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Evolutionary interplay of single nucleotide polymorphisms at the promoter region of TNF-α gene in different clinical outcomes of malaria in India

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ABSTRACT

Host genetic factors are frequently ascribed to differential malaria outcomes as a by-product of evolutionary adaptation. To this respect, Tumor Necrosis factor alpha (TNF-α), a human cytokine, is known to be associated with malaria through its differential regulation in diverse malaria manifestations. Since diversity in differential malaria outcome is uncommon in every endemic settings, possible association of TNF-α and malaria is not commonly established. In order to check for association between the occurrence of Single Nucleotide Polymorphisms (SNPs) in the TNF-α gene with different malaria manifestations, we have sequenced a 4011 bp region constituting the promoter and the whole gene of human TNF-α in 61 patients [(16 cerebral plus severe (SCM), 21 severe (SM) and 24 uncomplicated (UM)] samples in a highly malaria endemic state (Odisha) of India. Multiple sequence alignment revealed presence of six SNPs (−1031 T > C, −863C > A, −857C > T, −308G > A, −806C > T, +787C > A), out of which the -806C > T and +787C > A are novel in malaria patients in general and the +787C > A was detected for the first time in humans. Although alleles due to six different SNPs segregate differentially in the three groups of malaria (SCM, SM and UM) in the present study, interestingly, for the −1031 T > C position, the frequency of individuals possessing the homozygous rare allele was higher in the SCM group with a higher number of heterozygotes in the UM group. The Tajima's D values considering all the SNPs in a defined group were positive and statistically insignificant conforming no evolutionary constraint. However, statistically significant deviation from expectation under Hardy-Weinberg equilibrium for -1031 T > C SNP in the UM group points towards the probable role of natural selection providing some kind of protection to malaria in Odisha, India.

1. Introduction

Malaria is one of the major parasitic diseases causing morbidity and mortality in tropical and subtropical regions around the globe. WHO estimates around 3.2 billion people worldwide live under the risk of malaria. In 2016, 216 million malaria cases with 445,000 deaths were estimated globally wherein India contributed to ~8% of the malaria cases and ~47% of deaths worldwide (WHO, 2017). According to WHO about 75% of malaria cases in South-east Asia are from India and about 95% of the country's population resides in malaria endemic areas. An important observation by the Indian National Vector Borne Disease Control Program (NVBDCP) that around 80% of cases are reported from areas where only about 20% of the population live. The major cause of suffering in malaria is due to *Plasmodium falciparum* and the spectrum of disease manifestations can range from uncomplicated malaria to fatal outcomes, which includes cerebral malaria and other severe complication. Uncomplicated malaria is defined febrile illness without any of vital organ failure whereas complicated forms involve the central nervous system (cerebral malaria), pulmonary system (respiratory distress), renal system (acute kidney injury) and hematopoietic system (severe anemia) (Wah et al., 2016). Most of the cases of only cerebral malaria are treatable whereas the cases of cerebral malaria along with multi-organ failure are mostly fatal (Mishra et al., 2007).

Malaria due to *P. falciparum* is caused by the red cell parasitization and destruction by asexual parasites along with the host's response to this process. Cytoadherence of *P. falciparum* matured blood stage

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parasite to vascular endothelium is mediated by strain specific adhesive proteins called *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1) which results in the sequestration of parasitized RBC's to the capillaries (Udomsangpetch et al., 2002). This adherence further interferes with the microcirculation and metabolism of the vital organs like the brain, kidneys, liver and lungs which is central to the pathogenesis of severe malaria (White et al., 2013). The current definition of severe malaria does not necessarily reflect a unique mechanism of the disease and is only based on clinical outcome (WHO, 2000). The diversity of clinical manifestations of severe malaria reflects a diverse patho-physiological mechanism which needs specific therapy beyond anti-malarial drugs. In order to provide new insights into this, a network-based approach was used to identify biologically meaningful phenotypes that differ from the current clinical definition of severe malaria in 2915 Gambian children from a hospital setting and identified heart failure as one of the mechanisms in the pathogenesis of severe malaria (Cominetti et al., 2018).

Host response to *P. falciparum* infection determines the course of the disease to a certain extent. Several studies have provided evidences on an imbalance between the pro and anti- inflammatory cytokines leading to severe and cerebral malaria (Kurtzhals et al., 1999; Othoro et al., 1999; Ouma et al., 2008; Achidi et al., 2013). Tumor necrosis factor α (TNF-α) affects the outcome of *P. falciparum* malaria in more than one ways. Firstly, it acts as a pro-inflammatory cytokine secreted by activated macrophages in response to *P. falciparum* infection. Secondly, TNF-α forms an important component of the immune response to *P. falciparum* malaria due to its pyrogenic characteristics and also in activating the cascade of pro-inflammatory cytokines (Gichohi-Wainaina et al., 2015). Furthermore, it promotes fever that can inhibit parasite growth and at the same time also contributes to the over-expression of adhesion molecules and pro-inflammatory cytokines (Cabantous et al., 2006). Most importantly, when at high concentrations, TNF-α is believed to provoke severe clinical features like cerebral malaria; while at lower concentrations, TNF-α becomes protective against severe malaria (Awasthi et al., 2008). There has also been a report on the circulating high levels of the cytokine being directly proportional to disease severity (Clark et al., 2006) pointing towards the dual nature of TNF-α in relation to malarial pathogenesis.

The transcription of the TNF-α gene is tightly regulated and the polymorphisms present in the 5′ region have been associated with a number of infectious and inflammatory diseases (Heel et al., 2002; Asghar et al., 2004). Some commonly identified variants *viz.*, 1031 T > C, −863C > A, −857C > T, −575G > A, −376G > A, −308G > A, −244G > A and −238G > A present within the TNF-α promoter region are thought to modulate production of the cytokine. This kind of regulation may in turn affect the course of a certain disease (D'Alfonso and Richiardi, 1994, Higuchi et al., 1998, Uglialoro et al., 1998, Brinkman et al., 1994, Wilson et al., 1992, Brinkman et al., 1995, Hamann et al., 1995). These widely reported variants of the TNF-α gene are thought to control the binding of transcription factors, which in turn, affect the transcription and hence protein production. The rare allele at −308 position, *i.e.* −308AA (present upstream of the transcription initiation site) has been associated with enhanced production of the cytokine in both *in vitro* and *in vivo* conditions (Mira et al., 1999). Several studies have proved an association of these factors with the disease and several others have shown the other way round. For example the TNF-α −308AA genotype has been associated with cerebral malaria in Gambian children (McGuire et al., 1999), while the same genotype was not associated similarly in a Thai population (Hananantachai et al., 2001). Very similarly, while the −1031 CC genotype was found to be associated with severe malaria along with high levels of the cytokine in the serum of patients in India (Sinha et al., 2008), no such association could be detected in Kenyan and Malawian populations (Clark et al., 2009). Also, an allele formed due to the Single Nucleotide Polymorphisms (SNPs) −1031C, −863C, and −857C was found to be significantly related to cerebral malaria in a Thai population (Hananantachai et al., 2007). Although a number of studies have been conducted to signify the role of SNPs present in the promoter region of the TNF-α gene in malaria through genotyping, no study to detect/associate other SNPs that might be present in the TNF-α gene has thus far been conducted in malaria patient samples, barring the lone study reporting on the association of two SNPs in the first intron with mild malaria (Flori et al., 2005).

The state of Odisha is situated in the eastern region of India constitutes about 4% of the land area and 3% of the country's population, but report about 26.9% of the cases and 17.6% deaths due to malaria in the country (NVBDCP, 2013). Malaria is definitely one of the most challenging health issues faced by Odisha with some estimates of over 50,000 deaths per year (Dhingra et al., 2010).The clinical manifestations of the disease are dependent on several factors like overall health status of the patients, parasite diversity *etc.*; although, 25% of the risk associated with malaria is influenced by host genetic factors (Mackinon et al., 2005). Considering the fact that host genetic factors regulate varied clinical manifestations and also evolution of the disease (Mangano and Modiano, 2014), the fact that why only a small proportion (1–3%) of *P. falciparum* infections progress to severe or fatal episodes while others remain asymptomatic or develop an uncomplicated illness (Malaria, 2008) is a challenging question. Also, since Odisha is highly endemic to malaria and that too with differential outcomes, the consequence of infection and its association with a particular SNP might also vary if only genetic susceptibility of individuals is considered (Laishram et al., 2012). In order to test this hypothesis, we planned the present study in 61 malaria patients with three different clinical manifestations [(severe (SM), severe and cerebral (SCM) and uncomplicated (UM)] in a hospital setting of Odisha, India and sequenced the whole TNF-α gene including its promoter region. We performed evolutionary analyses of the generated DNA sequences of the TNF-α gene, independently in three different groups of malaria manifestations. The present analyses provide baseline information on the association of TNF-α genotype with malaria phenotype and the possible role of natural selection providing some kind of protection to malaria in Odisha, India.

2. Materials and methods

2.1. Study site and sample collection

The study was carried out involving patients from Ispat General Hospital (IGH), Rourkela, Odisha, India (latitude: 22.12°N; longitude: 84.53°E). Rourkela is a hyper endemic region for malaria and is located in the western part of Odisha with *P. falciparum* being the major disease-causing pathogen. In order to decipher any role of nucleotide substitution in the TNF-α gene with differential clinical manifestations of malaria, approximately 2 ml of venous blood was collected from 61 individuals before administration of any specific antimalarial treatment. Clearance from the ethical committee of National Institute of Malaria Research, New Delhi (where the laboratory work was performed) was obtained prior to the study. Also, written informed consents from each individual patient donating blood samples at the IGH, Rourkela were obtained. The presence of *P. falciparum* infection was initially diagnosed by microscopy and Rapid Diagnostic Test (RDT) kits, and was later confirmed by PCR-based amplification of the 205 bp region of the 18srRNA gene of *P. falciparum* using standard method (Gupta et al., 2010). According to the criteria given by WHO based on manifestations of malaria (WHO, 2014), patients were divided into three groups, (i) severe malaria with cerebral involvement(SCM; $n = 16$), (ii) severe malaria without cerebral involvement(SM; $n = 21$) and (iii) uncomplicated malaria (UM; $n = 24$). Severe malaria (SM) was classified as the presence of high parasitemia (> 100,000 parasites/ml or 10% parasite infected erythrocytes), hypoglycemia (glucose level < 22 nmol/l or 40 mg/dl), severe anemia (Hb level < 7 g/dl), acute kidney injury (serum creatinine > /= 3 mg/dl), malarial jaundice

Table 1
Details of primers used in amplification of TNF-α gene.

Primers			
Fragment	Forward (5′-3′)	Reverse (3′-5′)	Amplicon size
TNF 1	ACACGGAGGCATCTGCAC	CCCTCTACATGGCCCTGTCT	457
TNF 2	TGACCACAGCAATGGGTA	CTGTGTTGAGTCCTGAGGC	450
TNF 3	CCAGTGGGGTCTGTGAATT	CTCGGTTTCTTCTCCATCG	496
TNF 4	CAGCCTCCAGGGTCCTAC	GTCCTTTCCAGGGGAGAGA	474
TNF 5	GAGAAGCAACTACAGACCCC	CCATCTCTTGCCACATCTC	482
TNF 6	GATGGGTGAAAGATGTGCG	GTCTGGCCTGCGCTCTTA	464
TNF 7	GAAAGAGCTGTTGAATGCCTG	CAAGTTCCAAGACACATCCTCA	470
TNF 8	GAGTGACAAGCCTGTAGCC	TGAAGAGGACCTGGGAGTA	471
TNF 9	CTCCAGCAAACCCTCAAG	TGAGCCAGAAGAGGTTGAG	520
TNF 10	CTGGGCAGGTCTACTTTGG	GCTCCGTGTCTCAAGGAAG	447
TNF 11	GCAGGACTTGAGAAGACC	GAGAGAGGACTTAGGGAGC	558

(serum bilirubin > /= 3 mg/dl) and unrousable coma. Those patients with fever and without any other symptoms of severe malaria were grouped into the category of uncomplicated malaria (UM).

2.2. Selection of the TNF-α gene

A 4011 bp genetic region of the TNF-α gene spanning promoter and the entire gene was selected to determine if any association exists between the SNPs of this gene and malaria outcome. Eleven sets of primers (forward and reverse) were designed for PCR amplification and sequencing after downloading the DNA sequence of the gene and promoter region from the NCBI database (accession no. NG_007462.1). Novel primers were designed with the help of the Sigma Oligoanalyser (www.oligoevaluator.com) (Table 1).

2.3. DNA isolation, PCR amplification and Sequencing of the TNF-α gene

Genomic DNA was extracted from each of the blood samples using the Axyprep Blood Mini kit (Axygen) as per the manufacturer's instructions. PCR amplification was carried out in a final volume of 25 µl using 0.5 µM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 unit of Taq DNA Polymerase (Bangalore Genei) and 1× Taq DNA Polymerase buffer. Thermal cycling conditions included initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 1 min, then annealing at a temperature standardized for each primer pair and extension at 72 °C for 45 s. A final extension step at 72 °C for 7 min was also carried out. The PCR amplicons were then subjected to enzymatic purification using ExoNuclease and Shrimp Alkaline Phosphatase (Fermentas, USA). Following purification, samples were subjected to cycle sequencing using Big Dye Terminator kit (Applied Biosystems, USA). The conditions for cycle sequencing included; initial denaturation at 95 °C for 5 min, then 25 cycles of denaturation at 96 °C for 10s, annealing at 50 °C for 5 s, followed by extension at 60 °C for 4 min. Post cycle sequencing, the samples were sequenced on an ABI 3730xl DNA Analyzer (an in-house facility of the ICMR-National Institute of Malaria Research, New Delhi). The samples were sequenced in 2× coverage (both from forward and reverse directions). All the newly generated sequences have been submitted to the GenBank with accession numbers MH180323-MH180383.

2.4. DNA sequence editing and statistical analyses

Each DNA fragment sequenced on the DNA analyzer was obtained in the form of a chromatogram and then manually checked for the presence of peaks using the SeqMan module of the Lasergene (DNASTAR, USA) computer program. Observation of single peaks with minimum background noise in the chromatogram indicated the presence of homozygotes, whereas the occurrence of double peaks at any particular site indicated the presence of heterozygotes. In order to identify SNPs, the sequences were aligned using the ClustalW algorithm implemented in the MegAlign module of the DNASTAR computer program. The aligned sequences were then used to carry out various statistical analyses. Since the TNF-α gene is located on chromosome number 6 in humans; allele phase of each of the sequences generated was ascertained using the PHASE module of the DnaSP 5.10 computer program (Rozas et al., 2003). This module generated two sequences for each of the input sequences giving a total of 122 sequences for 61 samples. This was particularly useful for heterozygous samples wherein each sequence contained either of the nucleotide at a particular segregating site. In order to check if frequencies of alleles of all the SNPs occurred at random or whether there was any selection pressure on any of the groups, chi-square tests using genotype data of all the segregating sites from each of the three groups were performed. Since statistically significantly different expected values from the observed ones in a contingency table indicate deviation from the expectations of Hardy-Weinberg equilibrium, we have calculated the chi-square value using the genotype frequencies of each SNP in each of the three groups.

2.5. Determination of genetic association among SNPs using Linkage Disequilibrium tests

Since the association between different genetic polymorphisms is known to provide combined phenotypic effects, we wished to test if such association between SNPs located in the promoter region and in the gene of TNF-α with different malaria manifestations exists in our study. For this, Linkage Disequilibrium (LD) tests were performed separately for each group using the Haploview computer program (Barrett et al., 2005). The r^2value was determined for each of the SNP pairs, and the output was indicated by the colour of the blocks. The degree of association between two SNPs is indicated by the intensity of shade in the closed block. While an open block indicated no association between two SNPs, a closed block indicated a statistically significant association.

2.6. Determination of haplotypes, haplotype diversity and nucleotide diversity

The alleles of the different SNPs in a particular gene are organized into haplotypes. In order to pinpoint the association of any of the haplotypes of the TNF-α gene with different malaria manifestations, their frequencies were calculated in each of the three groups. The haplotype diversity was also determined for all groups. In order to check whether the TNF-α gene conforms to the neutral model of molecular evolution, two measures of nucleotide diversity (θ_W and π) were estimated for each of the three malaria manifestation groups. While θ_W (Watterson, 1975) takes into account the number of segregating sites, π is based on the pair wise differences between two sequences (Tajima, 1983). In order to know if the genetic fragment evolve under the

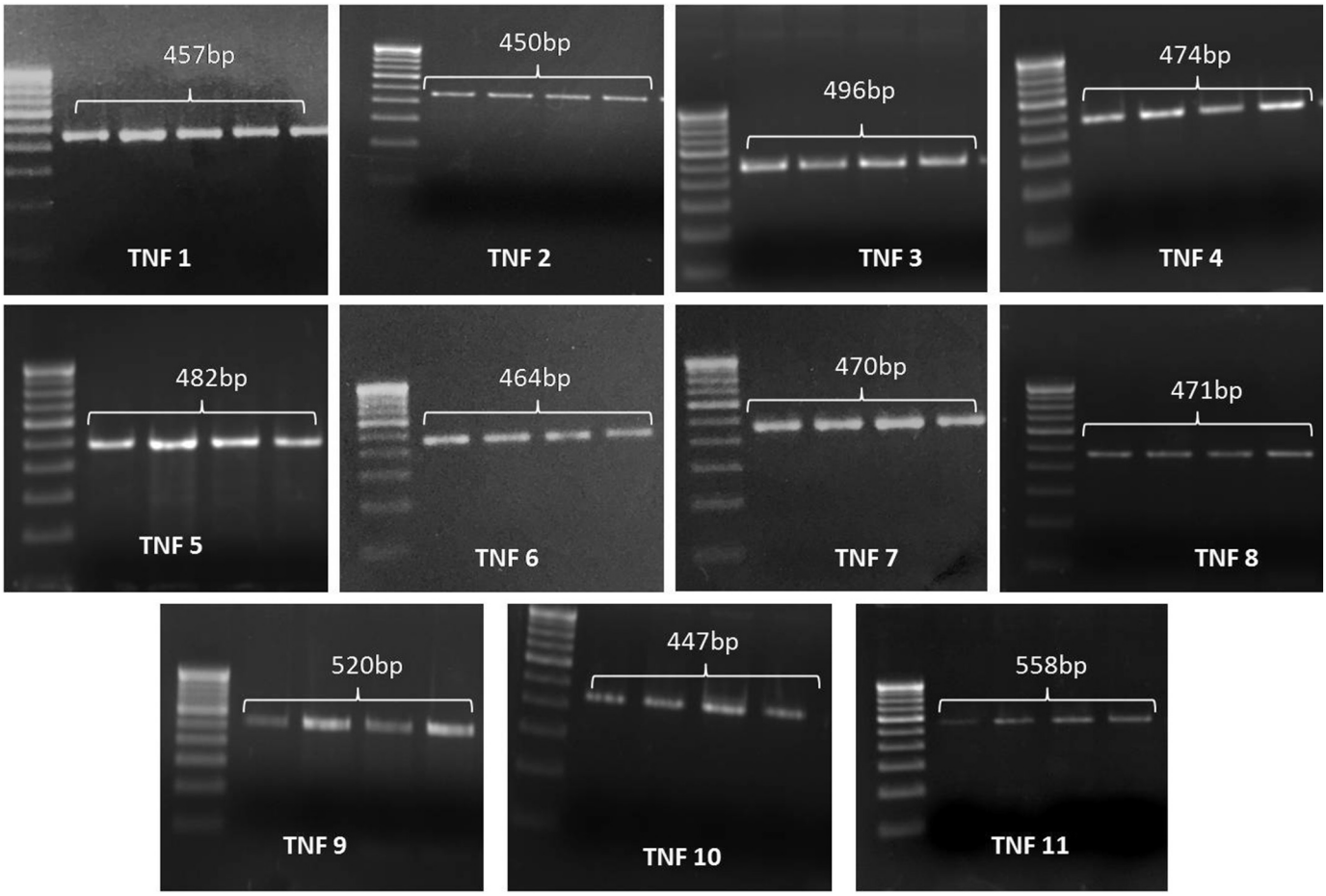

Fig. 1. Agarose gel image of the amplicons obtained after amplifying the fragments of the 4011 bp region of the TNF-α gene (including its promoter) using the primers designed in this study. The names of the amplicons along with their sizes have been mentioned in the image.

neutral model of molecular evolution, Tajima's D values (Tajima, 1989) were calculated. Tajima's D calculates the normalized differences between θ_W and π. While the negative value of Tajima's D indicates an excess of low frequency polymorphisms pointing towards directional selection, a positive value indicates presence of a low level of high frequency polymorphisms pointing towards balancing selection. The Tajima's D value was calculated using the DnaSP 5.10 computer program.

3. Results

PCR amplification of the TNF-α gene was successfully carried out for all of the 61 samples using the eleven set of novel primers designed in this study (Fig. 1). The same sets of primers were further used for sequencing of the 4011 bp region of the TNF-α gene. The sequences were edited and multiple sequence alignment was performed, which revealed the presence of five point mutations upstream of the transcription start site and one point mutation in the first intron of the TNF-α gene in both homozygous and heterozygous conditions. Four of the six mutations have already been reported by other studies and bear dbSNP identification numbers *viz.* −1031 T > C (rs1799964), −863C > A (rs1800630), −857C > T (rs1799724), −308 G > A (rs1800629). The other two SNPs, −806C > T and +787C > A were found to be novel, detected for the first time in the present study (Fig. 2).

Since the TNF-α gene is located in an autosome (chromosome no. 6), the haplotypic phase of each of the generated sequences was ascertained (as either homozygous or heterozygous) for a particular SNP (Table 2). As depicted in Table 2, the frequency of individuals possessing the rare allele (CC) at the −1031 position was much higher than the wild type (TT) in the SCM group. The occurrence of the same rare allele was significantly lower in the SM and UM categories. In the UM group the heterozygotes (TC) at −1031 position were found in a much higher frequency compared to the homozygous mutants (Table 2). The SM group contained an equal frequency of the homozygous wild type alleles and heterozygotes at the −1031 site. The frequency of the homozygous rare allele at the −863 position was much higher in the SCM group when compared to the other two groups (SM and UM; Table 2).The occurrence of heterozygous alleles at −863 position was the highest in the UM group (Table 2). A very high frequency of homozygous wild alleles along with a very low frequency of heterozygous ones at −857 position was observed in case of the SCM and UM groups. This was not the case of SM group where the alleles had similar frequencies (Table 2). In case of the novel mutation at −806 position, individuals from the SM category mostly had the homozygous wild allele *i.e.*CC. For the same mutation, the distribution of individuals possessing the CC and CT allele was almost equivalent in the SCM and UM groups (Table 2). In the SM category with respect to the −308 site, the frequency of homozygotes for the wild allele was much higher than the heterozygotes. However, similar phenomenon did not hold true on comparison with the other two groups (Table 2). The rare allele, −308AA was found only in the SM group at a very low frequency (Table 2). In the SCM group the frequency of homozygous wild alleles at +787 position was much higher than the heterozygous ones (Table 2). However, such a wide difference between the homozygous wild and heterozygous alleles could not be found in the other two categories with respect to the same position (Table 2). It was also observed that at sites −857, −806 and +787, the homozygous rare allele

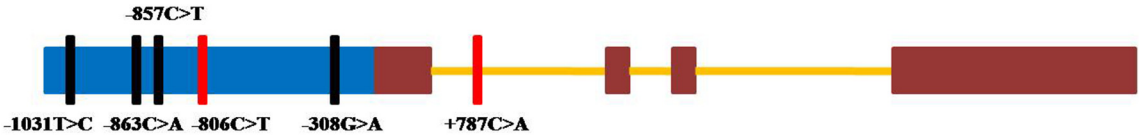

Fig. 2. Schematic representation of the structure of the 4011 bp fragment representing TNF-α gene and promoter showing the location of different SNPs. The blue region is the promoter, maroon regions are the four exons and yellow strips are introns. The black bars represent the already reported SNPs and the red bars indicate the novel ones. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was absent (Table 2). The occurrence of these mutations across the three groups of malaria manifestations has been illustrated in Fig. 3.

For each of the three groups, the distribution of alleles at six different SNPs was arranged and the null hypothesis of random distribution of different alleles (18 alleles altogether) was tested. In the SCM, SM and UM groups the chi-square values indicated a non-random distribution, signifying the fact that alleles of certain SNPs have skewed frequencies ($p < .05$) (Table 3). On close observation, it was found that the SCM group had a higher frequency of homozygous mutant alleles at the −1031 position, as compared to their homozygous wild counterparts, which caused non-random distribution of alleles. In the SM group, skew in the frequency distribution was due to the higher homozygous wild alleles of the novel SNP at −806 position. It was interesting to note that the skewed frequencies in the UM group was due to the higher number of heterozygotes observed at the −1031 position and also an excess of homozygous wild alleles at −857 position.

In order to know if the distribution of different alleles of each particular SNP in a particular group conforms to the expectation under Hardy-Weinberg equilibrium, a chi-square contingency table was prepared and values were calculated (Table 4). Deviation from the equilibrium model for the mutation at the −1031 position in the UM group was observed. Also, statistically significant deviation from expectations under Hardy-Weinberg equilibrium could be detected in other mutations (−863 position in the SCM group; −308 and +787 positions in the SM group; −308 and +787 positions in the UM group), indicating biasness in the frequency distribution of the different alleles at that particular SNP.

Genetic association (LD) between each pair of SNPs was checked by performing tests for linkage disequilibrium across all three groups of malaria manifestations. The strength of LD was determined by r^2 values as depicted in Fig. 4. A significant association between SNPs at −1031 and −863 sites in all three groups (with the highest being in the SCM group followed by SM and UM; Fig. 4) could be observed. The mutation present at the −863 position was found to have mild association with the mutation present at the −806 position in all the three groups; strongest being in UM and weakest in SM group (Fig. 4). There was a weak association between the SNPs −308 and +787 positions in the SM group (Fig. 4). A moderate association between the mutations at −1031 and −806 positions was found along with a weaker association between mutations at −1031 and −308 positions in the UM group (Fig. 4). Very weak associations of −1031 with −857 and −806, −863 with −806 and −308 with +787 were also detected in the SM group (Fig. 4).

The phasing out of alleles formed seventeen haplotypes based on the six mutations (Table 5). The H3 comprising of the wild alleles at all the six segregating sites was the most common haplotype in the SM and UM groups with a frequency of 33.3% and 29.2% respectively (Table 6). The H4 haplotype which had the mutated alleles at −1031 and −863 positions had a higher frequency in the SCM (25%) group, followed by SM group (14.3%) with least frequency in the UM group (4.2%) (Table 6).The H2 haplotype was present in the SM and UM groups while it was absent in the SCM group (Table 6). It may be noted here that the H2 haplotype possesses the rare alleles at −1031 and −308 positions. Similar was the case of H16 haplotype, which contains the rare alleles at −863 and −857 positions (Table 6).The H12 haplotype was present in similar frequencies across the SCM and UM groups, however, its frequency in the SM group was fairly low. There were a few haplotypes that were unique to a particular group like H13 and H17 in SCM, H9 and H14 in SM, H5 and H6 in UM groups (Table 6).There was no major difference in the distribution of the rest of the haplotypes across the three groups (Table 6). The estimation of haplotype diversity did not reveal a noticeable difference among all the groups *viz.*CM (0.92), SCM (0.87), SM (0.86) and UM (0.87) (Table 6).

The two measures of nucleotide diversity, *i.e.* θ_W and π were estimated in order to check the effect of selection acting at the TNF-α gene. Tajima's D value normalized the differences and gave a value which would point towards the effect of selection, if any. The Tajima's D values for the SCM and SM groups were between zero and one whereas it was slightly more than one in the UM group (Table 6). Interestingly, none of the Tajima's D value was found to be statistically significantly different from expectation under neutral molecular evolution.

4. Discussion

TNF-α is undoubtedly one of the most widely studied host factors in relation to malaria susceptibility. The association of TNF-α polymorphisms and different malaria outcomes has been a major topic of interest particularly in malaria endemic regions of the world. Although the effect of promoter polymorphisms on the level of the cytokine remains debatable, yet several studies have ascertained their influence in a number of autoimmune and infectious diseases including malaria (Yang et al., 2006). Here we report a possible association of the homozygous rare allele at −1031 position of the TNF-α promoter with cerebral malaria; with the heterozygous allele protecting against the same. It has also been shown in a haplotype analysis that many variants of the TNF gene are independent of each other and a variant could influence disease susceptibility in spite of the others being neutral (Ackerman et al., 2003).The sequencing of the promoter and further alignment revealed the presence of a novel SNP, −806C > T in malaria infected individuals. The rare allele was also found to exist only in heterozygous condition. This observation is similar to a report from Taiwan on the presence of this heterozygous allele at −806 position in individuals suffering from naso-pharyngeal carcinoma (Ho et al., 2006). This rare SNP, however, was found in almost equivalent proportions in the SCM and UM groups. The role of the entire TNF-α gene has not been evaluated much with respect to malaria. Analysis of the genetic region revealed an intronic polymorphism at +787 site which has never been reported before. The rare allele was found to occur in heterozygous condition only and the frequency of heterozygotes was highest in the SM group followed by UM and least in the SCM group. Flori and coworkers (Flori et al., 2005) had looked into the entire TNF-α gene and have reported two intronic polymorphisms at +851 and +1304 sites that show an association with increased risk of mild malaria. The effect these SNPs have on the severity of malaria is not conclusive from the above results. It can, however, be checked with a larger sample size.

In order to pinpoint the allele contributing to such a distribution, we looked at the observed and expected values from the chi-square contingency table (Table 3). In the SCM group, the higher number of homozygous mutants as compared to their wild counterparts at −1031 position was responsible for the non-random distribution. For the same position in the UM group the number of observed heterozygotes was

Table 2
Details of SNPs observed along with their genotype frequencies in different groups of malaria manifestations.

	−1031 T > C			−863C > A			−857C > T			−806C > T			−308 G > A			+787C > A		
Category	TT^w	TC^h	CC^m	CC^w	CA^h	AA^m	CC^w	CT^h	TT^m	CC^w	CT^h	CC^m	GG^w	GA^h	AA^m	CC^w	CA^h	AA^m
SCM (n = 16)	3 (18.7)	6 (37.5)	7 (43.7)	6 (37.5)	5 (31.2)	5 (31.2)	14 (87.5)	2 (12.5)	0	9 (56.2)	7 (43.8)	0	11 (68.7)	5 (31.2)	0	11 (68.7)	5 (31.2)	0
SM (n = 21)	10 (47.6)	10 (47.6)	1 (4.8)	10 (47.6)	9 (42.9)	2 (9.5)	12 (57.1)	9 (42.9)	0	19 (90.5)	2 (9.5)	0	15 (71.4)	4 (19)	2 (9.5)	9 (42.9)	12 (57.1)	0
UM (n = 24)	8 (33.3)	15 (62.5)	1 (4.1)	9 (37.5)	13 (54.2)	2 (8.3)	21 (87.5)	3 (12.5)	0	14 (58.3)	10 (41.7)	0	13 (54.1)	11 (45.9)	0	13 (54.1)	11 (45.9)	0

The numbers in parenthesis indicate the % frequency of each of the genotype in the respective groups. w: Homozygous wild, h: Heterozygous, m: Homozygous mutant mentioned as superscript with the category of alleles.

much higher than the expected numbers which could be one of the reasons for the non-random distribution observed. In a study conducted in Tanzania, it was ascertained that there was an increased incidence of malaria among children who possessed the −1031 CC allele (Gichohi-Wainaina et al., 2015).We have observed a higher frequency of the homozygous rare allele at −863 site in the SCM group as compared to the other groups. In corroboration with a study including samples from an endemic region of Odisha had found an association of the TNF −1031CC and −863AA alleles with high levels of the cytokine circulating in the body (Sinha et al., 2008). Along with this there was also a correlation between the high TNF levels and severe *P. falciparum* malaria in the same study. A positive correlation between increased TNF-α expression mRNA level and the −1031 CC genotype has been observed in Iranian patients suffering from chronic inflammatory bowel disease (Nourian et al., 2017). An association of the rare allele at the −1031 and −863 positions of the TNF-α promoter with multi-organ failure in acute pancreatitis has already been reported in a study (Bishehsari et al., 2012). This may also mean that these alleles may play a role in the progress of a disease into its severe forms along with the increased susceptibility. There has been a contradicting view on TNF-863 mutation wherein the homozygous rare allele has been associated with lower levels of the cytokine in individuals from Saudi Arabia and hence the homozygous wild allele is associated with the disease (Nasr et al., 2014). Our results not only point to an association between −1031CC and -863AA with cerebral and severe malaria but also towards the dual nature of TNF- α. There has been a report of the wild allele at −857 position (*i.e.*−857C) together with −1031C and −863C was significantly associated with cerebral malaria in northwest Thailand (Hananantachai et al., 2007).We have detected a higher proportion of homozygous wild alleles at −857 position with a complete absence of homozygous rare alleles across all three groups of malaria manifestations. This higher number of observed homozygous wild type alleles than the expected ones at the −857 position could also have been a reason for the non-random distribution of alleles in the UM group. Since our study has involved samples from a hospital setting, there are chances that few of the UM patients might have progressed into severe forms had they not been treated on time. So the role of the homozygous wild allele at −857 remains debatable from our results. The non-random distribution in the SM group was due to a very low number of observed heterozygotes at −806 position than the expected ones. This was in contrast to the occurrence of heterozygotes in the other two groups. Another important reason for the non-random distribution of alleles in the SM group could be because of the lower frequency of heterozygotes observed at −308 position in that group. The SNP at −308 position has been one of the most extensively studied promoter polymorphisms of TNF-α. Results from a recent study suggest that the−308AA allele is associated with a decrease in malaria rates (Gichohi-Wainaina et al., 2015). In our study, only two patients in the SM group were found to possess the homozygous rare allele at −308 position. Our results are also in lines with a recent study where the allele frequency of -308A was found to be higher in the *P. falciparum* malaria patients than in controls in Ethiopia, although the difference was not statistically significant (Tegenaw et al., 2016). Another recent report has also associated the presence of -308AA with malaria infected individuals from Nigeria (Ojurongbe et al., 2017).

The presence of quite a few heterozygotes across the groups also indicates the action of balancing selection (Penrose, 1955). An analysis of the TNF-308 locus revealed a deviation from Hardy-Weinberg equilibrium due to the selective advantage provided by a high number of heterozygotes producing intermediate levels of TNF-α in West Africa (Santovito et al., 2012). In the present study, deviation from the Hardy-Weinberg equilibrium at −1031 position in the UM group was observed, which might be due to the high number of heterozygotes. These results point towards a probable selective advantage of heterozygotes at the −1031 site of the TNF-α promoter. The deviation from the Hardy Weinberg equilibrium at −863 position in the SCM group may again be

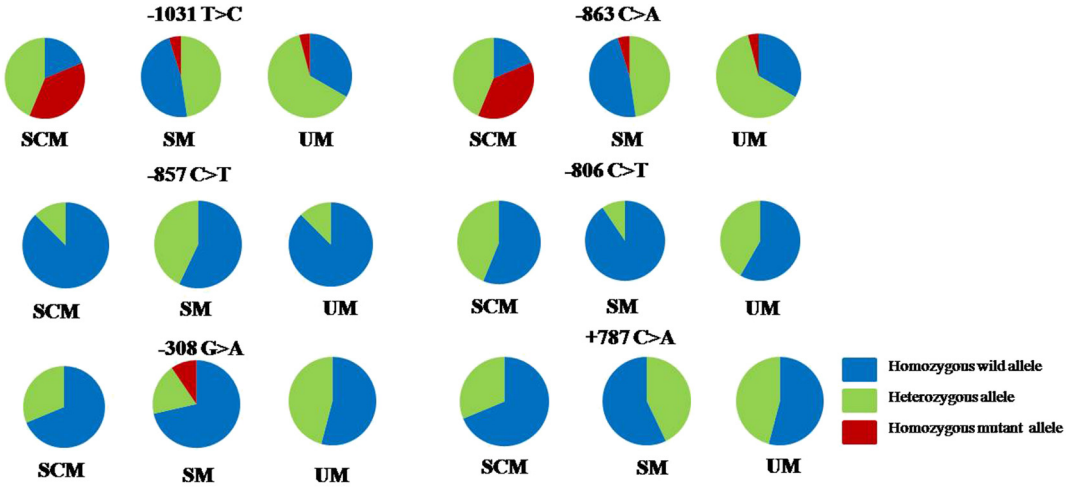

Fig. 3. Pie chart representing frequencies of the six SNPs in the TNF-α gene across the three groups of malaria manifestations. The blue region of the pie charts represents the frequencies of homozygous wild alleles; the green region represents the frequencies of heterozygous alleles; the maroon region represents the frequencies of homozygous mutant alleles of the six SNPs across the three groups of malaria manifestation *viz.* Severe malaria with cerebral involvement (SCM), Severe malaria only (SM) and Uncomplicated malaria (UM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Chi-square tests to check the random/non-random distribution of alleles for each group.

Malaria manifestation category	SCM	SM	UM
Chi-square value	36.5 ($p < .01$)	21.2 ($p < .025$)	23.1 ($p < .025$)

due to the higher number of homozygous mutants at the position further supporting the involvement of that SNP with cerebral malaria. In the SM and UM groups there was a deviation from the expectation of Hardy-Weinberg equilibrium at −308 and +787 positions. In the SM group the deviation was due to the lower frequency of heterozygotes and higher frequency of homozygous wild type alleles observed at the −308 position. Whereas, the deviation from Hardy-Weinberg equilibrium at −308 position in the UM group was due to the higher frequency of heterozygotes. This observation is in consensus with the study by Santovito and co-workers (Santovito et al., 2012) pointing towards the selective advantage of heterozygotes at −308 position. The deviation from equilibrium of the novel SNP at +787 position in the SM and UM groups might be due to a slightly high number of heterozygotes. However, finding of statistically insignificant and positive Tajima's D values across all three groups indicate no deviation from the neutral model of molecular evolution. This justifies no role of natural selection acting on any of the three groups of malaria manifestations (Chauhan et al., 2014). Our study revealed a higher number of associations in patients with SCM and UM than patients with SM. The genetic association between −1031 and −863 positions is indicated by comparatively stronger association in the SCM followed by SM and UM groups. This is in lines with the study wherein a strong association was found between these two SNPs (Sinha et al., 2008). A very strong association of −863 and −806 positions was detected in the UM group followed by SCM and SM groups. It has also been reported that these −1031 and −863 polymorphisms are present in high frequency in India and may have a substantial role in dictating the immune response and outcome of several infectious diseases (Sinha et al., 2008). Our study revealed most of the associations are linked to −1031 or −863 which could probably be because of their higher frequencies in India. A possible reason for the genetic association between two SNPs is their physical distance, which means that an association between two positions can be observed if they are closely located (Chauhan et al., 2014). This could also be a possible reason for the higher number of associations among SNPs in this study.

The genetic variation existing in a population is organized into haplotypes that are likely to be transmitted together (Clark, 2004). Haplotype analysis has shown that the H4 haplotype that has mutated alleles at both −1031 and −863 positions had a high frequency in the SCM group and comparatively low frequency in the other two groups. This haplotype could have a possible association with high levels of the cytokine resulting in cerebral and severe malaria. A study has reported that the haplotype containing the mutated alleles at −1031 and −863 positions was associated with high levels of TNF-α (Sinha et al., 2008). The H3 haplotype consisting of the wild alleles at all the five positions had high frequency across all the three groups making it the most common haplotype of the region. However since the H4 haplotype is quite prevalent in the SCM group because of the higher frequency of the homozygous mutants at −1031 and −863 it can be predicted that this haplotype may have an association with severe and cerebral malaria.

5. Conclusion

The present study is a first of its kind with clinical samples from a hospital setting characterized into three groups as per WHO criteria and also involved DNA sequencing and sequence analyses of the entire TNF-

Table 4
Chi-square values for the test of H–W equilibrium.

Malaria manifestation category	−1031 T > C	−863C > A	−857C > T	−806C > T	−308 G > A	+787 C > A
SCM	1.28	4.44*	0.142	2.51	1.1	1.1
SM	1.17	0.3	3.12	0.105	6.14**	6.72***
UM	6.44**	1.63	0.213	3.32	4.24*	4.24*

*: $p < .05$; **: $p < .025$; ***: $p < .01$.

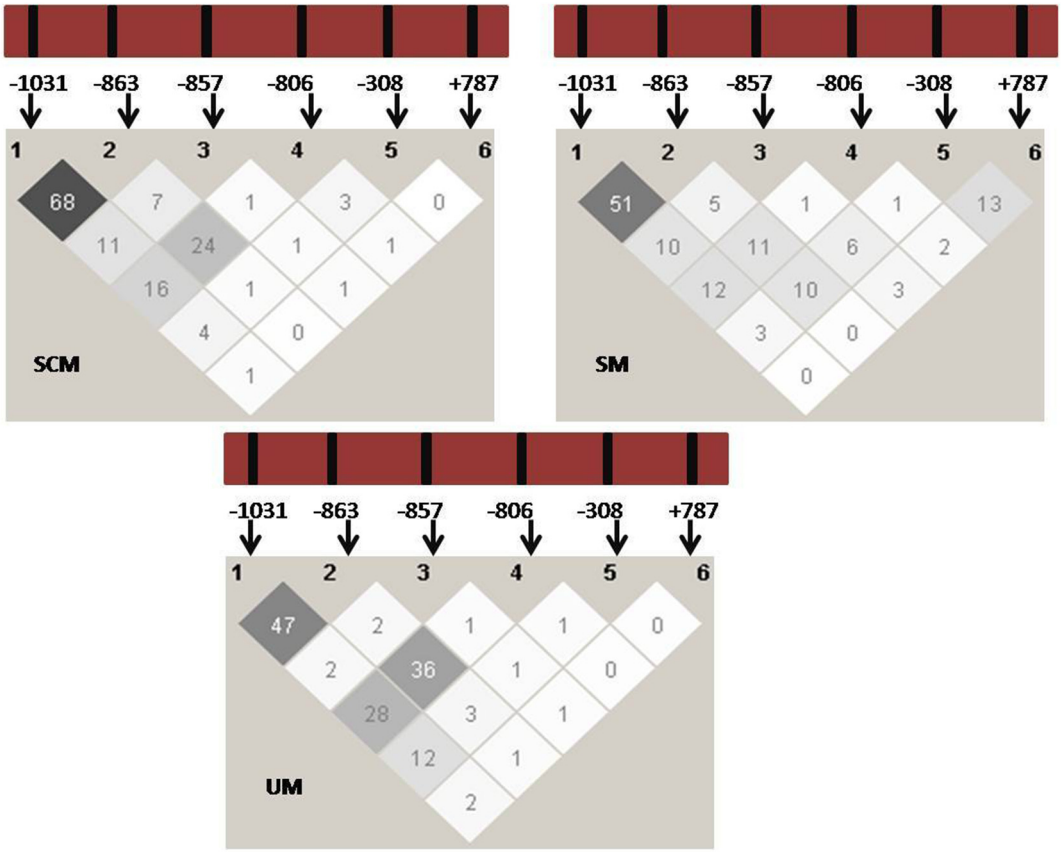

Fig. 4. Tests for pair-wise linkage disequilibrium between the SNPs in SCM, SM and UM groups. Strength of association between a pair of SNPs is indicated by the colour intensity of the boxes such as the darker boxes represent stronger and the lighter ones represent weaker ones. The numbers within the boxes are the r^2 values, stronger the association between two SNPs, higher is the r^2value.

Table 5
Haplotypes formed after phasing of alleles.

Haplotype	−1031	−863	−857	−806	−308	+787
H1	T	C	C	C	A	A
H2	T	C	C	C	A	C
H3	T	C	C	C	G	C
H4	C	A	C	C	G	C
H5	T	C	C	T	A	C
H6	T	A	C	T	G	C
H7	T	C	C	C	G	A
H8	C	A	C	C	G	A
H9	T	C	T	C	G	A
H10	C	C	C	C	G	C
H11	T	C	T	C	G	C
H12	C	A	C	T	G	C
H13	C	A	C	T	A	A
H14	T	A	C	C	G	C
H15	C	C	C	C	A	A
H16	T	A	T	C	G	C
H17	C	A	C	T	A	C

α gene instead of directly genotyping the SNPs. The results indicated possible interactions between SNPs at the TNF-α promoter region with different malaria manifestations. To be more specific, the already reported SNP at −1031 seems to play some role in complicated malaria in general, particularly cerebral malaria. The two novel mutations found in this study (−806 newly found in India and +787 for the first time in TNF-α) appear to be associated with only cerebral or cerebral malaria with other complications. The deviation from Hardy-Weinberg equilibrium for many of the SNPs in different malaria manifestations indicate the possible role of natural selection in providing protection against malaria. This condition looks to hold true in case of −1031 position.

Although unique, this study has several limitations. Whereas (i) very limited (but clinically well characterized) sample size was used, (ii) the sampling locality was also confined to a single center resulting in an almost homogenous population. Therefore, results from the present study may be specific to this particular malaria endemic region but nonetheless they add significant weightage to the already existing evidences on such associations. Although a direct association study of TNF-α levels and disease manifestation could not be detected justifying

Table 6
Frequency of haplotypes, haplotype diversity and tests of neutrality.

Frequency of haplotypes (%)																	Hd[a]	Nucleotide diversity		Tajima's D
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17		Π	θ_W	
SCM	6.25	–	21.9	25	–	–	3.1	6.25	–	6.25	6.25	15.6	3.1	–	3.1	–	3.1	0.87	0.0005	0.00037	0.9999
SM	7.1	7.1	33.3	14.3	–	–	2.4	4.8	9.6	2.4	7.1	2.4	–	2.4	2.4	4.8	–	0.86	0.00035	0.00047	0.93
UM	8.3	12.5	29.2	4.2	2.1	2.1	6.2	6.3	–	6.3	4.2	14.6	–	–	2.1	2.1	–	0.87	0.0005	0.0034	1.29

[a] Haplotype diversity.

the dual role of TNF-α in malaria pathogenesis, and concrete evidence on the exact role of genetic determinants in any type of malaria could not be established in the study due to limitations mentioned above, the preliminary data as presented here might serve as a "step forward" in future search for genetic determinants of cerebral/severe malaria.

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Declarations of interest

None.

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