Advanced Research Journal of Immunology and Virology Vol. 1 (3), pp. 049-053, November, 2013. Available online at www.advancedscholarsjournals.org © Advanced Scholars Journals

Full Length Research Paper

Interleukin-10 gene promoter polymorphism as a potential host susceptibility factor in Pakistani patients with pulmonary tuberculosis

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Accepted 24 October, 2013

Tuberculosis (TB) is an infectious disease, which causes the death of nearly three million humans and eight million cases worldwide annually. Pakistan ranks the seventh position globally in terms of *mycobacterium tuberculosis* infection. TB susceptibility has been associated with cytokines polymorphism in different populations. Single nucleotide polymorphisms within the promoter region of interleukin-10 (IL-10) gene have been associated with altered levels of circulating IL-10, a Th2 cytokine that plays a key role in the pathogenesis of TB. We analyzed the frequencies of IL-10 promoter polymorphisms in 82 TB patients and 99 healthy Pakistani subjects using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) and results indicate that the allelic frequencies for the IL-10 -1,082 G/A, -819 C/T and -592 C/A did not differ significantly between the two groups. However, an association was found between TB occurrence and the IL-10 −1,082 GG genotype (p = 0.006, OR = 6.03), while the -1,082GA was predominant in healthy subjects (p = 0.013, OR = 0.29). Haplotype frequencies were similar in sick and health people. The diplotype GCC/ATA (intermediate IL-10 producer) predominates in Pakistani population, while the GCC/GTA (high) was associated with TB (p = 0.03, OR = 6.7) and GTA/ATA (intermediate) with healthy subjects (p = 0.03, OR = 0.23). Our findings therefore corroborates that the polymorphisms in IL-10 gene affect susceptibility to TB and increase risk of developing the disease.

Key words: Interleukin-10 **(**IL-10), polymorphism, tuberculosis, susceptibility.

INTRODUCTION

Tuberculosis (TB), a lung infection caused by *mycobacterium tuberculosis*, is a leading cause of morbidity and mortality worldwide (Rahman et al., 2009) leading to 1.3 million deaths annually and approximately 8.9 to 9.9 million people are infected with *M. tuberculosis* every year (WHO, 2010). In the recent decades, the number of reported TB cases has increased in both industrialized and developing countries (Zaman, 2010). Pakistan is one of the largest country in terms of the tuberculosis burden, being ranked seventh globally (Ansari et al., 2009). In Pakistan, the incidence and prevalence of the disease is 181 and 329/100,000 habitants per year, respectively (Akhtar et al., 2007). Among the infected patients, only approximately 5 to 10% will develop clinical disease (WHO, 2008). Host genetic factors including cytokine genes are important determinants of TB susceptibility and progression of the disease (Yim and Selveraj, 2010). Increased circulating level of different cytokines, including both the pro inflammatory and the down regulatory cytokine, have been reported to play key role in pathological severity of TB (Ferraz et al., 2006). Shifting of immunity from protective to pathogenic may involve a shift in Th1/Th2 balance (He et al., 2010). Interleukin-10 (IL-10) has an inhibitory effect on Th1 cytokine synthesis and secretion and it functions by inhibiting macrophage, monocyte and T lymphocyte replication (Shin et al., 2005).

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Table 1. Primers used in the study and their amplicon size.

Polymorphism/ Allele location	Primer	Sequence	Product Size (bp)
-1082	Common primer (antisense)	5'-cagtgccaactgagaatttgg-3'	258
	Primer G (sense)	5'-ctactaaggcttctttgggag-3'	
	Primer G (sense)	5'-actactaaggcttctttgggaa-3'	
-819*/ -592*	Common Primer (antisense)	5'- aggatgtgttccaggctcct-3'	233
	Primer C (sense)	5'-cccttgtacaggtgatgtaac-3'	
	Primer T (sense)	5'-acccttgtacaggtgatgtaat-3'	
Internal control	Primer 1	5'-gccttcccaaccattcectta-3'	429
	Primer 2	5'-tcacggatttctgttgtgtttc-3'	

*IL-10 -819 polymorphism is in linkage disequilibrium with -592 polymorphism; allele C at -819 is always present when at position -592 in allele C and allele T is always present when at position -592 in allele A.

According to recent report by Jamil et al. (2007), low level of IL-10 favors the immune response against the mycobacterium, while high levels are associated with disease progression. Therefore, the identification of host genes responsible for susceptibility and resistance to TB should provide a significant contribution for understanding the pathogenesis of the disease and may lead to the development of new prophylaxis and treatment strategies.

Single nucleotide polymorphisms (SNP) in the promoter or coding region of different cytokine genes may alter their transcriptional activation and results in differential cytokine production. Interindividual variations in IL- 10 production are genetically contributed by polymorphisms within the IL-10 promoter (Afzal et al., 2011). Three of these SNP found on the promoter region of IL-10 gene (-1082 G/A, -819 C/T and -592 C/A) exhibit strong effect on IL-10 gene transcription. These SNP show linkage disequilibrium and three main haplotypes (GCC, ACC and ATA) segregate in most populations (Turner et al., 1997; D'Alfonso et al., 2000). These haplotypes are associated with high (GCC), intermediate (ACC) and low (ATA) expression of IL-10 (Turner et al., 1997). Moreover, IL-10 gene promoter polymorphic sites and frequencies vary among populations. Therefore, we investigated here the frequencies of the SNP of IL10 promoter region at -1082 G/A (rs1800870), -819 C/T (rs1800871) and -592 C/A (rs1800872) position and its possible association with the occurrence of TB in Pakistani population.

MATERIALS AND METHODS

Patients and control

For determining the association between IL-10 promoter polymorphisms and TB incidence, blood samples were collected from 82 pulmonary TB patients admitted in Sambli Sanitorium, Murree, TB hospital Rawalpindi, NCVI Diagnostic Lab Islamabad in storage tubes coated with ethylene diamine tetraacetic acid (EDTA). TB was diagnosed on the basis of radiography, clinical presentation, positive smear test and *M. tuberculosis* culture. 99 healthy control subjects were enrolled in the study, none of whom had any history of TB. Patients and healthy volunteers were of same ethnicity and from the same geographical area. The study was approved by the Ethical Committee of NUST Center of Virology and Immunology (NCVI), Islamabad, Pakistan and written consent was obtained for each participant.

DNA extraction

Genomic DNA of both the patients and the healthy control subjects' venous blood samples was extracted using genomic DNA extraction kit (Gentra, USA) according to the manufacturer's protocol. Quantity of DNA was confirmed by Bio Photometer (Eppendorf, USA) and DNA was stored at -20°C.

Genetic analysis

The IL-10 promoter polymorphism at -1082 G/A, -819 C/T and -592 C/A position was genotyped by amplification refractory mutation system- polymerase chain reaction (ARMS-PCR) method as described by Perry et al. (1999). Two separate reactions were performed for each polymorphism. Each reaction contained a common anti-sense primer and one of the two allele-specific forward primers. PCR amplification was performed in 20 µl reaction volume containing 50 ng genomic DNA, 150 µM dNTP, 1 µl 10 pmol each primer, 2 mM $MgCl_2$ and 0.7 units of Taq polymerase in 1X reaction buffer with cycling conditions of 95°C for 5 min, followed by 35 cycles at 95°C for 40 s, 58°C for 45 s, 72°C for 50 s and finally for 7 min extension at 72°C. To ensure PCR success, an internal control region was amplified from the human growth hormone (Table 1). The amplified products were analyzed on 2% ethidium bromide stained agarose gel.

Statistical analysis

Statistical analysis was performed by Study Result Software Version 1.0.4 (CreoStat HB Frolunda, Sweden). The frequencies of alleles, genotypes and haplotypes of the IL-10 polymorphic sites in TB patient and healthy control groups were determined by counting

Table 2. Frequencies of IL-10 promoter polymorphic sites in TB and healthy Pakistani population.

IL-10 locus	Control (n = 99)	Frequency (%)	Patient (n = 82)	Frequency (%)	P-value* (Yates corrected)	OR (CI95%)
-1082 G/A						
G/G	3	3.03	13	15.9	0.006	6.03 (1.52-20.01)
G/A	92	92.93	65	79.2	0.013	0.29 (0.10-0.70)
A/A	4	4.04	4	4.9	0.784	
Allele Frequency						
G	98	49.49	91	55.5	0.256	
A	100	50.51	73	44.5		
819C/T (592C/A)						
T/T (A/A)	15	15.15	6	7.3	0.101	
C/T (C/A)	81	81.82	74	90.3	0.108	
C/C (C/C)	3	3.03	2	2.4	0.809	
Allele frequency						
T(A)	111	56.06	86	52.4	0.491	
C(C)	87	43.94	78	47.6		

and compared by the $\chi 2$ or Fischer's exact test and p-values smaller than 0.05 were considered significant.

RESULTS

Characteristics of patients

Mean age for the patients and control was 42.5 ± 14.3 and 45.7 ± 13.4 years, respectively. Among the 82 patients, 59 were males (43.4 ± 15.1 years) and 23 were females (40.2 ± 16.8 years). The control group consisted of 99 healthy subjects none of whom had any history of mycobacterium infection. There were 60 males (43. 9 ± 12.3 years) and 39 females (48.5 ± 15.1 years) in the healthy group.

Genetic analysis

In this study, the frequencies of alleles at -1082 G/A and −819 C/T polymorphic sites did not differ significantly between TB patients and healthy Pakistani subjects (Table 2). The heterozygous genotypes were predominant for both -1082 and -819 position at the IL-10 promoter. We also found that frequency of GG genotype at IL-10 −1082 polymorphic site was higher in TB patients then in the controls (16 vs. 3%, respectively), suggesting an association with TB susceptibility (p = 0.006, OR = 6.03); whereas the GA genotype was more common in healthy subjects than in TB patients (93 vs. 79%, respectively) suggesting a protective association with TB (p = 0.013, OR = 0.29). There was no association between TB and −592 CC, CT or TT genotypes (Table 2). Our results suggest a lack of association of IL- 10 promoter GCC, ACC, GTA and ATA haplotypes with TB (Table 3). The inheritance of GTA haplotype (high producer) was about 9 and 11% in healthy and diseased subjects. In Pakistani local population, we already reported the prevalence of GTA haplotype (Afzal et al., 2011), which was previously only reported from China (Mok et al., 1998).

It was also observed that the frequency of the GCC/GTA diplotypes was higher in TB compared with healthy controls (12.2 vs. 2%, respectively), with a significant difference (p = 0.03, OR = 6.7), while the GTA/ATA diplotypes were lower in TB compared with healthy controls (3.66 vs. 14.4%, respectively) and a significant difference (p = 0.03, OR = 0.23) was also observed (Table 3). There was, however, no significant association found in GTA/GTA, GCC/ACC, GCC/ATA and ACC/ATA diplotypes in controls and TB. We did not find any GCC/GCC, GTA/ACC and ATA/ATA haplotypes inherited in our local Pakistani population (Table 3). All the SNPs studied followed the Hardy Weinberg equilibrium.

DISCUSSION

Single nucleotide polymorphism in number of candidate genes have been linked to relatively increased risk of *M. tuberculosis* infection (Hill, 1998). IL-10, a T regulatory cytokine seems to have a pivotal role during the chronic/latent stages of TB. With its increased production, it plays a potentially central role in promoting reactivation

Table 3. Relationship between IL10 haplotypes, diplotypes and TB.

IL-10 locus	Control (n = 99)	Frequency (%)	Patient (n = 82)	Frequency (%)	P-value (Yates corrected)	OR (CI95%)
Haplotypes						
GCC (high)	83	41.92	72	43.9	0.704	
GTA (high)	18	9.09	19	11.6	0.435	
ACC (intermediate)	4	2.02	5	3.1	0.532	
ATA (low)	93	46.97	68	41.4	0.293	
Diplotypes						
GCC/GTA (high)	4	2.02	10	12.2	0.03	6.7 (1.1 -16)
GTA/GTA (high)	1	1.01	3	3.66	0.227	
GCC/ACC (intermediate)	3	3.03	1	1.22	0.409	
GCC/ATA (intermediate)	74	75.76	61	74.39	0.830	
GTA/ATA (intermediate)	14	14.14	3	3.66	0.03	0.23 (0.06-0.83)
ACC/ATA (low)	4	4.04	4	4.88	0.785	

of TB (Turner et al., 2002). Modulation of T-cell responses by IL-10 influences the host susceptibility to TB (Fortsch et al., 2000). So determining the IL-10 polymorphism that is involved in its differential production may be beneficial for predicting the probability of disease occurrence. Studies so far done for the association of IL-10 polymorphism with TB shows ambiguous results. In the polymorphism association study, we investigated the significance of the relationship between IL-10 gene promoter polymorphism at -1082 G/A, -819 C/T and 592 C/A and susceptibility to TB in local Pakistani population. When studied independently, there was no significant association between all three investigated IL-10 polymorphic alleles (-1082 A/G, -819 C/T, and -592 A/C) and TB as in the previous report from Pakistan by Ansari et al. (2009). In other recent reports by Mosaad et al. (2010) and Akgunes et al. (2011), IL-10 -1082 G/A alleles showed no association with TB (Mosaad et al., 2010; Akgunes et al., 2011), while in Tunisian population, it showed significant association with TB susceptibility (Ben-Selma et al., 2011). However, analysis of genotypes from this study showed that *-1082 GG* genotype (homozygous *G* allele) was susceptible to TB (p = 0.002). Previous investigations showed that *IL-10 -1082 G/A* polymorphism was associated with TB in the Hong Kong Chinese (Tso et al., 2005), Colombian (Henao et al., 2006), Spanish (López-Maderuelo et al., 2003), Turkish (Akgunes et al., 2011; Oral et al., 2006; Ates et al., 2008), Cambodian (Delgado et al., 2002), but not in the Tunisian (Ben-Selma et al., 2011), Iranian (Amirzargar et al., 2004), West African (Thye et al., 2009), Macedonian (Trajkov et al., 2009), Gambian (Bellamy et al., 1998), Spanish (López-Maderuelo et al., 2003) and a separate study from Korean population (Shin et al., 2005). Another independent studies from Korean (Shin et al., 2005), Spanish (López-Maderuelo et al., 2003) and Turkish (Akgunes et al., 2011) populations showed that *IL-10 -592 A/C* promoter polymorphism was found to have significant association with TB susceptibility.

Our results show that IL-10 haplotypes were not significantly different between patients and controls. In contrast to this report, it has been previously reported that GCC (Ates et al., 2008; Ben-Selma et al., 2011), ACC (Ben-Selma et al., 2011; Ates et al., 2008; Trajkov et al., 2009) and ATC (Trajkov et al., 2009) are distributed differently between healthy controls and TB cases in different studies from different ethnic areas. However diplotypes analysis showed that individuals with *IL-10/GCC: GTA* diplotype was genetically predispose of developing TB (p = 0.006). *IL-10/ GTA: ATA* diplotype was more common in the controls then patients (p = 0.016) and the individuals with this genetic makeup have less tendency of developing TB. Previous data showed that *IL-10/GCC: GCC* or haplotype having G allele correlates with higher level of IL-10 after stimulation, which leads to the suppression of IFN-g, and hence favors development of TB (Turner et al., 1997; Tso et al., 2005). In our study, *IL-10/ GTA: ATA* (intermediate IL-10 production) was a preventive genotype from TB but Macedonians GCC/ATC (intermediate IL-10 production) have association with TB susceptibility (Trajkov et al., 2009).

Although the mechanism associated with change in gene expression due to polymorphism are still poorly understood, it is a well known fact that TB is partly under polygenic control. The genetic components, which are involved in host defense or susceptibility to TB en-compass not only alleles of different genes and even on different chromosomes, but also genes environment interaction as well. Pattern of many of these

polymorphism are in ethnic specific manner. From Pakistan there are no previous reports on this issue, hence the identification of these kinds of interactions is of great importance in clarifying the genetic role in TB pathogenesis and may open the new ways of treatment. Our results demonstrate that the polymorphisms of IL-10 gene may be valuable markers to predict the risk for the development of TB. Since the sample size in this study was small, further studies with larger sample sizes are therefore necessary to clearly elucidate the discrepancies.

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