ABSTRACT

Toll like Receptors (TLRs), an integral part of the innate immune system, was first described in drosophila and later in mammals, playing an important role in recognizing the invading pathogen including malaria parasite *Plasmodium*. This recognition of the pathogen by the TLRs is a crucial step in infectious diseases which further orchestrates the downstream events, as the pattern of cytokines being expressed influences the type of adaptive immune response. This mechanism of counteraction is very complex, and various factors including the genetic makeup of the exposed population can be of utmost importance for its predisposition to malaria. Thus polymorphic studies on immune genes have gained importance recently and the polymorphisms at position 753 and 677 in TLR2, at positions 299 and 399 in TLR4, at positions -1237 and -1486 in TLR9 and at residue position 180 in TIRAP have been associated with various diseases including malaria which prompted us to study their role in malaria. In this study, malaria patients from seven different eco-epidemiological regions of India were genotyped for TLR2, TLR4, TLR9 and TIRAP polymorphisms using DNA sequencing methods. No variation was observed at residue positions 677 and 753 in TLR2 whereas residue positions 299 and 399 in TLR4, nucleotide positions -1486 and -1237 in TLR9 and residue position 180 in TIRAP were highly polymorphic. In TLR4 the mutant allele G for residue position 299, had the lowest frequency in Nicobar (0.0454) and highest in Vizianagaram (0.2). On the other hand, the lowest frequency of the mutant allele T for residue position 399 was observed in East Singhbhum (0.060) and highest in North Goa (0.190). Four haplotypes, AC (Asp/Thr), AT (Asp/Ile), GC (Gly/Thr) and GT (Gly/Ile) were observed in all populations except in Kolkata, Dakshin Kannada and Nicobar district where only three haplotypes AC (Asp/Thr), AT (Asp/Ile) and GT (Gly/Ile) were observed and GC haplotype was completely absent. The GC haplotype in TLR4 (Asp299Gly/Thr399Thr) was observed at the highest frequency in
populations of East Singhbhum, Vizianagaram and North Goa. Upon comparison between malaria patients and healthy subjects no significant difference was observed (P > 0.05) at both residue positions Asp299Gly and Thr399Ile of TLR 4 except in samples from Dakshin Kannada at Thr399Ile where it was found to be statistically significant (p =0.003). On the other hand, though the distribution of the two polymorphisms in TLR9 was heterogeneous amongst the malaria patients and the healthy subjects no significant association was observed between malaria and TLR9 promoter polymorphism. However, upon comparison between malaria patients and healthy subjects the population of East Singhbhum showed a highly statistically significant (p=0.0001) difference between malaria patients and healthy subjects at position -1237. In TLR9, the C allele for locus -1237 was absent in Nicobar district and its highest frequency was observed in Kolkata (0.217). In contrast, the C allele for locus -1486 was observed in all populations at similar frequencies with East Singhbhum having the lowest at 0.167 and both North Goa and Dakshin Kannada having the highest at 0.382. In TLR9, four haplotypes (-1486/-1237) were observed in malaria patients TT, CT, TC and CC except in the population of Nicobar district where only two haplotypes were observed TT and CT, suggesting the complete absence of polymorphism at -1237 nucleotide position. In TIRAP, the mutant T allele for residue 180, had lowest frequency in Nicobar district (0.045) and highest in Kolkata (0.207) followed by North Goa (0.203). In case of TIRAP the heterozygous genotype (Ser180Leu) was observed highest in Kolkata and Sundergarh districts where the mutant genotype was completely absent. Thus, maybe in TIRAP the mutant genotype is under negative selection in the Sundergarh and Kolkata populations. All polymorphisms were in Hardy Weinberg equilibrium.

In addition, functional studies were carried out to see the effect of these polymorphisms on the immune response upon *Plasmodium falciparum*
infection. It was observed that in TLR4 the wild type genotype (Asp299/Thr399) gave a pronounced proinflammatory response than the double heterozygous (Asp299Gly/Thr399Ile) genotype upon Plasmodium falciparum infection as well as against conventional ligand LPS. Moreover, in TLR9 the wild type genotype (T-1486/T-1237) and the double heterozygous genotype (T-1486C/T-1237C) expressed high levels of proinflammatory cytokines against Plasmodium falciparum infection as well as against commercially available ligand ODN2006 as compared to the single heterozygous genotypes (T-1486/T-1237C and T-1486C/T-1237) for either position. In TIRAP, all the three genotypes including wild type (Ser180), heterozygous genotype (Ser180Leu) and mutant genotype (180Leu) expressed moderate levels of cytokines except in IL-12 by the heterozygous genotype upon Plasmodium falciparum infection.

Thus, combined with the genotypic and functional results it can be suggested that TLR2 polymorphism was absent in the Indian population and an overall heterogeneous pattern of TLR4, TLR9 and TIRAP polymorphism can be attributed to genetic drift. The presence of mutation at 399 in TLR4 may have a deleterious effect on the proinflammatory phenotype of the 299 SNP when present alone and it can be inferred that GC haplotype confers protection from mortality and Plasmodium falciparum predominance may be one of the factors responsible for its ongoing selection in malaria endemic regions in India. Conversely, presence of mutations at both positions -1237 and -1486 in TLR9 may have an additive effect on the proinflammatory phenotype of the mutant allele C at -1237 even though at the genetic level no association with malaria was observed. Finally in TIRAP, at the genetic level the mutant genotype may be undergoing negative selection pressure in the malaria endemic populations of Kolkata and Sundergarh and at the functional level it has been observed that Ser180Leu heterozygote does produce an early proinflammatory response thereby protecting the individual from disease severity.