7] (32.6% vs. 5.6% in India, 9.3% in the present study). TNF-308A was also found to be associated with several infectious diseases with excessive TNF α production and TNF α plays a critical role, such as cerebral malaria and mucocutaneous leishmaniasis[14]. Interestingly, in the present study, TNF-238A was higher in PB leprosy than MB leprosy and normal controls, although not significant which may be from the small sample size. However, in a Brazilian population, TNF-238A was found to be increased with higher bacteriological index. TNF-238 A was also found to be associated with chronic hepatitis C[15]. In functional analysis, the results are still controversial. TNF-308A was found to be associated with enhanced TNF α level and associated with development of LL[6], but in another study, this induces a stronger DTH skin response in PB leprosy and restricts *M. leprae* growth in MB patients[16]. For TNF-238, the allele A was found to be associated with lower levels of TNF α[17]. In a recent study, it was suggested that -308 and -238 polymorphisms are not functional, but in linkage disequilibrium with other genes influencing transcription of TNFA[18]. Further studies are needed for better understanding in the functional basis of TNF polymorphisms. For NRAMP1, no association of the three polymorphisms studied with leprosy was observed in the presented populations. This confirmed a study in India[13]. However, in Mali, association between 3′ UTR deletion and leprosy type was observed[19], whereas in a Brazilian population, association between (GT) n promoter repeat was observed[20]. The discrepancies were found in the polymorphisms of NRAMP1 associated with the disease and the functional significance of the polymorphisms is still unclear. The difference of results can also be from the sample size. As the relative risk of NRAMP1 in the previous report was small, this may not be detected in the presented small sample size. Further studies in a larger sample size are needed. Functional studies have demonstrated pleiotropic effects of NRAMP1 on macrophage activation, regulation of expression of HLA class II and regulation of cytoplasmic cation levels, especially iron[21]. However, the impact of this gene on leprosy pathogenesis is still a question and requires further study. In summary, the present study provides consistent evidence for the involvement of TNF gene in susceptibility to leprosy.

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ความหลากหลายทางพันธุกรรมของยีน TNF α และ NRAMP1 ในผู้ป่วยโรคเรื้อน

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วัตถุประสงค์*: ศึกษาความสัมพันธ์ของยีน TNF α และ NRAMP1 ในผู้ป่วยโรคเรื้อน*
วัสดุและวิธีการ*: ทำการศึกษายีน TNF α ตำแหน่ง -238, -308 และ NRAMP1 ตำแหน่ง INT4, D543N, 3′ UTR ในผู้ป่วยโรคเรื้อน 37 ราย โดย 24 รายเป็นชนิด multibacillary และ 13 รายเป็นชนิด paucibacillary และในคนปกติ 140 ราย การตรวจยีน TNF α และ NRAMP1 ทำโดยวิธี PCR-SSP และ PCR-SSO ตามลำดับ*
ผลการศึกษา*: พบว่า TNF-308 G/A เพิ่มขึ้นอย่างมีนัยสำคัญในผู้ป่วยโรคเรื้อนทั้งหมด เปรียบเทียบกับคนปกติ (p = 0.04, OR = 2.69) เมื่อแบ่งกลุ่มของโรคเรื้อนพบว่า TNF-308A เพิ่มขึ้นอย่างมีนัยสำคัญในผู้ป่วยโรคเรื้อนชนิด multibacillary เปรียบเทียบกับคนปกติ (p = 0.04, OR = 2.93) แต่ไม่พบมีความแตกต่างอย่างมีนัยสำคัญของจีโนไทป์และอัลลีลของยีน TNF -238 และ NRAMP1 ระหว่างกลุ่มผู้ป่วยและกลุ่มคนปกติ*
สรุป*: TNF-308A มีความสัมพันธ์กับโรคเรื้อนชนิด multibacillary*