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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Genetic polymorphism in association with susceptibility to tuberculosis: a study in a Pakistani population

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Abstract

Tuberculosis is becoming a global issue with raising occurrences; particularly in developing countries, the situation is alarming. Besides environmental factors, host genetic factors are vital in disease development. A demographical and genotypic analysis in relation to tuberculosis commencement is conducted in a Pakistani population, and genotypic frequency of *EBI3* (rs4740) was analyzed. Allelic frequencies of *EBI3* (rs4740) were significantly associated with disease susceptibility in the reviewed population. Analysis for *EBI3* (rs4740) genotyping showed a significant association of "GG" with reduced risk for disease. Moreover, females and older age found to be more perilous to develop TB while smoking and a family history of TB are additional risk factors for disease development. Further work with a larger population is necessary to identify the true causative variants of tuberculosis.

Keywords Tuberculosis · Polymorphism · *EBI3* (rs4740) · Smoking

Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) and is one of the most devastating chronic infectious diseases, which remains the leading cause of death in developing countries [1]. Worldwide, there is a heavy burden of TB with 9.6 million new cases besides the 1.5 million deaths reported in the year 2014 [2]. It is estimated that nearly one-third of the world's population is infected with *M. tuberculosis* while a large number of the population are left with no clinical symptoms of this infectious disease. Since merely 5–15% of individuals will develop the active disease [3, 4], it is assumed that susceptibility and progression to active TB are partly regulated by the host genetic factors [5]. In this regard, the identification of host genes and genetic variations would lead to a better understanding of the pathogenesis of TB and undoubtedly lead to novel strategies of treatment or prophylaxis.

It has already been acknowledged that innate and adaptive immune responses are imperative in the control of TB infection [6]. At some point during the infectious cycle, immune competent–infected humans will show the presence of *Mycobacterium* and start to generate an immune response, destroying macrophages containing bacilli. This leads to the presentation of *Mycobacterium* antigens to the host immune system resulting in the generation of a specific immune response against *M. tuberculosis* 7.

Numerous immune regulatory genes involved in host immune responses have been proven to contain multiple polymorphisms thus contributing to TB susceptibility among different populations [8]. Among them, *EBI*3 has been found to be an important regulator in inflammation and infection. It has

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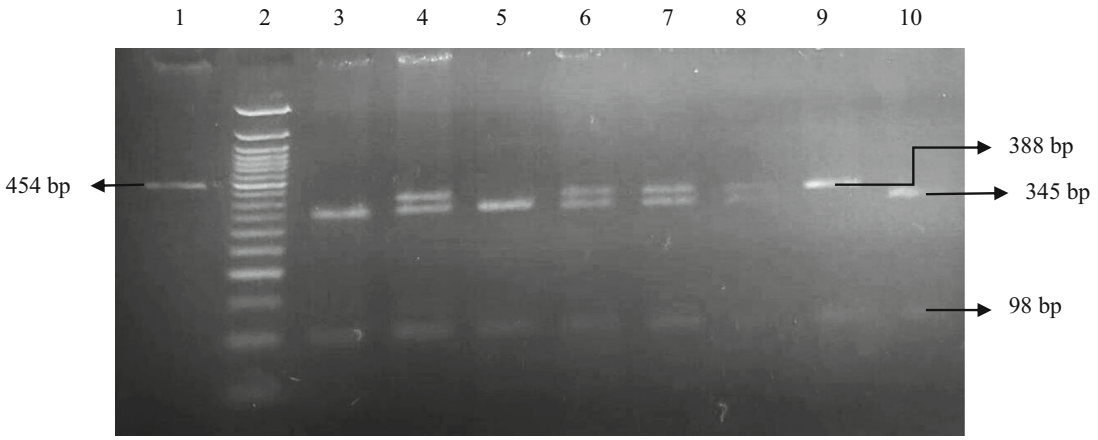

Fig. 1 Electrophoresis of PCR and restriction digestion products for *EBI3 rs4740*. Ethidium bromide–stained electrophoresed representative RFLP products samples: 50-bp ladder (lane 2); *EBI3 rs4740*; PCR product (lane 1) 454 bp; homozygous wild A/A (lane 9) 388 bp, 98 bp; homozygous mutant G/G (lanes 3, 5, 10) 98 bp and 345 bp; heterozygous A/G (lanes 4, 6, 7, 8) 98 bp, 345 bp, and 388 bp

been shown to modulate differentiation of hematopoietic progenitor cells and regulate activation of immune cells as well as chemokines and cytokine production [9]. It was identified as a susceptible gene for pulmonary TB (PTB) and its deficiency protected mice against mycobacterial infection [10]. The G allele at *EBI3* (rs4740) reduced the risk of developing tuberculosis in the Chinese population [10]. *EBI3* is a member of the *IL-12* heterodimeric cytokine family [11, 12]. Deficiency of *EBI3* caused a reduction in bacterial burden and histopathological injury in lungs infected with *Mycobacterium bovis*. *EBI3* was also found in higher abundance in the granuloma of PTB patients and in lung tissues infected with BCG. Mycobacterial infection extraordinarily induced the expression of *EBI*3 at both mRNA and protein levels. Consequently, polymorphism in the *EBI3* gene rs4740 is closely linked with PTB susceptibility [10]. The expression of *EBI3* is significantly upregulated in a variety of cancers such as breast cancer [13], gastric cancer [14], and pancreatic ductal adenocarcinoma [15]. Moreover, *EBI3* polymorphisms were also reported to be closely associated with susceptibility to other diseases such as allergic rhinitis [16] and chronic rhino sinusitis [17].

In order to investigate the role of *EBI3* in accordance with the reported findings, we performed a study to evaluate the association of *EBI3* (rs4740) with PTB susceptibility in a Pakistani population through genotyping.

Methods

Study population

A total of 292 PTB patients and 199 healthy controls were analyzed in this study. Patients were consecutively recruited from Nishtar Hospital, Multan, after complete microbiological (smear positive and/or culture positive) and radiographical (x-ray) examination. Subjects showing either only a positive smear culture or only positive radiographical reports were excluded. Moreover, subjects under anti-TB medication were also omitted from the study. The control subjects were unrelated adults selected through the population without recent sign, symptom, or history of TB, and they were living in the same region as the patients with PTB. Inclusion criteria for controls were no history of previous TB or anti-mycobacterial treatments, no evidence of TB-related infiltrates in chest x-rays and no microbiological finding of *Mycobacterium* in their sputum. All cases and controls were HIV negative. Written informed consents were obtained from all the participants of the present study, and they donated a blood sample for genotyping analysis. The Ethical Committee of Bahauddin Zakriya University, Multan, approved the study.

Table 1 Enzymes and primers used in the study

SNPs studied	Sequence of primers	Product size	Restriction enzyme used	Restriction digestion patterns for different alleles and their band sizes
EBI3 A/G (rs4740)	5′-GCTCCGTTGTGTGGTTCTGT-3′ 5′-AGTGACAGTTCAGTCAGCCC-3′	486 bp	HpyCH4IV	A allele: 98 + 388 bp G allele: 98 + 43+ 345 bp

Table 2 Influence of various demographic factors on phenotype

Factors	Categories	Cases ($n = 292$)	Control ($n = 199$)	Significance	OR (95% CI)
Gender	Male	121 (41.4%)	141 (70.8%)	$\chi^2 = 41.148$, df = 1,	3.44 (2.34–5.05)
	Female	171 (58.6%)	58 (29.1%)	P value = 0.000	
Age	11–30	132 (45.2%)	90 (45.2%)	$\chi^2 = 9.954$, df = 2,	0.69 (0.53 0.90)
	31–50	97 (33.2%)	86 (43.2%)	P value = 0.007	
	51–70	63 (21.6%)	23 (11.5%)		
Smoking	Smokers	57 (19.5%)	15 (7.5%)	$\chi^2 = 13.580$, df = 1,	0.34 (0.18 0.61)
	Non smokers	235 (80.4%)	184 (92.5%)	P value = 0.000	
Family history of TB	TB present in family history	137 (46.9%)	10 (5%)	$\chi^2 = 99.019$, df = 1,	0.06 (0.03–012)
	No TB history in family	155 (53.1%)	189 (95%)	P value = 0.000	

Genomic DNA preparation and genotyping

Genomic DNA was isolated from peripheral blood mononuclear cells and granulocytes obtained from the blood of patients and controls using salting out procedure. *EBI3* A/G (rs4740) polymorphism was studied using PCR-based restriction fragment length polymorphism (RFLP) methods. The sequences of primers, restriction digestion enzyme used, and restriction digestion pattern (Fig. 1) for different alleles are given in Table 1. Some randomly selected samples were sequenced (TSINGKE Biological Technology) for the conformation of PCR-RFLP, which showed complete matching of results.

Statistical analysis

SPSS 17.0 was used to carry out statistical analysis. The association between phenotype (TB) and various demographic and genotypic parameters was determined using cross-tabulation in complex samples. Analysis with χ^2 was used to test the statistical significance of the association. Stratified analyses were used to explore the correlation between phenotype and genotypes. The odds ratios (OR) and 95% confidence limits (CL) were calculated as an estimate of the relative risk and strength of association using logistic regression analysis. The result was considered significant when its associated P values were less than 0.05.

Results

Overall, this study consists of 292 cases (41.4% males, 58.6% females) and 199 controls (70.8% male, 29.1% female). The age (mean ± SD) was 36.36 and ± 17.17 for cases and 30.35 ± 10.87 years for controls. Despite the difference in number, significant differences in the distribution of gender and age between cases and controls were observed (Table 2) as a result of frequency matching. Despite the different ratios of males:females in the selected population, females tend to be more prevalent in the case group. Furthermore, patients aging 51–70 years had a higher prevalence of the disease (21.6%) compared with the same age group from the controls (11.5%). In addition to this, smoking and a family history of TB were also found to be significantly associated with the disease (P value < 0.0001) as the frequency distribution showed both of these factors are more prevalent in cases (Table 2).

Genotypic and allelic frequency of various genotypes in controls or cases

On exploring *EBI3* (rs4740 A/G) polymorphism in controls and PTB subjects, significant difference was observed among case and control groups (Table 3). Frequency of "AA" and "AG" genotypes is higher in cases (53.1% and 36.6% respectively) than in controls (48.2% and 29.6% respectively) while the "GG" genotype is higher in controls (22.1%) than in cases

Table 3 Distribution of genotypic and allelic frequencies among cases and controls and their possible association with tuberculosis

Genes	Genotypes/allele	Cases ($n = 292$)	Control ($n = 199$)	P value	OR (95%CI)
EBI3	AA	155 (53.1%)	96 (48.2%)	0.292	1.21 (0.85–1.74)
(rs4740 A/G)	AG	107 (36.6%)	59 (29.6%)	0.108	1.17 (0.97–1.42)
	GG	30 (10.2%)	44 (22.1%)	0.0005	0.74 (0.62–0.87)
	A	417 (0.71)	251 (0.63)	0.0066	1.4624 (1.1148–1.9184)
	G	167 (0.29)	147 (0.37)		0.6838 (0.5213–0.897)

Table 4 Association of genotypes and tuberculosis stratified by gender

Genes	Genotypes	Male			Female		
		Cases (121)	Control (141)	Significance	Cases (171)	Control (58)	Significance
EBI3 (rs4740 A/G)	AA	65 (53.7%)	72 (51%)	$\chi^2 = 5.215$, df = 2,	90 (52.6%)	24 (41.3%)	$\chi^2 = 6.324$, df = 2,
	AG	41 (33.8%)	37 (26.2%)	*P* value = 0.074	66 (38.5%)	22 (37.9%)	*P* value = 0.042
	GG	15 (12.3%)	32 (22.6%)		15 (8.7%)	12 (20.6%)	
	A	0.71	0.64		0.72	0.60	
	G	0.29	0.36		0.28	0.40	

(10.2%). This showed a significant association of "GG" with reduced risk for developing tuberculosis ($P < .0001$; Table 3). Similarly, allelic frequency of the "G" allele is also higher in the control group. These results display that *EBI3* genetic polymorphism is associated with susceptibility to PTB and allele "G" denotes protection against infection.

Effect of stratification by gender and age on the incidence of tuberculosis

To explore studied genes to environment interactions, we examined the association between genotype and TB; the data was stratified by selected characteristics such as sex and age (Tables 4 and 5). While stratifying rs4740 with gender, although we found a higher frequency of "GG" genotype and "G" allele in controls of male and female subjects, we only found a significant association in females only ($\chi^2 = 6.324$, df = 2, $P = 0.042$; Table 4). It depicts the protective role of the "G" allele against TB, while a higher frequency of "AA" genotype (52.6%) and "A" allele (0.72) in female cases illustrates that the "A" allele is involved in increasing the risk of TB in females.

On stratification of patients with different age groups, it was observed that subjects 11–30 years and 51–70 years of age had a significant interaction with *EBI3* ($\chi^2 = 7.262$, df = 2, $P = 0.026$ and $\chi^2 = 6.124$, df = 2, $P = 0.047$, respectively; Table 5), while the interaction of genotypes with age group of 31–50 years was not significant.

Discussion

The magnitude and complexity of the human immune response to *Mycobacterium* have historically been underestimated [18]. It is vital to determine whether those who remain healthy have a genetically endowed high level of resistance to tuberculosis or whether the resistance is affected by environmental or other exogenous factors [19].

The genome-wide association studies (GWA) identified several susceptibility loci for tuberculosis in sub-Saharan African, Russian, and Moroccan populations [20–22]. However, follow-up studies reported conflicting results [23]. In the present study, we explored the genetic polymorphism of *EBI3* (rs4740) in association with pulmonary tuberculosis in a Pakistani population. *EBI3* is a soluble glycosylated protein initially identified as a transcriptionally activated gene in Epsteine-Barr virus (EBV)-infected human B lymphocytes [24]. Our results were in agreement with a previous finding [10] that the "G/G" genotype was significantly associated with a reduced risk of TB where the allele "G" located in rs4740 protects against the disease. In addition to this, "AA" genotypes increase the risk of TB in our female population. This type of association in females was not found in previous studies. To the best of our knowledge, no data of this SNP have been published yet in association with TB except in the Chinese population [10] making us the pioneer to explore the role of genetic polymorphisms in this region. Variant rs4740 is a non-synonymous SNP (ns SNPs) and "G" allele replaced by "A" allele leads to substitution of valine with isoleucine at

Table 5 Association of genotypes and tuberculosis stratified by age

Genes	Genotypes	11–30 years			31–50 years			51–70 years		
		Cases (132)	Cont. (90)	Significance	Cases (97)	Cont. (86)	Significance	Cases (63)	Cont. (23)	Significance
EBI3 (rs4740 A/G)	AA	72 (54.5%)	48 (53.3%)	$\chi^2 = 7.262$,	49 (50.5%)	37 (43%)	$\chi^2 = 2.478$,	34 (54%)	11 (47.8%)	$\chi^2 = 6.124$,
	AG	50 (37.8%)	25 (27.7%)	df = 2,	34 (35.05%)	29 (33.7%)	df = 2,	23 (36.5%)	5 (21.7%)	df = 2,
	GG	10 (7.5%)	17 (18.8%)	*P* value = 0.026	14 (14.4%)	20 (23.3%)	*P* value = 0.290	6 (9.5%)	7 (30.4%)	*P* value = 0.047

position 201 which is located in fibronectin type III domains of *EBI3*. This missense mutation of rs4740 affects the stability, structure, or biological function of the *EBI3* gene disturbing the bacterial processing during infection. Although, in the present study, we only focused on rs4740, other SNPs of *EBI3* are also involved in various other conditions and we cannot rule out their role. For instance, rs428253 is associated with the risk of coronary heart disease [25] while has protective effects against allergic rhinitis [16]. Similarly, rs568408 and not rs2243115 is associated with asthma [26].

Furthermore, a significant association of the incidence of TB was observed with demographic factors such as gender, age, smoking pattern, and the presence of TB in a family history. Like a previous study from Pakistan [27], we observed that females are at higher risk of TB development, divergent to the Taiwanese population [28], since females in our society have worsened conditions concerning TB diagnosis, treatment, and cure. The interaction of genders with the *EBI3* (rs4740 A/G) revealed that the females carrying "AA" genotypes were at significantly higher risk of disease development than males while the "GG" genotype plays a protective role in males only. Old age was recorded as the risk factor for the disease development, as in aged people, the immune system is compromised. Age-related factors enhance TB susceptibility as well as increase the possibility of TB reactivation [29]. The incidence of TB among older people is almost three times higher than that of young adults worldwide [30].

We additionally observe smoking as a risk factor for TB susceptibility as described by other studies [31, 32]. Smoking results in malfunctioning of alveolar macrophages and tuberculosis leads to apoptosis of these cells [33–35]. Antigen presentation of alveolar macrophages is impaired by nicotine present in cigarette smoke; thus, prolonged acquaintance to smoking diminishes the expression of surface proteins involved in antigen presentation [36–39] consequently resulting in disease development. Lastly, a noteworthy association of TB sensitivity was observed in relation to the presence of TB in a family history strengthening the perception that host genetic factors are equally contributive.

In summary, our data suggest that allelic frequencies of *EBI3* (rs4740) are associated with the risk of TB in a Pakistani population. In the present study, heterogeneity was found, which is possibly due to the ethnic origin of the included TB patients as ethnicity-specific genetic variations may influence the host immunity to bacterial infection. Further studies of SNPs in high linkage disequilibrium covering a larger cohort are under process in our institutes for further investigation.

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Compliance with Ethical Standards

Written informed consents were obtained from all the participants of the present study and they donated a blood sample for genotyping analysis. The Ethical Committee of Bahauddin Zakriya University, Multan, approved the study.

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