Association of Interferon- γ Receptor-1 Gene Polymorphism with Nontuberculous Mycobacterial Lung Infection among Iranian Patients with Pulmonary Disease

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Abstract. Nontuberculous mycobacteria (NTM) cause significant pulmonary infections in humans. Researchers have reported an association between interferon-gamma receptor-1 (IFN- γ R1 or IFNGR1) deficiency and susceptibility to NTM, but the relevance of polymorphism within these genes is not yet clear. In this study, a single nucleotide polymorphism (SNP), T to C, at position-56 in NTM patients with pulmonary disease was investigated. Molecular identification of *Mycobacterium* isolates was performed with *hsp65* genes using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Then, the host genomic DNA from confirmed NTM patients ($N = 80$) and control subjects ($N = 80$) were screened for SNPs of IFNGR1 (T-56C) by PCR-RFLP. The results indicated that NTM patients had higher TC (26/80; 32.5%) or CC (46/80; 57.5%) genotypes in comparison with control groups (TC genotypes [22/80, 27.5%]; CC genotypes [6/80, 7.5%]) ($P < 0.05$). In this regard, all the patients infected with rapid-growing *Mycobacterium* (RGM, i.e., *Mycobacterium chelonae* and *Mycobacterium fortuitum*) had CC genotypes (100%). In contrary, only 50.7% (35/69) of infected patients with slow-growing *Mycobacterium* (i.e., *Mycobacterium simiae*, *Mycobacterium kansasii*, and *Mycobacterium avium-intracellulare*) had CC genotypes. Thus, patients with CC mutation in IFNGR1 at position-56 are more likely to develop RGM infection. In overall, there is a significant association between SNP of IFNGR1 at position-56 and susceptibility to NTM infection. Based on these data, we propose SNP of IFNGR1 at position-56 as a suitable “biomarker” for identifying populations at higher risk of infection.

INTRODUCTION

Nontuberculous mycobacteria (NTM) are free-living organisms that commonly occur in different environmental reservoirs, including water, soil, and biofilms.^{1–3} They cause a variety of diseases in humans, notably severe, protracted lung disease in patients with underlying lung disorders.⁴ Conditions such as human immunodeficiency virus (HIV) infection, acquired immune deficiency syndrome (AIDS), immunosuppressive therapy, cancer, autoimmune disease, and diabetes mellitus can predispose a person to NTM infection.^{5–10} However, it is not clear why some infected people become ill or why certain individuals have a particular presentation of NTM diseases.⁶ Normally, the occurrence of NTM infections in a “well-defined population” suggests an immune deficiency in the host genetic system.⁶ During the recent years, a considerable number of candidate-based association studies have been performed to demonstrate the association of predicted functional DNA variations with NTM infection.⁶ For example, polymorphisms of tumor necrosis factor (TNF- α) gene, interleukin-12-receptor-1 (IL-12R1), and interferon-gamma (IFN- γ) signaling were shown to be associated with susceptibility to *Mycobacterium*.^{7–15} Among host genetic factors, IFN- γ plays a key role in protective immune response against mycobacterial infection.^{16,17} IFN- γ executes its antimycobacterial effector mechanism by the activation of macrophages and dendritic cells via interaction with its receptor complex.^{12,17,18} Generally, IFN- γ mediates its action through a receptor complex (IFN- γ R or IFNGR) composed of two heterologous subunits, a ligand-binding subunit (IFNGR1), and an accessory subunit (IFNGR2) on the cell surface.^{17–19} The

gene coding for IFNGR1 is located on the long arm of chromosome 6 at position 6q23.3, which consists of seven exons.^{12,19,20} Patients with complete or partial IFNGR1 receptor deficiency are highly susceptible to infection by NTM, *Mycobacterium tuberculosis*, and some *Salmonella* species.^{10,21} In 2006, Sampaio and others provided detailed evidences on the genetic mechanisms underlying polymorphisms of IFNGR1 and susceptibility to pulmonary tuberculosis (PTB).²² They showed that the substitution of wild type (T) to variant (C) forms in IFNGR1 at position-56 caused 3-fold lower expression of a receptor gene on the cell surface.^{10,23} This will consequently lead to decrease in the binding site for the transcription factor of activator protein 2 and 4 (AP-2 and AP-4) on both strands.^{10,23} His findings support the hypothesis that variation in IFN- γ gene might increase the risk of TB development.¹⁰ In a study performed by Varahram and others, a correlation between single nucleotide polymorphisms (SNPs) of IFNGR1 and susceptibility to Iranian PTB patients were demonstrated.¹⁵ In a convincing report, Velayati and others showed an association between polymorphisms of IFNGR1 at position-56 with susceptibility to leprosy in children of the same family.²⁴ Even though most of the available data outlines the importance of polymorphisms and susceptibility to *M. tuberculosis* species, but it is expected that polymorphisms in IFNGR1 at position-56 might possibly affect the NTM development and outcomes, which need to be investigated.^{15,20,24} In the present study, we aimed to study the IFNGR1 T-56C SNP in patients with pulmonary disease that were infected with NTM. Our results showed a significant correlation between IFNGR1 T-56C SNP and NTM infection among studied populations.

MATERIALS AND METHODS

Study group. The investigation was conducted from December 2012 to June 2016 at the National Research Institute of Tuberculosis and Lung Diseases (NRITLD). NRITLD is the

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only World Health Organization–approved center for the detection and diagnosis of *Mycobacterium* infection in Iran.¹⁵ A total of 80 patients with pulmonary disease with confirmed culture-positive NTM results and 80 healthy volunteers (referred to as normal controls) were included in the study. Patient's confirmation with NTM infection was based on the American Thoracic Society and the Infectious Disease Society of America guidelines.²⁵ In brief, the diagnosis was based on the presence of clinical symptoms, radiologic abnormalities, and microbiologic cultures in conjunction with the exclusion of other potential etiologies. Patients and control subjects were matched for age, sex, and nationality.

Ethics. The Institutional Review Board at the NRITLD approved the study and all the patients signed informed consent (MRC-2013/028a/sbmu).

Mycobacterial isolation. Collected sputum samples from each patient were digested and decontaminated by Petroff's method.¹⁵ Lowenstein–Jensen media were used for bacterial growth. The extracted DNA from culture-positive samples was used for identification.²⁶

Mycobacterial identification. The isolates were characterized using *hsp65* genes spacer polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). For *hsp65* gene, a PCR reaction was amplified in 50-μL mixtures containing 4 pmol of specific primers (TB15 [59-CGT AYG ACG AAG AGG CCC GT-39] and TB17 [59-WAS GGR TCC TCS AGG ACS GC-39], 1 mL deoxynucleotide triphosphates (dNTPs), 1.5 mL MgCl₂, 0.25 mL (1 U) *Taq* polymerase, 2.5 mL (1%) dimethyl sulfoxide, 5 mL PCR buffer, and 5 mL (20 ng) of extracting DNA.^{27,28} The reaction mixture was subjected to 30 amplification cycles (20 seconds at 95°C, 1 minute at 60°C, 40 seconds at 72°C) followed by a 5-minute extension at 95°C.^{27,28} The PCR product of the first step (470 base pairs [bp]) was used for the second amplification using primers TB11 (59-ACC AAC GAT GGT GTG TCC AT-39) and TB12 (59-CTT GTC GAA CCG CAT ACC CT), which produced a segment of 439 bp (26). PCR products were digested with 5 U of restriction enzyme *Hae*III and *Bst*EII for 24 hours at 37°C. The pattern of digested products was analyzed using 8% polyacrylamide gel.^{27,28}

IFN-γ genotyping. SNPs in IFN-γ receptor-1 (T-56 C) was performed using PCR-RFLP.^{10,24} The target DNA was amplified in a PCR reaction mixture containing 1× reaction buffer (50 mM KCl, 2 mM MgCl₂), 67 mM Tris-HCl (pH 9.0), 0.2 U of *Taq* DNA-polymerase (Roche, Basel, Switzerland), 2 mM of dNTPs, and 20 pmol of each primer. For IFN-γ receptor-1 (T-56 C), the following primers were used to amplify a 455-bp product: 5'-GGTGACGGAAGTGACGTAAGG, reverse 5'-GGTCGCTGGCTCAA. The PCR products of IFN-γ receptor-1 (T-56 C) were digested with 2 U enzymes of *Bst*110.^{22,29,30} Digested products were run on 8% polyacrylamide gel and were stained with silver nitrate.²⁴

Statistical analyses. Statistical analysis was performed using χ^2 test to determine the statistical difference between studied groups. A *P* value less than 0.05 was considered statistically significant. Data were analyzed using SPSS version 18 software (IBM, Chicago, IL).¹⁵

RESULTS

Participants and demographics. During the study period, 100 patients with confirmed NTM infection were hospitalized

FIGURE 1. The pattern of *Mycobacterium* isolates that were characterized using *hsp65* genes spacer polymerase chain reaction restriction fragment length polymorphism.

to NRITLD for diagnosis and treatment. Twenty patients were excluded because they either had NTM and TB (6/100; 6%) or NTM and lung cancer (3/100; 3%) together. Few patients (11/100; 11%) were not ready to donate their blood for research purposes. Thereby, in total, 80 cases and 80 controls enrolled in this study. The mean ages of patients and control were 48.2 ± 21.2 and 45.5 ± 20.16 years, respectively. Majority of patients (51; 63.7%) were over 45 years of age, and five were more than 70 years of age (5; 6.25%). Forty-eight (60%) patients were male and thirty-two (40%) were female. Fisher's exact test showed a correlation between old age and susceptibility to NTM (*P* value < 0.001).

NTM isolation and IFN-γ receptor genotyping. Majority of patients with pulmonary disease (69/80; 86.2%) were infected with slow-growing *Mycobacterium* (SGM) (Figure 1). Among identified SGM, *M. simiae* (62/80; 77.5%) and *Mycobacterium kansasii* (4/80; 5%) were the most frequent isolates (Table 1). Eleven patients (11/80; 13.75%)

TABLE 1

The allele and genotyping frequencies of IFN-γ receptor-1 (T-56 C) among patients with different NTM species

Isolated <i>Mycobacterium</i>	Total = 80	IFN-γ receptor-1 (T-56 C)
<i>Mycobacterium simiae</i>	62	TT = 8 (12.9%) TC = 25 (40.3%) CC = 29 (46.7%)
<i>Mycobacterium kansasii</i>	4	TT = 0 TC = 1 (25) CC = 3 (75%)
<i>Mycobacterium avium-intracellulare</i>	3	CC = 3 (100%)
<i>Mycobacterium chelonae</i>	6	TT = 0 TC = 0 CC = 6 (100%)
<i>Mycobacterium fortuitum</i>	5	TT = 0 TC = 0 CC = 5 (100%)

IFN = interferon; NTM = nontuberculous mycobacteria.

FIGURE 2. The electrophoresis pattern of polymerase chain reaction (PCR) restriction fragment length polymorphism of single nucleotide polymorphisms of interferon-gamma receptor-1 (IFNGR1) at position-56. The PCR product is 455 base pairs (bp) (line 1). After digestion with *Bst*I, three genotypes were obtained: TT (line 3 = 313 + 215 + 142 bp), TC (lines 2 and 5 = 455 + 313 + 216 + 142 bp), and CC (lines 4 and 6 = 455 + 215 bp).

had been infected with rapid-growing *Mycobacterium* (RGM) (Table 1). For IFN- γ receptor genotyping, one type of T to C substitution at position-56 occurred which resulted in three types of genotypes (TT, TG, and GG) (Figure 2). As shown in Table 2, the frequency of the C allele (112 [70.0%]) was significantly more common in the NTM group (odds ratio = 0.6; 95% confidence interval = 0.4–0.9; $P < 0.05$), whereas the frequency of the C allele in the control group was as low as 27.5%. In overall, we found only eight patients (10%) with TT genotypes; 72 patients (90%) had either heterozygous (TC: 32/80; 40%) or homozygous (CC: 40/80; 50%) mutations. Comparison of IFN- γ receptor genotyping among patients revealed that all cases with RGM infection had CC genotypes (11/11; 100%), whereas 50.7% (35/69) of patients with SGM, showed CC genotypes. In control subjects, TT genotypes was more frequent (56/80; 65%) than TC and CC genotypes (Table 2).

DISCUSSION

To our knowledge, this is the first case-control study which demonstrates association of IFN- γ receptor-1 (-56 CC) with susceptibility to NTM infection among Iranian patients with pulmonary disease.

TABLE 2

The allele and genotyping frequencies of IFN- γ receptor-1 (T-56 C)

Genotypes	Control (80)	NTM cases (80)	OR (95% CI)	P value
IFN- γ receptor-1 allele				
T	126 (72.5%)	48 (30.0%)	0.6 (0.4–0.9)	0.05
C	44 (27.5%)	112 (70.0%)		
Genotype				
TT	52 (65%)	8 (10.0%)	0.6 (0.3–1.2)	S
TC	22 (27.5%)	32 (40.0%)	1.3 (0.5–1.8)	S
CC	6 (7.5%)	40 (50.0%)	1.5 (1.4–1.7)	S

CI = confidence interval; IFN = interferon; NTM = nontuberculous mycobacteria; OR = odds ratio.

As shown in Table 2, majority of patients ($N = 72$) had either TC (40%) or CC genotyping (50%). Normally, a switch from T to C at position-56 in the IFNGR1 promoter leads to a lower expression of luciferase in a standard test system.¹⁰ Therefore, lower levels of IFNGR1 expression would lead to reduced IFN- γ -mediated immunity against intracellular microbes by at least partially blocking the pathways which activate macrophages or process antigens.^{10,11,19,29,31} In this regard, Velayati and others showed an association between autosomal recessive disorders of IFN- γ receptor-1 (T-56 C) with susceptibility to leprosy in children of the same family.²² He investigated an influence of SNPs of various candidate genes, that is, vitamin D receptor, natural resistance-associated macrophage protein 1 gene, TNF- α , IL-10, IL-12 receptor (IL_12R1). However, polymorphism of IFN- γ receptor-1 (T-56 C) was shown to be the most effective SNP.²⁴ In the present study, all patients ($N = 11$) with RGM infection (i.e., *Mycobacterium chelonae* and *Mycobacterium fortuitum*) had CC alleles. This might explain the correlation of IFN- γ receptor-1 (T-56 C) polymorphisms with RGM infection. Indeed, the increased susceptibility to NTM in a host with defective immunity was first reported in four children of the same family in 1995. These children were infected with *M. chelonae*, *M. fortuitum*, and two different subspecies of *Mycobacterium avium-intracellulare*.³² A whole genome search for homozygosity in three of the affected children showed a mutation in the coding region of IFNGR1.³¹ This mutation then resulted in the complete absence of IFN- γ R1 expression at the cell surface and was subsequently identified as the cause of the defect in these children. Thereafter, other investigators outlined the importance of molecular defects in IFN- γ -dependent signaling with susceptibility to disseminated NTM infection.^{32–37} They showed that mutations in each of the IFN- γ signaling genes have either complete or partial deficiency of the encoded protein.^{32,35,36} The partial gene deficiency negatively affects the expression of aberrant proteins and as a result IFN- γ cannot be mediated.³² Complete deficiency of IFN γ R1 has a much worse prognosis than partial deficiency. Hence, mutated genes in IFN γ R1 can play a critical role in immunity against mycobacterial infection.³⁸ In most previous studies,^{32–36} the NTM-infected patients were either children or had underlying diseases (e.g., HIV/AIDS). In the present study, the majority of patients were above 45 years of age, without any underlying disorder. These data might suggest a high risk of NTM infection in elderly people. A recent study by Mirsaedi and others has highlighted the risk factors that are associated with NTM infection in North America.³⁹ They demonstrated that while the U.S. population is aging, NTM diseases are increasing in an elderly populations.³⁹ Therefore, they proposed that the elderly population in any community may be more vulnerable to NTM infection. Addressing the importance of polymorphisms of IFN γ R1 at position-56 with susceptibility to mycobacterial infection, Wang and others performed an extensive meta-analysis of 56 CC in *M. tuberculosis*.²⁰ They analyzed 1,497 TB cases and 1,742 healthy controls from six case-control studies that were reported from African, Asian, and Caucasian populations. They showed that IFNGR1 T-56C polymorphism is possibly associated with increased TB risk in Africans, but not in Asian or Caucasians.²⁰ Wang and others had examined the Korean and Chinese

populations as representatives of Asian communities,²⁰ whereas the Iranian ethnicity is different from other Asians and the results may be variable. For example, Biranvand and others reported an association between SNPs in IFNGR1 T-56C polymorphisms and susceptibility to TB.⁴⁰ Today it is known that reported discrepancies between studies regarding the impact of IFNGR1 polymorphisms on susceptibility to *Mycobacterium* infection may be due to population genetic and/or environmental factors. In this study, *Mycobacterium simiae* was the most prominent NTM among PTB-infected patients (62/80; 77.5%). Previous reports also documented *M. simiae* as the most frequent SGM species in Iran and in the Middle East region.^{41,42} Of 62 infected patients with *M. simiae*, 54 (87.0%) had SNP in IFNGR1 T-56C position. We also found eight *M. simiae*-infected patients with no polymorphisms in IFNGR1 at position-56 (TT genotyping), five of which were more than 60 years of age and three had diabetes mellitus. Therefore, we propose a further investigation in determining the associated risk factors with NTM infection.³⁹

In conclusion, the 56 CC genotypes of IFN- γ receptor-1 were significantly associated with NTM infection. Although the number of studied population was low, patients belonged to a different geographical region of Iran that were referred to NRITLD for diagnosis and treatment. The high prevalence of CC genotypes among randomly selected patients with pulmonary disease outlines the correlation of this mutation with susceptibility to NTM infection.

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