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Comparison of Nramp1 Gene Polymorphism among TB Health Care Workers and Recently Infected Cases; Assessment of Host Susceptibility

Parisa Farnia¹, Omid Pajand¹, Saber Anoosheh¹, Payam Tabarsi^{1,2}, Mehdi Kazampour Dizaji¹, Forozan Mohammadi^{3,4}, Mohammad Varahram¹, Parvaneh Baghaei², Moslem Bahadori³, Mohammad Reza Masjedi⁵, and Ali Akbar Velayati⁶

¹ Mycobacteriology Research Center, ² Department of Infectious Disease, ³ Department of Clinical Anatomical Pathology, ⁴ Lung Transplantation Research Center, ⁵ Department of Pulmonary Medicine, ⁶ Department of Pediatrics, NRITLD, Shahid Beheshti University M.C., TEHRAN-IRAN

ABSTRACT

Background: A link between polymorphisms in the natural resistance –associated macrophage protein gene 1 (Nramp) and susceptibility to tuberculosis (TB) has been demonstrated worldwide. This study aimed to investigate the Nramp1 gene variants among workers exposed to TB bacilli (1-2 hours per day for 1 to 20 years) who did not develop the diseases with those who developed the disease through recent transmission.

Materials and Methods: The polymorphism of Nramp1 at INT4, D543 and 3'UTR was examined in 71 newly smear-positive TB cases and 39 healthcare workers exposed to TB. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used to genotype Nramp1 polymorphism. Patients' clinical and demographical data were collected.

Results: The heterozygote patterns of INT4 (G/C), D543 (G/A) and 3'UTR (+/del) occurred more frequently in control subjects than in patients ($P=0.012$), respectively (odds: 1.9 $CI_{95\%}$ [1.13-3.12]). Although, the homozygous patterns of INT4 (C/C; 8.5%), D543 (A/A; 1.4%) and 3'UTR (del/del; 1.4%) were only seen in patients (sensitivity 11% and specificity 100%). The other risk factors like gender, age, resistance and PPD were not associated with Nramp1 gene polymorphism.

Conclusion: Individuals with homozygous type mutation have an increased risk of developing tuberculosis. Therefore, we suggest detection of Nramp1 variants in high-risk groups i.e., health workers and close contact cases. (**Tanaffos 2008; 7(1): 19-24**)

Key words: Tuberculosis, Nramp gene polymorphism, Healthcare workers

INTRODUCTION

Modern technology has identified numerous outbreaks, relapsed infection, dissemination of multi

drug-resistant strains and nosocomial cases of tuberculosis (TB), worldwide (1). Recently, DNA fingerprinting of *Mycobacterium tuberculosis* (M.TB) demonstrated a high degree of similarity among isolated M.TB strains in Iran (2, 3, 4). At present, the nature of the force that contributes to the

Correspondence to: Farnia P

Address: NRITLD, Shaheed Bahonar Ave, Darabad, TEHRAN 19569, P.O:19575/154, TEHRAN- IRAN

Email address: pfarnia@hotmail.com

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selection or dissemination of particular M.TB strains is unknown (5, 6). Although, the possibility of host genetic control and susceptibility to tuberculosis is possible, the idea is highlighted by the fact that clinical forms of TB occur in a small proportion of those infected (7). The candidate gene involved with susceptibility to tuberculosis is SLC11A1 (solute carrier family 11 member 1), formerly known as natural resistance- associated macrophage protein gene1 (*Nramp1*). *Nramp1* plays a critical role in early innate macrophage responses to intracellular infection (8, 9). Its pleiotropic effects include the activation of microbial responses, including the production of reactive oxygen and nitrogen intermediates and pro-inflammatory cytokines (9, 10). Initial studies demonstrated an association between four different *Nramp1* polymorphisms (5' CA, INT4, D543 and 3' UTR) and pulmonary tuberculosis (11). In contrast, a few reports found no relation between *Nramp1* polymorphisms and susceptibility to tuberculosis (12, 13). Therefore, controversies exist regarding the exact role of *Nramp1* in pathogenesis of tuberculosis in different human populations. In the present study, the frequency of 3 different polymorphisms of *Nramp1* gene (INT4, D543, 3'UTR) in TB healthcare workers exposed to TB bacilli who did not develop the disease was compared with those cases whom were recently infected. The control subjects were selected among nurses, doctors and technical staff working in TB wards or laboratory. The duration of contact for healthy subjects was 1-2 hours per day for a minimum of 1 to 20 years. In the patients group, the TB cases were selected among either close or passive contact cases.

MATERIALS AND METHODS

Study population

Seventy-one newly smear-positive TB patients referred to the National Research Institute of

Tuberculosis and Lung Diseases (NRITLD) in Tehran for diagnosis on June 2006 to July 2007. For each case, information was obtained on gender, age, family or close contact, previous TB history, duration of contact, present address and associated medical data such as HIV infection, and tuberculin skin test [positive: more than 10mm, negative: below 5mm, equivocal: between 6-10mm]. Thirty-nine healthy personnel working in the TB laboratory or TB wards (for 1 to 20 years) were included as healthy control subjects. The Institutional Review Board at the NRITLD approved the study, and all patients signed an informed consent).

DNA isolation

Genomic DNA was extracted using the standard protocol with slight modifications (14). Briefly, peripheral blood leukocytes (PBLs) were separated from two milliliters of the whole blood using RBC lysis buffer (0.155 M NH_4Cl , 0.01 M NaHCO_3). Thereafter, PBLs were re-suspended in 500 μl of SE buffer (NaCl 3M, EDTA 0.5M, pH=8), containing 40 μl of 10% SDS and 3 μl of 20 mg/ml of proteinase K. The suspension was incubated at 60 $^{\circ}\text{C}$ for 30 minutes. After incubation, 200 μl of equilibrated phenol (pH=8) was added to the mixture and centrifuged for 10 min at 12,000g. The aqueous phase was transferred to a new tube and the DNA was precipitated using cold propanol.

Nramp1 genotyping

Nramp1 genomic polymorphisms were determined using the polymerase chain reaction (PCR) and then restriction fragment length polymorphisms (PCR-RFLP). Each polymorphism was 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.

Polymerase chain reaction

PCR was performed in a total volume of 50 µl of solution, containing 0.1 µg of genomic DNA, 5 µl free Mg²⁺ 10X PCR buffer (Roch Diagnostic GmbH, Germany), 200 µM dntps, 1.5mM MgCl₂, 0.4 µM of each primer and 2.5 units Taq DNA polymerase (Roch). Thermal cycling was performed on a TC-412 device (Techne, Cambridge, UK). For D543 and 3'UTR Nrampl polymorphism, the reaction was allowed to continue for 3 min at 94⁰C, denaturation for 1min at 94⁰C, annealing for 1 min at 55⁰C, extension for 1 min at 72⁰C repeated at 30 cycles and 3 min at 72⁰C, and then stored at 4⁰C. With INT-4, annealing was done at 56⁰C for 1 min (15). All other procedures were identical. The primers' characterization was as follows: 5'-CTC TGG CTG AAG GCT CTC C-3' and 5'-TGT GCT ATC AGT TGA GCC TC. For D543N and 3'UTR 5-GCA TCT CCC CAA TTC ATG G-3' and 5'-AAC TGT CCC ACT CTA TCC TG-3' 15. A region of 244 bp for D543 and 3'UTR, and 623bp of DNA fragment for INT-4 were amplified.

Restriction fragment length polymorphisms

ApaI was used for INT-4 and with the G→C mutant type; two bands of 455bp and 169bp were verified. *AvaII* was used for D543N with allele G(Asp) showing three bands of 126bp, 79bp and 39bp, and A(Asn) showing two bands of 201bp and 33bp. *FOKI* was used for 3UTR with allele TGTG⁺ showing 211bp and 33bp fragments and del showing a 240bp fragment. Digested products were run on 8% polyacrylamide gel, and were stained with silver-nitrate (16).

Computer –assisted analyses of fingerprints

The 8% polyacrylamide gel of *ApaI*, *AvaII*, and *FOKI* was scanned with a HP jet 4850 scanner. Bionumerics Software version 2.5 (Applied Maths, Kortrijk, Belgium) was used to analyse the molecular patterns generated by *ApaI*, *AvaII*, and

FOKI –RFLP. The similarity metrics were constructed using Jacquard index with a liner error tolerance of 1% proportional to the size of the bands.

Statistical analysis:

The continuous variables were expressed as a group means ± standard deviation (SD). The primary variable was polymorphism in Nrampl gene in pulmonary TB patients and control subjects. Secondary variables included gender, age, family / close contact, pattern of drug resistance, PPD test and duration of contact. Statistical analyses were performed when appropriate. All P-values were two-tailed. A P-value <0.05 was considered statistically significant. Data were analyzed using SPSS version 11 Software.

RESULTS

All patients had pulmonary tuberculosis with cavity formation and negative HIV serology test. The patients were divided into two groups; those who had family history or close contact with TB patients (n=18; 25.3%) for a time period of 1-20 years and the remaining (n=53; 74%) who could not recall any contact with TB patients. Among patients, 39 (54%) were females and 32 (45%) were males; while in controls 19 (48%) were females and 20 (51%) were males. The mean age was 46.7 and 34.1 years in the patients and control groups, respectively. Frequencies of PPD results in patients were 19(26.8%) negative, 16 (22.5%) equivocal and 36(50.7%) positive. In control subjects 7(18%) had negative, 11 (28%) had equivocal and 21(53%) had positive PPD results. Forty-seven patients (50.7%) were susceptible to all four drugs tested. Twelve (16.9%) had multi-drug resistant tuberculosis (MDR –TB), and the remaining had other forms of resistance.

RFLP of INT4, D543 and 3' UTR in controls and patients

Based on RFLP-INT4 banding pattern (formed by

APAI restriction –enzyme), the individuals in the control group were divided into two clusters, a cluster of 13 subjects (33%) with heterozygote pattern (G/C), and a cluster (66%) of 26 cases with uncut pattern (G/G). In contrast, the TB patients were grouped into three types of clusters, homozygous pattern (C/C, 6; 8.4%), heterozygous pattern (G/C, 19; 26.7%) and those that remained uncut (G/G, 46; 64%). As shown in Table 1, the allele frequencies of INT4(C) were 33% and 35% in controls and TB patients, respectively (P=0.5). The INT4 (C) variant was observed more in patients with close contact (7/18; 38%) than those without close contact (18/53; 33.9%). By RFLP- D543 (formed by AVAII restriction –enzyme) the control cases clustered into two clusters of mutant (G/A; 82%) and wild type (G/G; 17.9%). The frequency of mutant type of D543 (G/A) was lower in patients (49.3%) than controls (82.1%, P >0.005). Although, the frequency of mutant type (G/A) in patients with close contact

(16/18; 88.8%) was higher than patients without close contact (25/53; 47.2%), the difference was not statistically significant. No homozygous patterns were found for INT4 (C/C) and D543N (A/A) in controls, whereas, 8.4% and 1.4% of patients had homozygous patterns for INT4 (C/C) and D543N (A/A), respectively. Comparison of distribution of *Nramp1* variants at the 3'UTR loci, showed no significant difference between TB patients and controls. Indeed, 94% of control subjects and 95% of patients had similar banding patterns (TGTG +/+) by RFLP –3'UTR (formed by FOKI restriction –enzyme). The allele frequencies of 3'UTR deletion (del) were 5.1% and 4.2% in controls and patients, respectively (P=0.58). We found only one patient that showed homozygous pattern for all *Nramp1* variants; C/C for INT4, A/A for D543 and for del/del 3' UTR. In general, no association was found between *Nramp1* and gender, age, resistance and PPD.

Table 1. The allele frequencies of *Nramp1* gene in patients with pulmonary tuberculosis and controls.

Genotype	Patients =71		Controls=39	P value
	Family or Close contact = 18	Passive cases = 53		
INT4				
G/G (uncut)	11(61.1%)	35 (66%)	26 (66.7%)	0.5
G/C(heterozygote)	5 (27.8%)	14 (26.4%)	13 (33.3%)	
C/C (homozygous)	2 (11.1%)	4 (7.5%)	0	
D543				
G/G (wild type)	7(38.9%)	28(52.8%)	7(17.9%)	0.001
G/A(heterozygote)	10(58.6%)	25(47.2%)	32 (82.1%)	
A/A (homozygous)	1(5.6%)	0	0	
3'UTR				
TGTG+/+(wild type)	17(98.2%)	51(97.8%)	37 (94.9%)	0.58
TGTG+/del (heterozygote)	0	2 (2.2%)	2 (5.1 %)	
TGTG del/del (homozygous)	1(1.8%)	0	0	

DISCUSSION

It is known that natural mutation in Nrampl1 gene can impair early immunity to several intracellular pathogens including *Mycobacterium. Tuberculosis* (9, 10). Nrampl1 gene acts at the macrophage level and is thought to control susceptibility to macrophage priming for activation. Several studies showed an increased frequency of specific Nrampl1 mutation among patients versus subjects (11, 15, 16). Here, we found no association between, 3'UTR (del) alleles of Nrampl1 variant and susceptibility to tuberculosis. In fact, a mutant pattern by RFLP-3'UTR was higher in control subjects (5.1%) than patients (4.2%). Other studies showed higher mutant frequency of 3'UTR compared to our study e.g. it was 43% in African, followed by 11% in other Asian populations (11, 12). To date, the functional significance of 3'UTR of Nrampl1 has not been demonstrated. The investigators showed that in Thais, Chinese and Koreans the allele of 3'UTR is in linkage disequilibrium with D543 N loci (12, 17). In this study, both 3'UTR and D543 loci were less diverse among patients than controls (Table 1). D543N is aspartic acid to asparagines substitution at codon 543 in the predicted cytoplasmic carboxyl terminal end of the Nrampl1 protein. The substitution of a negatively charged aspartic acid by an uncharged asparagines residue has been thought to affect protein function. In Japanese and Chinese TB patients, D543N variant was associated with the presence of cavity lesions and severe form of tuberculosis (16, 17, 18). In contrast to these reports, the mutant type of D543N (G/A) in our study occurred more frequently in control subjects (82%) than in patients (49.3%). Therefore, our results showed no association between polymorphism of D543, 3'UTR and progression of disease. However, more patients (35%) with a history of close contact had either hetero or homozygous patterns for Nrampl1 gene polymorphisms. Thereby, we suggest that the natural mutant may occur randomly in Nrampl1 gene, but

other risk factors like socio-economical status, duration of contact, underlying disease and even other genes can increase the susceptibility to TB. Similar to 3'UTR and D543, the INT4 mutant type (G/G) more frequently occurred in control subjects than in patients. However, 8.5% of patients showed homozygous patterns of RFLP-INT4 (C/C) that was not present in control cases. Indeed, none of the control subjects, showed homozygous patterns by RFLP in INT4, D543 and 3'UTR. Hence, the homozygous pattern of Nrampl1 gene polymorphisms might be associated with susceptibility to TB. This hypothesis became stronger when we found no homozygous pattern for INT4, D543 and 3'UTR, in controls. In conclusion, in this study, only homozygous patterns of Nrampl1 gene were associated with pulmonary TB. This might be one of the reasons why the TB-exposed healthcare workers did not develop TB, even after long periods of working with TB bacillus. The duration of contact was between 1-20 years, with an average of 7 years for TB laboratory staff and 10 years for nurses working in TB wards.

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