Asian Biomedicine Vol. 6 No. 3 June 2012; 337 - 342
DOI: 10.5372/1905-7415.0603.064

Original article

Association of interleukin-10 polymorphism and malarial susceptibility in Pakistani population

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Background: Malaria is a leading cause of morbidity and mortality worldwide resulting in approximately 350 to 500 million clinical cases and up to two million deaths. In Pakistan, 1.5 million cases of malaria are reported annually. The genetic factors of both host and pathogen are related to the severity of the disease. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that can play a key role in *plasmodium falciparum* infection. Variations in IL-10 production are genetically related to polymorphisms within the IL-10 promoter region.
Objective: We investigated the association of IL-10 gene promoter -1082 G/A, -819 C/T, and -592 C/A polymorphism with malarial susceptibility in Pakistani individuals.
Methods: Ninety malarial patients and 99 healthy control subjects were enrolled. IL-10 genotyping was performed by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR).
Results: There was no significant difference observed in inheritance pattern of studied single nucleotide polymorphisms. All the alleles, genotypes, and haplotypes had almost similar frequencies among diseased and healthy control groups. IL-10 -1,082 homozygous G was comparatively higher in healthy subjects but difference was not statistically significant.
Conclusion: We have found no significant association between IL-10 promoter polymorphism and *plasmodium falciparum* infection in Pakistan. Our result from Pakistani population confirm previous association in studies from Thailand, Gambia, Republic of Mali, Tanzania, and contradict one from Kenyan population.

Keywords: IL-10, malaria, polymorphism, susceptibility

Malaria remains a leading cause of morbidity and mortality worldwide, resulting in approximately 350 to 500 million clinical cases and up to two million deaths [1]. *Plasmodium falciparum* (*P. falciparum*) malaria is responsible for over one million annual deaths; mostly among children in sub-Saharan Africa [2]. There are 1.5 million estimated malaria deaths in Pakistan. In 2005, falciparum malaria represented 33% of reported confirmed malaria cases; they decreased to 24% in 2008 [3]. Most of the individuals with malaria experienced persistent subclinical infection, but only a few developed severe case. The genetic background of both host and pathogen are related to the clinical variations and other factors. Significant evidence shows that the immune response genes influence the clinical outcome of malaria [4].

Despite the fact that pediatric severe malarial anemia (SMA) results from both inefficient red blood cell (RBC) production and enhanced RBC destruction, the pathophysiology is largely unknown [5]. IL-10 is a type 2-cytokine anti-inflammatory produced primarily by monocytes and lymphocytes. It has a pleotropic effects on immunological regulation [6]. Dysregulation of inflammatory mediators such as IL-10 appear to play an important role in determining disease outcomes [5]. IL-10 down regulates the expression of type-1 pro inflammatory cytokines [6] and disturbs the type-1 and type-2 cytokine balance. The dysregulation between type-1 and type-2 cytokine balance is associated with the pathogenesis of severe and cerebral malaria (CM). It is an established fact that single nucleotide polymorphisms (SNPs) in the promoter or coding region of different cytokine genes

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alters their transcriptional activation and results in differential cytokine production. Inter-individual variations in IL-10 production are genetically contributed by polymorphisms within the IL-10 promoter region [7]. IL-10 possesses a highly polymorphic promoter with variations at -1082 G/A (rs1800870), -819 C/T (rs1800871), and -592 C/A (rs1800872) that have been extensively studied and implicated in altering the rates of IL-10 gene transcription. The -1082G, -819C, and -592C (GCC) haplotype has been associated with elevated levels of IL-10 production [8] while ACC and ATA haplotypes exhibit intermediate and low IL-10 gene transcription respectively [9]. IL-10 polymorphisms have been associated with a range of inflammatory diseases including psoriasis [10], systemic lupus erythematosus [11], graft-versus-host disease [12] and rheumatoid arthritis [13]. In our current study, we have investigated the above mentioned three SNPs of IL-10 promoter that are involved in differential IL-10 production in a Pakistani population to determine if they play any role in the incidence and severity of *P. falciparum* infection in Pakistan.

Material and methods

Inclusion and exclusion criteria

Malarial patients with no evidence of other illnesses were selected. Malarial parasites were detected microscopically in blood smears and patients were excluded if they had developed other illnesses within three days of admission or if there was any other present infection. A control group of healthy individuals was selected from members of the community without malaria or any other febrile illness.

Patients and healthy individuals

To determine the association between IL-10 promoter polymorphisms and malaria, blood samples were collected from 90 malarial patients. These samples were stored in EDTA tubes. Ninety-nine age-matched healthy controls were enrolled in this study, none of whom had any history of *P. falciparum* infection in the past. Patients and controls were of the same ethnicity and from the same geographical area. The Ethical Committee of NUST Center of Virology and Immunology (NCVI), Islamabad, approved the study and written consent was obtained from each participant.

DNA extraction

Genomic DNA from venous blood of subjects was extracted using genomic DNA extraction kit according to manufacturer protocol (Gentra, USA). DNA quantification was done by Bio Photometer (Eppendorf, USA). DNA was stored at -20°C.

Genetic analysis

For IL-10 haplotypes determination, the Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) method was used as described by Perry et al [14]. PCR amplification was performed in 20 µl reaction volume containing 40 ng genomic DNA, 1.5 mM dNTP, 25 mM $MgCl_2$, 1 ul of 10 pmol each primer, and 0.7 units of Taq polymerase in 1X reaction buffer with cycling conditions 94°C for 4 minutes, followed by 30 cycles at 94°C for 45 seconds, 58°C for 40 seconds, 72°C for one minute, and finally 10 minutes extension at 72°C. To ensure PCR success, an internal control region was amplified from the human growth hormone. The amplified products were analyzed on 2% agarose gel.

Statistical analysis

Statistical analysis was performed using the Study Result Software Version 1.0.4 (CreoStat HB Frolunda, Sweden). The association of IL-10 promoter polymorphism in malaria patients and healthy individuals was compared using the χ^2 or Fischer's exact test. To elucidate whether there was an association between the age and gender of patients and healthy controls, analysis was done using the T-test and chi-square test of the online Graphpade software.

Results

Characteristics of patients

The characteristics of patients and healthy controls with respect to age and sex are mentioned in **Tables 1** and **2**.

A chi-square test was performed to test the null hypothesis regarding whether there was an association between gender and the number of subjects in the control and patient groups. No statistically significant association was found, c^2 (1, n = 188) = 0.559, $p = 0.4545$. Similarly, a chi-square test was performed to test whether there was any association between the gender and ages of subjects in the control group and in the patient group. Again no statistically significant

association was found, c^2 (1, n = 188) = 0.299, p = 0.5848.

Genetic analysis

The results of the IL-10 promoter polymorphism genotypic analysis did not show any significant association with disease occurrence. There was no significant difference found in patterns of inheritance of all IL-10 genotypes studied i.e. -1082 GG, GA, AA, -819/-592 CC/CC, CT/CA, and TT/AA (-819 and -592 are in linkage disequilibrium) shown in **Table 4**. The frequency of IL-10 -1,082 GG genotype that is associated with high production was comparatively higher in healthy control subjects than in the diseased group but this was not statistically significant.

The frequencies of -1082 G/A and -819 C/T alleles (-819 and -592 are in linkage disequilibrium) did not differ significantly between malarial patients and controls (**Table 3**). In our recent report [7], we identified a high IL-10 producing genotype, GTA in the Pakistani population, which was previously only reported in a Chinese Population [15]. Our results suggested a lack of association of IL-10, GCC, GTA, ACC, and ATA haplotypes with malarial susceptibility (**Table 4**). All the studied SNPs followed the Hardy Weinberg Equilibrium.

Table 1. Total number of patients and controls with mean ages.

Characteristic	Patients	Mean age	Control	Mean age	P value	±SD
Total number of subjects	90	22	99	26	0.29	0.8627
Adults >22 year	61		59			
Children <12 year	29		40			

Table 2. Patients and healthy individuals enlisted, sex, and mean ages.

Characteristic (n)	Patients	Mean age	Control	Mean age
Males	76	24	79	26
Females	14	20	20	27
Total	90	22	99	26

Table 3. Relationship between IL10 polymorphic genes, alleles, and severe malaria.

IL-10 locus	Control (%), n = 99	Patients (%), n = 90	P value
-1082 G/A (Genotype frequency)			
G/G (high)	11 (11.1)	8 (8.8)	0.64
G/A (Intermediate)	82 (82.9)	74 (82.2)	0.99
A/A (Low)	6 (6.1)	8 (8.8)	0.58
-1082 G/A (Allele frequency)			
G (High)	97 (49)	95 (52.7)	0.47
A (Low)	101 (51)	85 (47.3)	
819C/T (592C/A) (Genotype frequency)			
T/T (A/A)	15 (15.15)	11 (12.2)	0.67
C/T (C/A)	81 (81.82)	74 (82.2)	0.99
C/C (C/C)	3 (3.03)	5 (5.6)	0.48
819C/T (592C/A) (Allele frequency)			
T (A)	113 (57.1)	96 (53.3)	0.47
C (C)	85 (42.9)	84 (46.7)	

Table 4. Relationship between IL10 haplotypes and malaria.

IL-10 locus	Control (%), n = 99	Patients (%), n = 93	P value
Haplotypes			
GCC (high)	90 (45.5)	80 (43)	0.68
GTA (high)	17 (8.6)	13 (7)	0.57
ACC (intermediate)	15 (7.6)	14 (7.5)	0.99
ATA (low)	76 (38.4)	79 (42.4)	0.46

Discussion

Malaria is one of the strongest known factors for evolutionary selection in recent history of humans. This is demonstrated by the numerous signatures of selective pressure in the genome including some of the most common polymorphisms [16]. There is growing evidence of ethnic differences in susceptibility to malaria and of the diverse genetic adaptations to malaria that have arisen in different populations [4]. Cytokine production varies inter individually and this disparity is associated with certain SNPs in the coding as well as the regulatory regions of cytokine genes [17]. Cytokine IL-10 is produced by monocytes and lymphocytes [18]. There are several lines of evidence indicative of a IL-10 protective role against severe malaria and that IL-10 production is genetically controlled [19]. This suggested that being a key player in the differential expression, IL-10 polymorphism may be a crucial marker for predicting the probability of disease occurrence and disease expression. In this study, we have investigated the significance of IL-10 gene promoter polymorphism at -1082 G/A (rs1800870), -819 C/T (rs1800871), and -592 C/A (rs1800872) (-592 C/A is in linkage disequilibrium with -819 C/T) and susceptibility of malaria in Pakistani population.

Few studies evaluated the importance of IL-10 polymorphism in relevance to malarial susceptibility. We have found just five reports in the literature [5, 19, 20, 21, 23]. A large report from Thailand included 203 mild malaria, 164 non-cerebral severe malaria, and 109 cerebral malaria patients. The results of the investigation did not show any significant association of IL-10 promoter polymorphism -1082 G/A inheritance with disease susceptibility [20]. Wilson et al., (2005) studied a large number of Gambian children. They found no significant association of IL-10 individual alleles and genotypes with disease susceptibility [19]. In a study from the Republic of Mali, Western Africa, 2 related ethnic tribes differing in susceptibility to malaria were tested for IL-10 -1,082 G/A polymorphism but no association was found [21]. A Kenyan population, investigated by Ouma et al., also showed similar results with no significant association of IL-10 alleles/genotypes at -1082 G/A, -819 C/T, and -592 C/A with the expression and occurrence of the disease [5]. Our results are in accordance with these previous reports. There is no significantly different pattern of inheritance of IL-10 alleles and genotypes in patients and healthy controls. In Kenya, -1,082G/ -819C/-592C (GCC) haplotype was associated with protection against severe malaria anemia and increased IL-10 production. Although none of the other haplotypes were significantly associated with susceptibility to SMA, individuals having the -1,082A/-819T/-592A (ATA) haplotype had an increased risk of severe malaria and reduced circulating IL-10 levels [5]. Previous mice studies demonstrated that IL-10-deficient mice infected with *P. chabaudi chabaudi* leads to severe disease higher mortality than in heterozygotes or normal mice [22]. In a seven-year study of a Tanzanian population, the family based method of association was used to analyze genetic components for malarial susceptibility. In this study Carpenter et al., investigated the host genetic control of malaria susceptibility using parasite levels and clinical episodes. They evaluated IL-10, -1082G/A, -592 C/A, and IL-10 haplotypes but did not find any association of IL-10 allelic, genotypic, haplotypic variations with the malaria parasite density and clinical expression [23]. In our investigation, four haplotypes were found (GCC, GTA, ACC, ATA) in this Pakistani population, but none of these haplotypes showed association with disease expression. IL-10 is an important anti-inflammatory cytokine and murine malarial experiments showed that disruption of IL-10 gene results in increased disease severity including cerebral pathology [24]. Interestingly, a recent report by Wilson et al., [25] found higher IL-10 circulating levels associated with asymptomatic

malaria in pregnant women. While in Ghanaian children with *P. falciparum* malaria, low IL-10 circulating levels were found to be associated with severe anemia in malaria patients [26]. Several authors have used IL-10 to TNF circulating ratios to represent the balance of anti- and pro-inflammatory expression in malaria. The previous investigations suggested that appropriate regulation of IL-10 is essential for controlling *P. falciparum* infection and that dysregulation in IL-10 production may enhance pathogenesis. It is also a fact that IL-10 promoter polymorphism is linked with differential production and expression of IL-10 [27]. The results of our and previous studies raises the possibility that susceptibility to malaria is determined by IL-10 polymorphisms that were not typed in these investigations or that the effect depends on a specific combination of SNPs and microsatellite repeats with several allelic forms, being present. Our preliminary study is based on a small sample size and we believe that there is need for further investigation to assess the association of these polymorphisms along with other IL-10 polymorphisms and microsatellite repeats in malarial patients.

Conclusion

This is the first report from Pakistan of IL-10 polymorphism association with *P. falciparum* susceptibility. We found no association between IL-10 polymorphism and *P. falciparum* infection. This is in accordance with Ohashi et al. [20], Wilson et al. [19], Vafa et al. [21], and Carpenter et al [23]. However, it contradicts Ouma et al. [5] in which GCC and ATA haplotypes were associated with protection and increased risk against severe malaria. This is a preliminary report and further studies are needed to illuminate this important issue.

Acknowledgements

There is no conflict of interest to report in this study. Qadri I and Muhammad Sohail Afzal designed the study. Shakir Ullah, Zia Ur Rehman Farooqi, Talha Shafi, and Tahir Ahmed participated in data extraction and analysis. Muhammad Sohail Afzal and Sadia Anjum wrote the manuscript. All the work is being done under the supervision of Qadri I.

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