

**Original article****Association of Mycobacterium Tuberculosis Lineages with IFN- $\gamma$  and TNF- $\alpha$  Gene Polymorphisms among Pulmonary Tuberculosis Patient**

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**Abstract.** The six major lineages of *Mycobacterium tuberculosis* [MTB] are found to be strongly associated with specific geographical outbreaks. But whether these bacterial lineages influence the host genetic polymorphism is uncertain. The present study was designed to evaluate the relevance of strain diversity and host genetic polymorphisms in susceptibility to pulmonary tuberculosis [PTB]. For this reason, single nucleotide polymorphisms [SNPs] in interferon- $\gamma$  [IFN- $\gamma$ ] receptor-1[G-611A], IFNG [G+ 2109A] and tumor necrosis factors [TNF- $\alpha$ ] genes [at -238, 308,-857position] in patients [n=151] were analyzed and compared with controls [n=83]. The genetic diversity of *M. tuberculosis* isolates was performed using spacer oligonucleotide typing. Thereafter, the profile of IFN- $\gamma$  and TNF- $\alpha$  allele frequency were investigated in each subtype of *M. tuberculosis*. The results showed C allele of TNF 857 and A allele of TNF 238 were more frequent in PTB cases [[TNF 857 C allele OR [CI95%] 0.6[0.4-0.9], p= 0.02] for TNF 238 A allele OR [CI95%] 5.5[3.4-9.0], p= 0.00]]. Similarly, G allele in IFNG+ 2109 A/G polymorphism were significantly more in patients than control subject[OR[CI95%] 0.3; p< 0.05]. The major identified clinical isolates of *M. tuberculosis* were EAI[42; 27.8% ], Haarlem[ 31; 20.5% ], CAS [ 23;15.2% ], Beijing[14; 9.2%], and T [11; 7.2% ] lineages. No correction was observed between strains diversity and frequency of SNPs in studied PTB cases. In conclusions, we exclude the possibility of genetic mutation in IFN- $\gamma$  and TNF- $\alpha$  gene by different subtypes of *M. tuberculosis*. Although, our results supports a positive correlation between host SNPs and susceptibility to PTB.

**Introduction.** Tuberculosis [TB], caused by *Mycobacterium tuberculosis*, is a major cause of morbidity and mortality throughout the world. It is estimated that one third of the world's population is

infected with *M. tuberculosis*, and approximately 1 billion people will be added to this number till 2020.<sup>1</sup> Among those who are infected, only 5-10% will develop the active form of the disease with clinical

symptoms. Other infected individuals may remain noninfectious and symptoms free for many years.<sup>2</sup> Basically, the course of infection depends on a complex interaction of host, bacteria and environmental factors.<sup>3</sup> The genetic contribution of the host in the individual susceptibility and development of disease is well studied during recent years.<sup>3</sup> In this regards, both genes for interferon-gamma [IFN- $\gamma$ ] and tumor necrosis factor-alpha [TNF- $\alpha$ ] have been identified as a essential components of the host immune response.<sup>4,5</sup> IFN- $\gamma$  is the key cytokine involved in the protective response against *M. tuberculosis* infection and is required for control of this pathogen. TNF- $\alpha$  in synergy with IFN- $\gamma$  induce antimycobacterial activity of macrophages and increases its bactericidal activity.<sup>6</sup> Till date, several polymorphisms within the promoter region of TNF- $\alpha$  and IFN- $\gamma$  gene have been shown to be associated with susceptibility or resistance to TB in different ethnic groups.<sup>7,8,9,10</sup> In contrast, the role of genetic variability of *M. tuberculosis* in the outcome of the infection remains to be uncertain.<sup>11</sup> Most of immunological research on tuberculosis has been performed with laboratory strains i.e., H37RV and Erdman. But, with advances in molecular biology, it became apparent that *M. tuberculosis* is not a genetically conserved bacterium with limited phenotypic differences.<sup>11,12</sup> Additionally, studies on molecular epidemiology showed differences in transmissibility and virulence of various subtypes of *M. tuberculosis*. Lopez and his co-workers were among the first investigators who could represent different immunopathological events using different *M. tuberculosis* strains.<sup>11</sup> Later on, Tanveer *et al* studied the cytokine secretion in patients that were infected by CAS1 and Beijing subtypes of *M. tuberculosis*.<sup>12</sup> In other studies, the influences of *M. tuberculosis* lineages to innate immune responses were characterized.<sup>13,14</sup> However, association between host genetic polymorphisms and susceptibility to different lineages of *M. tuberculosis* strains was not reported. Recently, we showed the high prevalence of Beijing and Haarlem lineages among Iranian drug resistance TB patients.<sup>7,15</sup> Initially, Beijing was described in 1995 as a closely related group of tubercle bacilli from the People's Republic of China, and Haarlem were mainly found in Central America and Caribbean.<sup>16,17</sup> To date, the prevalence of Beijing and Haarlem have reported in several countries.<sup>16,18</sup> In the present study, the association of IFN- $\gamma$  and TNF- $\alpha$  polymorphisms with susceptibility to TB in genetically diverse subtypes of *M. tuberculosis* are investigated. To our knowledge this is the first report that investigates association of host genetic polymorphism with genotyping of *M. tuberculosis*.

## Material and Methods.

**Setting and study population.** The study was conducted from January 2010 to December 2012, in the Mycobacteriology Research Center [MRC]. MRC is the only WHO-approved center for the detection and diagnosis of TB patients in Iran. A total of 151 patients with culture-positive TB and 83 healthy volunteers [referred to as normal controls] were included in the study. Patients and control subjects were matched for age, sex and nationality [The Institutional Review Board at the NRITLD approved the study and all the patients have signed informed consent].

**Mycobacterial isolates.** Collected sputum samples from each patient were digested and decontaminated by Petroff's method.<sup>19</sup> Lowenstein-Jensen media were used for bacterial growth. The extracted DNA from culture positive samples was used for identification and spoligotyping.<sup>15,20</sup> Drug susceptibility testing was performed against first -line anti TB drugs using proportional method.<sup>21</sup>

**Spoligotyping of MTB isolates.** Spoligotyping was performed for all 151 clinical isolates according to the standard method.<sup>20</sup> Briefly, DR region of mycobacterial genome was amplified by PCR using following primers: DRa 5' - GGT TTT GGG TCT GAC GAC -3' [biotinylated at 5'end] and DRb 5'-CCG AGA GGG GAC GGA AAC-3' [Metabion, Martinsried, Germany]. The PCR amplicons were subsequently hybridized to a set of 43 different immobilized DR spacers covalently bound to the membrane. The hybridization signals were detected by chemiluminescence system [Amersham ECL detection kit, GE Healthcare Limited, UK] after incubation with a streptavidin-peroxidase conjugate [Roche, Germany]. DNA extracts of MTB H37Rv and *M.bovis* BCG were used as positive controls.

**Genetic evaluation.** Genomic DNA was extracted using the standard protocol with slight modifications.<sup>23,24</sup> Briefly, Peripheral Blood Leukocytes [PBLs] were separated from two milliliters of the whole blood using RBC lysis buffer [0.155 M NH<sub>4</sub>Cl, 0.01 M NaHCO<sub>3</sub>]. Thereafter, PBLs re-suspended in 500 $\mu$ l of SE buffer [NaCl 3M, EDTA 0.5M, PH=8], containing 40  $\mu$ l of 10% SDS and 3 $\mu$ l of 20 mg/ml of proteinase K. The suspension was incubated at 60<sup>0</sup>C for 30 minutes. After incubation, 200 $\mu$ l of equilibrated phenol [PH=8] was added to the mixture and centrifuged for 10 min at 12000g. The aqueous phase transferred to a new tube and the DNA was precipitated using cold propanol.

**TNF- $\alpha$  genotyping.** Polymorphisms in the TNF promoter region, namely TNF single nucleotide polymorphisms [SNP] 238, 308 and 857 were studied using PCR- RFLP. For TNF -308 polymorphisms, the following primers were used to amplify a 107bp product: 5' AGC AAT AGG TGG TTT TGA CTC



Most of the patients [54.0%] were Iranian, and remains were immigrants.

**Spoligotyping.** Of 151 MTB isolates for which spoligotyping was performed, 140 [92.6%] isolates were grouped into 13 different “shared type” that had been described in the SITVIT2 database and the remaining 11 [7.4%] isolates generated unknown spoligopatterns. The most frequent spoligotype in our populations belong to EAI [EAI1 and EAI3, n=42, 27.8%], Haarlem [H3 and H4, n=31, 20.5%] followed by CAS [CAS1 and CAS2, n=23, 15.2%], Beijing [n=14, 9.2%], and T [T, T3 and T4, n=11; 7.2%] lineages (**Table 2**).

**Drug susceptibility patterns.** As shown in **table 3**, drug susceptibility testing of 151 strains indicated that 99 [65.6%] were sensitive to all tested agents and 52 [34.4%] were resistant to at least one drug. The majority of drug resistant isolates were resistance to INH [6.6%] followed by PZA [2.6%] and ETM [2.0%] and STM [2.0%]. None of the investigated isolated were RMP monoresistant. Twenty three isolates were MDR-TB [15.2%]. In an investigation between MTB strains and drug resistance we found that Beijing genotype was highly associated with MDR [ $p < 0.05$ ].

**Association of IFN- $\gamma$  and TNF- $\alpha$  gene polymorphisms with TB.** Allele and the genotype frequencies of investigated IFN- $\gamma$  and TNF- $\alpha$  polymorphisms are enlisted in **Table 4**. In overall, three types of polymorphisms were observed in TNF- $\alpha$  gene: an A to G substitution at position -238, a G to A substitution at position -308, and a C to T substitution at position -857. Among these polymorphisms, C allele of TNF 857 and A allele of TNF 238 were more frequent in TB cases as compared to control group [TNF 857 C allele OR[CI95%] 0.6[0.4-0.9],  $p = 0.02$ ] for TNF 238 A allele [OR[CI95%] 5.5[3.4-9.0],  $p = 0.00$ ]. Additionally, TNF 857 C/C [85;56.2%] and TNF 238 A/A [127;84.1%] genotypes were associated with

increased risk of acquiring TB. Two types of polymorphisms, in an A to G substitution at position +2109 and -611, were observed for IFN- $\gamma$ . The result showed in -2109 A/G polymorphism, G allele were significantly more common in TB group [OR[CI95%] 0.3;  $p < 0.05$ ]. The investigation of the allele and genotype frequencies for TNF-308 and IFNR1 -611 polymorphisms revealed no significant association with resistance or susceptibility to TB [ $p > 0.05$ ; **Table 4**].

**Association of IFN- $\gamma$  and TNF- $\alpha$  polymorphisms with major lineages of *M. tuberculosis*.** As shown in **table 5**, three polymorphic variants [two in TNF- $\alpha$  gene, and one in IFN- $\gamma$ ] are associated with susceptibility to TB. Distributions of these alleles are similar in patients that were infected with different subtypes of *M. tuberculosis*. For example, out of 42, 31, 23, 19, 14 TB patients infected with EAI, Haarlem, CAS, T, Beijing lineages, 88.6%, 87.1%, 91.2%, 86.4% and 87.5%, had TNF 238A allele, respectively. Similarly, the frequency of IFN- $\gamma$  A allele was high in all TB patients [ranging from 61.1 to 86.4%]. Thereby, we found no correlation between host genetic polymorphisms and mycobacterial diversity.

**Discussion.** Pathogenesis in tuberculosis is dependent on many components of the host, pathogen and environment.<sup>27</sup> The present study was aimed to evaluate the possible correlation of host genetic polymorphisms with different genotypes of *M. tuberculosis*. Based on SIT from SITVIT2, the major identified clinical isolates of *M. tuberculosis* were EAI [42; 27.8%], Haarlem [31; 20.5%], CAS [23; 15.2%], Beijing [14; 9.2%], and T [11; 7.2%] lineages. Recently, it was shown that particular genotypes of *M. tuberculosis* could elicit different immune responses with high mortality rates in the course of experimental infection.<sup>11,12,13</sup> For

**Table 3.** Drug resistant patterns of MTB strains.

Type of resistance	No.[%] of strains									
	Haarlem	EAI	CAS	Beijing	T	MANU	U	LAM	Unknown	Total
Sensitivity to all drugs	19 [61.3]	28 [66.7]	19 [82.6]	4 [28.6]	8 [72.7]	6 [66.7]	4 [57.1]	2 [66.7]	9 [81.8]	99 [65.6]
MDR	2 [6.5]	5 [11.9]	1 [4.3]	10 [71.4]	1 [9.1]	2 [22.2]	1 [14.3]	1 [33.3]	0 [0.0]	23 [15.2]
INH resistance	2 [6.5]	4 [9.5]	1 [4.3]	0 [0.0]	1 [9.1]	0 [0.0]	1 [14.3]	0 [0.0]	1 [9.1]	10 [6.6]
STM resistance	1 [3.2]	1 [2.4]	0 [0.0]	0 [0.0]	0 [0.0]	1 [11.1]	0 [0.0]	0 [0.0]	0 [0.0]	3 [2.0]
PZA resistance	4 [12.9]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	4 [2.6]
ETM resistance	1 [3.2]	2 [4.8]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	3 [2.0]
INH/STM resistance	0 [0.0]	1 [2.4]	1 [4.3]	0 [0.0]	1 [9.1]	0 [0.0]	1 [14.3]	0 [0.0]	0 [0.0]	4 [2.6]
RMP/STM resistance	2 [6.5]	1 [2.4]	1 [4.3]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	0 [0.0]	1 [9.1]	5 [3.3]
Total	31 [100.0]	42 [100.0]	23 [100.0]	14 [100.0]	11 [100.0]	9 [100.0]	7 [100.0]	3 [100.0]	11 [100.0]	151 [100.0]

**Table 4.** Allele and genotype frequencies in TB cases

	Control [83]	TB cases [151]	OR [CI 95%]	P
<b>TNF857</b>				
Allele				
T	56 [33.7]	72 [23.8]	0.6 [0.4-0.9]	0.02
C	110 [66.3]	230 [76.2]		
Genotype				
TT	8 [9.6]	6 [3.9]	0.3 [0.4-1.1]	0.08
TC	40 [48.1]	60 [39.7]	0.7 [0.4-1.2]	0.2
CC	35 [42.1]	85 [56.2]	0.5 [0.3-0.9]	0.03
<b>TNF308</b>				
Allele				
G	154 [92.7]	272 [90.0]	0.7 [0.3-1.4]	0.3
A	12 [7.3]	30 [10.0]		
Genotype				
GG	71 [85.5]	122 [80.7]	0.7 [0.3-1.4]	0.3
GA	12 [14.5]	28 [18.5]	1.3 [0.6-2.8]	0.4
AA	0 [0.0]	1 [0.6]	1.5 [1.4-1.7]	0.4
<b>TNF238</b>				
Allele				
A	100 [60.2]	270 [89.4]	5.5 [3.4-9.0]	0.00
G	66 [39.8]	32 [10.6]		
Genotype				
AA	49 [59.0]	127 [84.1]	3.6 [1.9-6.8]	0.00
AG	2 [2.4]	16 [10.5]	4.8 [1.0-21.4]	0.02
GG	32 [38.5]	8 [5.2]	0.1 [0.03-0.2]	0.00
<b>IFNR1611</b>				
Allele				
A	118 [71.0]	237 [78.4]	1.4 [0.9-2.2]	0.07
G	48 [29.0]	65 [21.6]		
Genotype				
AA	47 [56.6]	97 [64.2]	1.3 [0.7-2.3]	0.2
AG	24 [28.9]	43 [28.4]	0.9 [0.5-1.7]	0.9
GG	12 [14.4]	11 [7.2]	0.4 [0.1-1.1]	0.07
<b>IFN2109</b>				
Allele				
A	152 [91.5]	241 [79.8]	0.3 [0.1-0.6]	0.001
G	14 [8.5]	61 [20.2]		
Genotype				
AA	69 [83.1]	97 [64.2]	0.3 [0.1-0.7]	0.002
AG	14 [16.9]	47 [31.1]	2.2 [1.1-4.3]	0.01
GG	0 [0.0]	7 [6.4]	1.5 [1.4-1.7]	0.04

**Table 5.** Allele and genotype frequencies in different genotypes of MTB

	TB cases [151]	Haarlem [31]	P	Beijing [14]	P	CAS [23]	P	EAI [42]	P	T [11]	P	MANU [9]	P	U [7]	P	LAM [3]	P
<b>TNF857</b>																	
Allele																	
T	72 [23.8]	15 [24.2]	0.9	10 [35.7]	0.1	13 [28.9]	0.4	18 [21.4]	0.6	5 [22.7]	0.9	4 [22.2]	0.8	5 [35.7]	0.3	0 [0.0]	0.3
C	230 [76.2]	47 [75.8]		18 [64.3]		33 [71.1]		66 [78.6]		17 [77.3]		14 [77.8]		9 [64.3]		3 [100.0]	
Genotype																	
TT	6 [3.9]	2 [6.5]	0.5	1 [7.1]	0.5	1 [4.3]	0.9	1 [2.4]	0.6	1 [9.1]	0.4	0 [0.0]	0.5	1 [14.3]	0.1	0 [0.0]	0.7
TC	60 [39.7]	11 [35.5]	0.6	8 [57.1]	0.2	11 [47.8]	0.4	16 [38.1]	0.8	3 [27.3]	0.4	4 [44.4]	0.7	3 [42.9]	0.8	0 [0.0]	0.1
CC	85 [56.3]	18 [58.1]	0.8	5 [35.5]	0.1	11 [47.8]	0.4	25 [59.5]	0.7	7 [63.6]	0.6	5 [55.6]	0.9	3 [42.9]	0.4	3 [100.0]	0.1
<b>TNF238</b>																	
Allele																	
A	270 [89.4]	54 [87.1]	0.5	26 [87.5]	0.5	42 [91.2]	0.6	76 [88.6]	0.7	19 [86.4]	0.6	15 [83.3]	0.4	14 [100.0]	0.1	5 [83.3]	0.6
G	32 [10.6]	8 [12.9]		2 [12.5]		4 [8.8]		8 [11.4]		3 [13.6]		3 [16.7]		0 [0.0]		1 [16.7]	
Genotype																	
AA	127 [84.1]	26 [83.9]	0.9	13 [92.9]	0.3	19 [82.6]	0.8	36 [84.5]	0.7	9 [81.8]	0.8	6 [66.7]	0.1	7 [100.0]	0.2	2 [66.7]	0.4
AG	16 [10.5]	2 [6.5]	0.4	0 [0.0]	0.2	4 [17.4]	0.3	4 [10.4]	0.8	1 [9.1]	0.8	3 [33.3]	0.04	0 [0.0]	0.3	1 [33.3]	0.2
GG	8 [5.2]	3 [9.7]	0.3	1 [7.1]	0.7	0 [0.0]	0.2	2 [4.8]	0.8	1 [9.1]	0.5	0 [0.0]	0.4	0 [0.0]	0.5	0 [0.0]	0.6
<b>IFN2109</b>																	
Allele																	
A	241 [79.8]	47 [75.8]	0.4	20 [68.7]	0.2	38 [82.6]	0.6	71 [84.5]	0.3	19 [86.4]	0.4	11 [61.1]	0.06	12 [85.7]	0.5	5 [83.3]	0.8
G	61 [20.2]	15 [24.2]		8 [31.3]		8 [17.4]		13 [15.5]		3 [13.6]		7 [38.9]		2 [14.3]		1 [16.7]	
Genotype																	
AA	97 [64.2]	17 [54.8]	0.3	7 [50.0]	0.2	16 [69.6]	0.6	31 [73.8]	0.2	8 [72.7]	0.5	4 [44.4]	0.2	5 [71.4]	0.6	2 [66.7]	0.9
AG	47 [31.1]	13 [41.9]	0.2	6 [42.9]	0.3	6 [26.1]	0.6	9 [21.4]	0.2	3 [27.3]	0.7	3 [33.3]	0.8	2 [28.6]	0.8	1 [33.3]	0.9
GG	7 [4.6]	1 [3.2]	0.7	1 [7.1]	0.6	1 [4.3]	0.9	2 [4.8]	0.9	0 [0.0]	0.4	2 [22.2]	0.02	0 [0.0]	0.5	0 [0.0]	0.7

example, in mice model Beijing subtypes, induced early and massive pneumonia with death. Whereas, Canetti strains induced limited pneumonia with sustained expression of TNF- $\alpha$ .<sup>27,29</sup> Likewise, other investigators showed a different level of cytokines production [TNF- $\alpha$ ,IFN- $\gamma$ ] in patients infected with genetically distinct *M. tuberculosis* subtypes.<sup>12</sup> Our results showed no association between the frequencies of SNPs in host and various lineages of *M. tuberculosis*. As shown in **table 5**, the TNF- $\alpha$  238A and 857C alleles were associated with susceptibility to TB infection, but their distribution was almost equal among patient infected with different subtypes of *M. tuberculosis*. Likewise, the frequency of IFN2109A allele was high in TB patients than control subject, but no statistical differences were observed among the allele distribution in different *M. tuberculosis* lineages. Therefore, our results demonstrate no correlation between genetic diversity of *M. tuberculosis* and host susceptibility to TB. On the contrary to our results, Tanveer *et al* showed a correlation between cytokine induction i.e., TNF- $\alpha$ , IFN- $\gamma$  and growth index of CAS1 and Beijing isolates in comparison to H37RV strain.<sup>12</sup> Furthermore, they suggested that the phenotypic and genotypic polymorphisms in clinical *M. tuberculosis* strains may in turn influence the persistence and dissemination of differing genotypes.

At present, we have no explanation for such discrepancy, but we need more detailed studies in order to outline the importance of *M. tuberculosis* genotypes with host genetic polymorphism. Basically, genetic contribution of the host is an important factor in determining susceptibility to TB. Today, several cytokine gene polymorphisms have been described in association with susceptibility or resistance to TB. In present investigations, we found two polymorphisms of TNF gene promoter [-857 and -238] that were significantly associated with TB patients (**Table 4**). For TNF- 238 A/G SNP, A allele was more frequent in TB cases as compared to control. Previously also, positive association of TNF- 238 A/G was reported among Iranian pulmonary tuberculosis cases.<sup>22,30</sup> TNF 238 A/G polymorphism has been extensively studied in TB cases of various ethnic groups.<sup>8,9,10</sup> However, studies that were conducted in Turkey, India and Columbia demonstrated no association of specific allele of TNF- $\alpha$  gene with susceptibility to TB.<sup>8,9,31</sup> There are also considerable variations in genotype frequencies of TNF 857 polymorphisms in different populations. TNF 857 T/C polymorphism in our study was significantly more frequent in TB cases as compared to control. However, conflicting reports are available about insignificant

association of 857T/C genotype in Asian TB patients i.e. Indians.<sup>4</sup> In fact, the contradictory data could be discussed in different ways; First of all, multiple polymorphisms within the TNF gene may have emerged during evolution in various ethnic groups to affect TB susceptibility or resistance. Second, the number of studied cases has a great impact on the outcome of the results. Generally, the large confidence intervals in some studies could be the result of the small sample size. For TNF 308 G/A, several studies on TB patients have produced approximately similar results. In recent surveys, no significant association in TNF 308 and TB were reported from Korea, Brazil and China, which is similar to our study.<sup>9,32,33</sup>

Another candidate gene for determination the susceptibility to TB is polymorphism in the IFN- $\gamma$  gene.<sup>3,26</sup> In different experimental set up, tuberculosis patients had deficient IFN- $\gamma$  production in their peripheral blood mononuclear cells. Also, it has been shown that partial or complete loss of function alleles of IL-12/IFN- $\gamma$  axis genes associated with diseases development.<sup>13,14,34</sup> In the present study, IFN2109G allele was significantly associated with increased susceptibility to TB. However, we found no significant association between IFNR1611 A/G SNP and TB patients. Previously, Mirsaeidi *et al*, also did not find any significant association between IFNR1395 SNP and susceptibility to TB among Iranian studied cases.<sup>35</sup> Also few studies declines the correlation of IFNGR1 polymorphism with *M. tuberculosis*, instead they proposed the correlation of IFNGR1 polymorphism with avirulent or *M bovis* BCG infection.<sup>25,36</sup> These observations may outline the alternative pathways for enhancing host immune response against *M. tuberculosis*.

**Conclusions.** Our findings showed that the polymorphisms in TNF- $\alpha$  promoter gene are likely associated with increased susceptibility to TB in Iranian patients. But, no significant association was found between frequencies of SNPs in host and genotyping of *M. tuberculosis*. However, further studies with multiple genes polymorphisms would be necessary to elucidate the exact role of *M. tuberculosis* genotyping.

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