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CLINICAL STUDY

Association of the *Nramp1* gene polymorphisms and clinical forms in patients with tuberculosis

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Abstract: *Background:* Recent studies have reported that *Nramp1* polymorphisms might have an important role in the development of tuberculosis in various populations. In this study, we aimed to determine *Nramp1* polymorphisms in our patients with tuberculosis population.
Methods: We enrolled 127 patients with active tuberculosis and 116 healthy adults with similar age and gender. Peripheral blood samples were taken for determining the *Nramp1* polymorphisms. By using Polymerase Chain Reaction (PCR) – Restriction Fragment Length Polymorphisms (RFLP) technique, we evaluated the polymorphisms of Nramp1 at the regions of D543N and INT4.
Results: We found that the *Nramp1* polymorphisms at the region of D543N (OR: 0.44, 95%CI: 0.09–2.06 for GA allele) were not a risk factor for tuberculosis. Furthermore, we could not able to detect *Nramp1* polymorphism at the regions of INT4 (OR: 0.97, 95%CI: 0.55–1.72 for GC allele and OR: 0.90, 95%CI: 0.21–3.77 for CC allele).
Conclusion: The findings of the present study do not support the hypothesis that *Nramp1* at the regions of D543 and INT4 might play a role in influencing the growth of bacilli and progression of cavitary tuberculosis rather than susceptibility to *M. tuberculosis* infection. Future studies are needed to elucidate the role of *Nramp1* variants in the pathogenesis of tuberculosis *(Tab. 3, Ref. 29)*. Full Text in PDF *www.elis.sk.*
Key words: tuberculosis, pathogenesis, *Nramp1* gene polymorphisms.

Tuberculosis (TB) is a major health problem throughout the world causing large number of deaths, more than that from any other single infectious disease. The global incidence of TB is rising, with 8.5–9.2 million new cases and 1.2–1.5 million deaths each year (1). Turkey is also a country with a moderate TB incidence. However, only 10 % of those infected are estimated to progress to active TB disease. Susceptibility to TB is multifactorial. Host genetic factors are important determinants of susceptibility to TB (2). The doubly high risk of disease in identical twins compared with nonidentical twins indicates a host genetic component in susceptibility (3). It is likely that host susceptibility to TB is at least partly under polygenic control. The importance of host genetic factors on the susceptibility or resistance to TB has been emphasized by many researchers. Moreover, it was suggested by the recent description of *Nramp1* susceptibility alleles in children with TB (4). Natural resistance-associated macrophage protein 1 (*Nramp1*), best characterized gene, is known to be associated with tuberculosis. *Nramp1* protein is a transmembrane iron transporter expressed mainly in phagocytes and located in the membrane of the phagolysome (5, 6). Although the function of *Nramp1* is not known in detail, one important function is probably to pump divalent cations (Fe^{2+}) across the phagosome membrane (7). This is interesting in relation to TB, because the Fe^{2+} concentration might affect the mycobacterial growth in the phagosome (8). In a much larger study on tuberculosis in Gambia, West Africa, Bellamy et al (9), identified a clear association between variants of the *Nramp1* gene and susceptibility to this mycobacterial disease, though the mechanism is still elusive. Potential malfunction of these aspects may affect innate and adaptive immune responses to *M. Tuberculosis* infection (6). In some studies performed in different ethnical population, weak and/or strong evidence of linkage was found between *Nramp1* and TB (10–18). Polymorphisms in the *Nramp1* gene have also been found in a number of genetic studies to be risk factors for the development of TB among adult populations (13–15, 18). However, except for the study of a TB outbreak in a Canadian community (12), no distinction was made between primary and reactivation TB for the patients enrolled in these previous studies. Such a study design might miss or underestimate the genetic control mechanisms that differ in the development of primary and reactivation tuberculosis. Human case-control studies have suggested that polymorphisms in the human homologue *Nramp1* modify the TB susceptibility in some subgroups of African and Asian populations (9, 19, 20). Soborg et al (21) indicate that variant alleles in the *Nramp1* gene are associated with increased mycobacterial replication rather than susceptibility for TB and may thus confer an increased risk of severe disease.

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Here we searched for the possible association between the polymorphisms of *Nramp1* gene in Turkish TB population.

Materials and methods

Patients

The study involved 127 patients with active tuberculosis referred from Department of Chest Diseases, School of Medicine, Cukurova University, Adana-Turkey and 116 healthy adults with similar age and gender. Also, it was granted ethical approval by the local health committee and permission from all patients and controls. Peripheral blood samples were taken from study groups for determining the *Nramp1* gene polymorphisms. By using the methods of Polymerase Chain Reaction (PCR) – Restriction Fragment Length Polymorphisms (RFLP), we evaluated the polymorphisms of *Nramp1* gene at the regions of D543N and INT4. The patient group consisted of 100 males and 27 females. Their ages ranged from 18 to 79 years with a mean age of 36 years. The control group consisted of 97 males and 19 females (ranging from 18 to 78 years with a mean age of 38 years). Collective data were taken for each patient (age; risk factors: d*iabetes, smoking, immunosuppressive therapy*; chest x-ray findings: c*avitary, localised pneumonic lesion, multilobar infiltration, miliary, pleurisy*; *d*iagnostic tools: s*putum and bronchoalveolar lavage smear, M.tuberculosis culture positivity, and histopathologic examination of pleural biopsy specimens*). Tobacco consumption of these patients ranged from 10–80 packs per year and the average tobacco consumption was 35.35 ± 22.33 packs per year. It was also recorded that four patients had never used tobacco. None of the patients received chemotherapy or radiotherapy before the present analysis. The diagnostic criteria for patients were culture confirmation of TB (127 cases) or clear clinical criteria of disease. The disease manifestation was classified as pulmonary (51.3 %), extrapulmonary (37.5 %), and both pulmonary and extrapulmonary (11.2 %).

Genotyping

A 3-ml sample of blood was taken from all subjects and referred to Department of Medical Biology and Genetics, Faculty of Medicine, Cukurova University. Genomic DNA was isolated from 0.2 ml of whole blood using QIAMP-DNA isolation kit (Qiagen). We selected two DNA polymorphisms in the *Nramp1* gene (*GenBank Accession no L32185*): D543N and INT4, and identified proper primers for all two sites from literatures (22, 23). D543N site, a nonconservative single-base substitution at codon 543 causes a change from aspartic acid to asparagine, was amplified using sense primer (5'-GCATCTCCCCAATTCATGGT-3') and antisense primer (5'-AACTGTCCCACTCTATCCTG-3'). Product size was 240 or 244 bp. PCR cycle conditions were 95 °C for 5 min, followed by 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s (30 cycles). The PCR mixture (25 µl) included 1XPCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 5 pmol primer, 100 ng DNA, and 2 U Taq Polimeraz (Fermantas). Similarly, INT4 (469 + 14G/C) was amplified using sense primer 5'-TCTCTGGCTGAAGGCTCTCC-3' and antisense primer 5'-TGTGCTATCAGTTGAGCCTC-3'. Product size was 624 bp. PCR cycle conditions were 95 °C for 10 min, followed by 94 °C for 30 s, 64 °C for 30 s, 72 °C for 30 s (30 cycles). The PCR mixture (25 µl) included 1XPCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 pmol primer, 100 ng DNA sample and 2 U Taq Polimeraz (Takara Taq™ Hot Start DNA Polimeraz, Takara Bio Inc.). The amplified DNA fragments surrounding the D543N and INT4 were incubated with 5 U of the restriction enzymes AvaII (for D543N) and ApaI (for INT4) at 37 °C for 2 h, and restriction digests were evaluated using 10 % polyacrilamid gels in 1XTBE, and visualised by ethidium bromide staining. To determine the size of the banding patterns, pUC18/HaeIII marker were loaded together with the digested samples and then compared with it.

Statistical analysis

Comparisons of continuous variables were applied using the student t-test between groups. The categorical variables between the groups were analyzed by using the Chi square test or Fisher's exact test. The Odds Ratio (OR) and 95% confidence interval (95%CI) was calculated by using SPSS statistical software15.0 (SPSS Inc.). Results were expressed as mean ± standard deviation, n (%), OR and CI. The results were considered statistically significant if $p<0.05$.

Results

We analysed a total of 127 patients with tuberculosis for polymorphic changes in the D543N and INT4 loci of the *Nramp1* gene, because these two loci were repeatedly evaluated by different laboratories for genetic variation and susceptibility to tuberculosis. The characteristics of our study groups are summarized in Table 1. There were no differences between study groups according to age and gender. While the most important risk factor is diabetes mellitus, the most frequent radiological lesion is cavitary lesion in our study group. The homozygous G/G variant in the D543N locus was

Tab. 1. Characteristics of patients with tuberculosis and healthy controls.

Features	Patients with TB (n=127) Number of patients (%)	Healthy controls (n=116) Number of patients (%]	p value
Male	100 (78.7%)	97 (83.6%)	0.3
Female	27 (21.3%)	19 (16.4%)	
Age, years (mean±SD)*	36 (18–79)	38 (18–78)	0.4
Risk factors;			
Diabetes	23 (18.1%)	–	
Smoking	21 (16.5%)		
Immunosuppressive therapy	1 (0.8%)		
Chest x ray findings;			
Cavitary	67 (52.8%)	–	
Localised pneumonic lesion	26 (20.5%)		
Multilobar infiltration	29 (22.8%)		
Miliary	1 (0.8%)		
Pleurisy	4 (3.1%)		
Diagnostic tools;			
Sputum smear	116 (91.3%)	–	
Culture positivity	5 (3.9%)		
Bronchoalveolar lavage	3 (2.4%)		
Pleural biopsy	3 (2.4%)		

*mean ±SD

Tab. 2. *Nramp1* gene polymorphisms and tuberculous.

Polymorphisms	Patients with TB (n=127) Number of patients (%)	Healthy controls (n=116) Number of patients (%)	Odds ratio (95% CI*) p value
D543N			
G/G	124 (97.6%)	110 (94.8%)	1
G/A	3 (2.4%)	6 (5.2%)	0.44 (0.09–2.06) 0.2
A/A	–	–	
INT4			
G/G	80 (63%)	72 (62%)	1
G/C	42 (33%)	39 (33.6%)	0.97 (0,55–1,72) 0.9
C/C	5 (4%)	5 (4.3%)	0.90 (0.21–3.77) 0.8

* CI = confidence interval

Tab. 3. Cavitary lesions and *Nramp1* gene polymorphisms.

Polymorphisms	Cavitary lesion (n=67) Number of patients (%)	No cavitary lesion (n=60) Number of patients (%)	Odds ratio (95% CI*) p value
D543N			
G/G	66 (52%)	58 (45.7%)	1
G/A	1 (0.78%)	2 (1.6%)	0.43 (0.03–4.97) 0.4
INT4			
G/G	44 (34.6%)	36 (28.3%)	1
G/C	21 (16.5%)	21 (16.5%)	0.82 (0.39–1.72) 0.5
C/C	2 (1.6%)	3 (2.4%)	0.54 (0.08–3.44) 0.5

* CI = confidence interval

detected in 124 (97.6 %) patients with tuberculosis and in 110 (94.8 %) control subjects who were matched to patients on the basis of ethnicity. The heterozygous G/A variants were found in only 3 (2.4 %) patients and 6 (5.2 %) control subjects (Tab. 2). Similarly, the G/C variant in the INT4 locus was identified in 42 (33 %) patients and in 39 (33.6 %) control subjects, whereas homozygous G/G and C/C variants were detected in 80 (63 %) and 5 (4 %) patients and 72 (62 %) and 5 (4.3 %) control subjects, respectively (Tab. 2). The homozygous G/G and C/C variants were identified in INT4 loci of 44 (34.6 %) and 2 (1.6 %) patients with cavitary lesion, and in 36 (28.3 %) and 3 (2.4 %) patients with no cavitary lesion, respectively, whereas heterozygous G/C variant was exhibited in INT4 loci of 21 (16.4 %) patients with cavitary lesion and 21 (18.7 %) patients with no cavitary lesion, respectively (Tab. 3). Statistical analyses indicated that these variants at the 2 loci were not significantly correlated with cavitary tuberculosis (p=0.2 and p=0.8, for variants at the INT4 and D543N loci, respectively), compared with control subjects. Similarly, no association was observed between cavitary and non-cavitary tuberculous lesions. Thus, the data suggested that allelic variants in INT4 and D543N loci of *Nramp1* gene were not associated with severe forms of cavitary tuberculosis in Turkish patients.

Discussion

Tuberculosis remains the single largest infectious disease causing high mortality in humans. Approximately 8.5–9.2 million people are infected with this pathogen every year (1). Turkey is a country with a moderate TB incidence, reaching 33.7 per 100,000 population in 1999 and 26.3 per 100,000 in 2000 (24, 25). The evidence suggests that genetic factors may be important determinants of increased susceptibility to progressive disease development (26). Numerous host genes are likely to be involved in this process. By using a variety of study methods, substantial progress has already been made in advancing our understanding of genetic susceptibility to TB. The initial study by Bellamy et al. (9) demonstrated that 4 *Nramp1* gene variants, including those in the INT4 and D543N loci, were each significantly associated with susceptibility to TB in West Africa. Case-control studies have also indicated that polymorphisms of human *Nramp1* modify host susceptibility to *M. Tuberculosis* among several major ethnic populations, including Asians (19). The human *Nramp1* gene has several polymorphisms (27). However, *Nramp1*-associated susceptibility to tuberculosis could not be confirmed by other studies involving patients living in Morocco, Japan, or Brazil (10, 19, 28). A study carried out in Taiwanese population revealed no association of *Nramp1* gene variants with the susceptibility to TB (29). Also in Turkey, Solgun et al (17) had the same results in Turkish pediatric patients with tuberculosis. Linkage between TB and the *Nramp1* locus has been shown in a large Canadian pedigree (12), but linkage was not seen in Brazilian, West African or South African populations (10, 11). One of the possible explanations for this inconsistency between these studies is that the genetic diversity among ethnicities could confer various genetic mechanisms underlying the TB susceptibility on the ethnic populations studied. By contrast to what has been suggested for other ethnic/racial groups, our studies on *Nramp1* gene polymorphisms (D543N G/G, G/A, A/A and INT4 G/G, G/C, C/C) in TB patients revealed no association with the susceptibility to cavitary TB in Turkish population (Tab. 1). A number of factors may be responsible for the discrepant results reported by different studies. Ethnic or racial backgrounds can certainly introduce some variation (27). In fact, the INT4 variant allele can be found in nearly 50 % of white Europeans, whereas the frequency of this allele is as low as nearly 20 % in the Chinese population (21, 23). Also, Stragas et al (18) reported that INT4 – CC allele was a risk factor for TB in Greeks. In China, D543N locus in Nramp1 gene was recorded as a risk factor in ethnic Han Chinese children (14). In another study, variant genotypes at 3' UTR locus in Nramp1 gene were associated with pediatric tuberculosis in Chinese patients (15). Severe forms of cavitary TB were defined as the presence of cavitary lesion on radiographs, because these clinical features represent the pathological consequences of high-level replicating or destructive *M. tuberculosis* infection. The severity of cases of tuberculosis in the West African study may help to explain, in part, why *Nramp1*-associated susceptibility to TB can be seen in West African patients but not in clinically heterogeneous patients from Denmark, Brazil, or Asian countries (21, 23, 28). The data obtained from the patients with advanced TB indeed implicate *Nramp1* polymorphisms as possibly being associated with severe forms of pulmonary tuberculosis as well as in West Africa. The studies from Japan, Korea, and Denmark also support the presumption that *Nramp1* variants are associated with the development of severe forms of pulmonary TB (19–21). The frequency of *Nramp1* variants was higher among patients in Denmark who had microscopy-positive TB (21). The

reason that no correlation between *Nramp1* variants and cavitary TB was noted in the Danish study might be attributed to the fact that although patients with cavitary TB were compared with patients without cavitary TB, they were not compared with the ethnically matched, healthy control subjects in the genetic study (23). The role of *Nramp1* in human resistance to TB is still ambiguous. The results from our study indicate that variations of *Nramp1* polymorphism at the region of D543 and INT4 were not a risk factor for TB infection among our TB patients in our region. Future studies are needed to elucidate the role of *Nramp1* variants in the pathogenesis of TB. There is still much work to be done as many more tuberculosis susceptibility genes are likely to be identified.

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