www.bti.org.in
ISSN 0974-1453
Research Article

TUMOR NECROSIS FACTOR ALPHA-308 GENE POLYMORPHISM AND RISK OF *PLASMODIUM FALCIPARUM* MALARIA AMONG PEOPLE LIVING IN DEMBIA WOREDA, NORTH WEST ETHIOPIA

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ABSTRACT

***Plasmodium falciparum* is the most dangerous species of *Plasmodium* parasites in terms of lethality and morbidity. In different studies, polymorphisms in the tumor necrosis factor alpha (TNF-α) gene have been associated with increased susceptibility to mild malaria infection and severe malaria. The aim of this study was to determine the frequency of TNF-α-308 G > A gene polymorphism in *P. falciparum* malaria infected patients that live in Dembia Woreda, North Gondar, North West Ethiopia and to assess the effect of TNF-α-308 gene polymorphism and different demographic factors on the risk of malaria infection. Two hundred blood samples were collected from November to December, 2014, from clinically confirmed *P. falciparum* malaria patients (n=100) and from *P. falciparum* sero-negative individuals (n=100) that live in the study area. *TNF-α-308* G > A polymorphism was detected using PCR-RFLP techniques. The allele frequency in malaria patient study subjects was 0.92 for TNF-α-308G (TNF-1) and 0.08 for TNF-α-308A (TNF-2). The distribution of TNF-α-308 genotypes in cases (P=0.065) and controls (P=0.677) were consistent with the Hardy-Weinberg equilibrium. There was no statistically significant association between TNF-α-308 genotypes and malaria infection (P=0.616). Further studies with large number of sample size and assessment in different malaria endemic areas of the country are recommended for generalization.**

Key words: Cytokine, Sequestration, Single Nucleotide Polymorphism, TNF-α-308 gene.

INTRODUCTION

Malaria is a protozoan disease caused by parasites of the genus *Plasmodium*. It is one of the leading causes of illness and death in the world. Globally, above three billion people are estimated to be at risk of malaria. Even though, there are many species of *Plasmodium* parasites, *Plasmodium falciparum* and *Plasmodium vivax* malaria pose the greatest public health challenge. *Plasmodium falciparum* is the dominant one and found in most tropical regions throughout the world and is the most dangerous species in terms of lethality and morbidity (Who, 2015). As a tropical country, the problem of malaria is very severe in Ethiopia where it has been the major cause of illness and death for many years. Seventy five percent of Ethiopia is malarious and about 68% of the total population lives in areas at risk of malaria (Dawit *et al*., 2012).

Female *Anopheles* mosquitoes are primary hosts and transmission vectors for *Plasmodium* parasites. Through the bite of an infected female *Anopheles* mosquito the parasite enters into the blood stream of the human host cell (Gunanidhi *et al.,* 2010) and penetrates hepatocyte to undergo growth and multiplication. As the host cell ruptures, the parasite will be released into the bloodstream and then infect specifically red blood cells in order to remain viable (Denise *et al.*, 2009). Then the parasite modifies the host to make it a more suitable habitat (Shi *et al.,* 2013). This modification causes alteration of cell membrane permeability, parasite sequestration (Charles *et al.,* 2000) and prevents immunological destruction of the parasite (Buffet *et al*., 2011).

Specific interaction between parasitized red blood cells (PRBCs) and the vascular endothelium causes sequestration of *P. falciparum* infected erythrocytes to endothelial cells (Dean, 2004). The adhesion *P. falciparum* erythrocyte membrane protein-1 (Pf EMP-1) of the parasite binds to several candidate endothelial receptors (Charles *et al.,* 2006), including intercellular adhesion molecule 1 (ICAM-1), CD54 and CD36 (Francischetti *et al.,* 2007).

Humans infected with malaria parasites experience a remarkable range of disease severity (Laura *et al.,* 2013). The treatment and control of malaria is dependent on the understanding of the causes of variation for the severity of malarial disease (Imad *et al.,* 2009). Parasite and host genetics, acquired immunity, and exposure levels are known to contribute to this variation (Alexandra *et al.,* 1997). According to a research done in Sri Lanka for clinical intensity of illness, 20% of the variation was explained by repeatable differences between patients, about half of which was attributable to host genetics. Based on this study, genetic control of *P. falciparum* infections appeared to modulate the frequency and intensity of infections (Mackinnon *et al.,* 2000). Malaria has been associated to gene selective pressure in the human genome, and it has been associated as an evolutionary force of some genetic diseases (Sunil and Philip, 2008).

Candidate chromosomal region analyses and candidate gene studies have been performed to find human genes involved in malarial disease and infection. Different studies have detected the association of severe malaria with several genes (Afridi *et al.,* 2012; Alphaxard *et al.,* 2015; Vinicius *et al*., 2012), including genes within the major histocompatibility (MHC) complex. In particular, polymorphisms in the *tumor necrosis factor* (*TNF-α*) gene have been associated with increased susceptibility to mild and severe malaria disease (Flori *et al.,* 2005).

Tumor necrosis factor alpha gene is located on the short arm of chromosome 6 within the major histocompatibility complex (Qidwai and Khan, 2011). TNF-α is a potent cytokine with a wide range of pro-inflammatory activities (Eva *et al*., 2013). TNF-α acts as a pyrogenic and proinflammatory molecule and is involved in the killing of *P. falciparum* mediated by neutrophils and monocytes in the presence of antibodies (Mary and Eleanor, 2004).

Different studies showed polymorphisms in the *TNF-α* gene are related with differences in phenotypes related to malaria infection. In a study performed in Burkina Faso, several *TNF-α* variants were found to be associated with phenotypes related to malaria infection and mild disease (Flori *et al.,* 2005). In the same way, in the ethnically diverse Indian population, higher TNF-α level was observed in patients with severe *falciparum* malaria (Swapnil *et al.,* 2008). Overproduction of TNF-α is considered a major pathogenic mechanism responsible for fever and tissue lesions in *P. falciparum* malaria (Qidwai and Khan, 2011).

Several polymorphisms have been identified inside the *TNF-α* promoter positioned at relative to the transcription start site which affects TNF-α production (Maqsood *et al.,* 2009). Among these variants, a polymorphism that directly affects *TNF-α* expression is located at nucleotide position-308. A single-base polymorphism within the promoter of the gene of *TNF-α* results in the formation of two allelic forms. One in which guanine defines the common allele (*TNFA-1*/*TNF -1*) and the other in which guanine is substituted by adenosine forms the rarer allele (*TNFA-2*/*TNF- 2*) at position -308 and the presence of the rarer *TNFA-2* allele has been found to correlate with enhanced spontaneous or stimulated TNF-α production in both *in vitro* and *in vivo (*Mira *et al.,* 1999).

Tumor Necrosis Factor-α up-regulates endothelial receptors such as ICAM-1 and probably redistributes other receptors, such as CD31 on endothelium, which may enhance parasite sequestration (Sri, 2003).Because of this reason the 308-A (*TNF-2*) allele has been associated with the risk of cerebral malaria and development of serious neurological consequences (Maqsood *et al.,* 2009). The aim of this study was to determine the frequency of *TNF-α-308* G>A polymorphism in malaria patients that live in malaria endemic area (Dembia Woreda).

MATERIALS AND METHODS

Study Area

The study samples for this research were collected from Dembia Woreda, North

Gondar, North West Ethiopia. According to 2007 population and housing census of Ethiopia the total population of Dembia Woreda have been 265,582 and the altitude ranges from 1800m-2300m above sea level (CSAE, 2007). This area is a continuous breeding focus for the malaria vector, *Anopheles* mosquito. It is also endemic for *P. falciparum* with high seasonal incidence after the major rainy season from September to November. A second peak of transmission follows during January to March (Abebe *et al.,* 2012).

Ethical Consideration

For this study, ethical clearance was obtained from the research and ethical committee of College of Natural and Computational Sciences of University of Gondar. Informed consent was taken from all study participants.

Study Design

The study design was case control study design.

Sample Collection

Three ml of blood samples were collected in EDTA coated test tubes from clinically confirmed one hundred *P. falciparum* malaria infected patients whose age is 15 years and above and with a previous history of frequent malaria infection that had visited Koladiba health center from November to December, 2014. In the meantime age and sex matched 100 blood samples were collected from *P. falciparum* sero-negative individuals. Demographic information for each study participant were collected using a questionnaire with information that deals about age, sex, insecticide treated mosquito net (ITN) usage, educational status, occupation, previous malaria history and special location of the patient area.

Genomic DNA Isolation

Genomic DNA from both cases and controls were isolated from whole blood by phenol chloroform DNA isolation method (Roe *et al*., 2014). The quantity and quality of the isolated DNA was analyzed through spectrophotometric analysis and agarose gel electrophoresis respectively before further processing.

Detection of *TNF-α- 308* G>A Polymorphism

Tumor Necrosis Factor-α-*308*G > A polymorphism was detected by using polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) techniques. This was done by amplifying a 107bp DNA fragment flanking the polymorphic site using primer pair, *TNF-α* Forward5'AGGCAATAGGTTTTGAGGGC CAT-3*TNF-α*-Reverse 5'TCCTCCCTGCTCCGATTCCG3' (Higham *et al.,* 2000).The PCR master mix was composed of 0.65µl 100pm of each primer(eurofins), 2.5µl of 25mM magnesium chloride, 2.5µl of 10X PCR buffer, 1µl of 20mM DNTP mixture and 0.2µl 5U Taq DNA polymerase (Soils Bio dyme), 17.5µl sterilized double distilled water and 1µl template DNA for 25µl reaction volume. PCR conditions were:

Initial denaturation at 94^0C for 8 min, 35 cycles of 94^0C as denaturation temperature for one min, 60^0C as annealing temperature for one min and 72^0C as extension temperature for one min and then a final extension temperature of 72^0C for 10 min. After PCR amplification 10µl of the PCR product was digested for 1 hour at

37^0C in a total volume of 25µl containing 1µl of 10U *Nco*I (Takara Bio Inc) enzyme, 2µl 10x buffer, 2µl BSA and 10µl sterilized double distilled water. The restriction enzyme digestion provides 107bp for the *TNF-2* and 87 and 20bps for *TNF-1* when visualized and photographed by gel documentation system after running on 2.5% agarose gel electrophoresis (Hingham *et al.,* 2000).

Data Analysis

Statistical analyses were performed with the SPSS version20. Data like age, sex, use of ITNs, educational status and occupation were analyzed for mean ± SD, frequencies and chi-square tests. Association between genotypes and malaria infection was assessed using chi-square and odds ratio with confidence intervals (CI 95%) which are interpreted as the relative risk of disease for malaria patient compared with control. Allele frequencies were calculated by genotype counting, and deviation from Hardy-Weinberg equilibrium was tested using a chi-square test with one degree of freedom. Probability (*P)*<0.05 was taken as the level of significance.

RESULTS

Demographics of Study Participant

Malaria Patients and Controls

An organized statistical data of demographical description of *P. falciparum* malaria patients (n=100) and *P. falciparum* sero-negative control participants (n=100) is presented in Table 1. The mean age of *P. falciparum* malaria patient study participants was 23.2 ± 8.36 years; the minimum age was 16years old and the maximum one was 53 years old. The highest incidence of the disease was found among the age group 16-25 years old which covers 68% of the study participants and the list affected age group was greater than 40 years old. Among the 100 malaria patient study participants 77(77%) were males and the remaining were females. Eighty seven percent of malaria patient study participants did not use insecticide treated nets. With respect to educational status and occupation the most malaria affected groups were illiterates (45%) and farmers (63%), respectively. Around 75% of malaria patients came from rural kebeles. On the other hand, the mean age of *P. falciparum* sero-negative control study participants was 25.97 ±10.64 with the maximum age of 58 years old and the minimum age of 17 years old. There was no statistically significant mean age variation between cases and controls. Sixty five percent of the control groups were within the age range of 16-25 years old. Sex wise 70% of the control group study participants were males. Fifty percent of the controls have a habit of using insecticide treated nets. From educational status point of view 43% of the controls were illiterates. When occupational status of controls was tabulated 52% were farmers, 27% were students and 21% were others (employed civil servants and construction workers).

Table 1. Demographic Characters of Study participants

Character		*P. falciparum* malaria patients (n=100)		*P. falciparum* sero-negative control groups (n=100)	
		Frequency	**Percent**	**Frequency**	**Percent**
Age	16-25 years	68	68.0	65	65.0
	26-40 years	24	24.0	26	26.0
	>40 years	8	8.0	9	9.0
Sex	Female	23	23.0	30	30.0
	Male	77	77.0	70	70.0
Use ITN	No	87	87.0	50	50.0
	Yes	13	13.0	50	50.0
Educational Status	Illiterate	45	45.0	43	43.0
	Grade 1-4	13	13.0	18	18.0
	Grade5-8	28	28.0	22	22.0
	Above grade 8	14	14.0	17	17.0
Occupation	Student	13	13.0	27	27.0
	Farmer	63	63.0	52	52.0
	Others	24	24.0	21	21.0

The Association of Different Demographic Characters (Risk Factors) and Malaria Disease

Different risk factors (age, sex, use of ITN, educational status and occupation) were considered to analyze their risk for malaria infection. Based on the result of this study, age difference, use of ITN and occupation altered the risk of malaria infection. Among those risk factors age (*P*=0.000), use of bed net (*P*=0.000) and occupation (*P*=0.046) were significantly associated with the risk of malaria infection. As shown in Table 2 age groups 16-25 years old (OR= 7.481, *P*=0.013) and 26-40 years old (OR=10.449, *P*=0.000) were more likely affected by malaria in comparison with age group greater than 40 years old. The risk analysis also showed ITN non-users were at higher risk for malaria infection than ITN users (OR=5.878, *P*=0.000). Prevalence of malaria was significantly higher for students (OR= 2.660, *P*=0.036) when compared with other workers (employed civil servants and construction workers). Based on this study sex (*P*=0.118) and educational status (*P*=0.667) were not associated to risk of malaria infection.

Table 2. Binary logistic analysis of risk factors for malaria infection in the study participants

Risk factors		B	*P*- value	Odd ratio
Age			0.000	
	16-25 years old	2.012	0.013	7.481
	26-40 years old	2.347	0.000	10.449
Sex	Female	-0.656	0.118	0.519
Use of Net	No	1.771	0.000	5.878
Educational Status			0.667	
	Illiterate	0.770	0.903	1.080
	Grade 1-4	-0.252	0.708	0.777
	Grade5-8	0.447	0.467	1.563
Occupation			0.025	
	Student	-1.322	0.036	2.660
	Farmer	0.391	0.427	1.478

Distribution of *TNF-α-308* Genotypes and Their Association with Malaria Infection in the Study Population

After screening of *TNF-α-308* polymorphism through PCR-RFLP techniques, the proportion of *TNF-α-308*gene genotypes of the study population was determined. After amplification of the specific region of *TNF-α* gene which contains fragment of interest, a 107bp product was found (Fig.1). By the *Nco*I restriction digestion the 107bp PCR products were cleaved into 87 and 20bp for *TNF-1*, but the *Nco*I enzyme does not cut the PCR product for *TNF-2* which provides 107bp after restriction digestion (Fig.2).The allele frequency in falciparum positive cases (n=100) was 0.92 for *TNF-α-308*G (*TNF-1*) and 0.08 for *TNF-α-308*A (*TNF-2*)while the allele frequency in control group (n=100) was 0.96 and 0.04 for *TNF-α-308*G (*TNF-1*) and *TNF-α-308*A (*TNF-2*), respectively. The distribution of *TNF-α-308* genotypes in falciparum positive cases (*P*=0.065) and control groups (*P*=0.677) were consistent with the Hardy-Weinberg equilibrium (Table 3). In falciparum positive cases 86 individuals were homozygote for *TNF-1* and only 2 individuals were homozygote for *TNF-2*. On the other hand, from the control groups 92 individuals were homozygote for *TNF-1*andno *TNF-2* homozygote was observed. Twelve individuals from falciparum positive cases and 8 individuals from controls were heterozygote for *TNF-α-308* gene

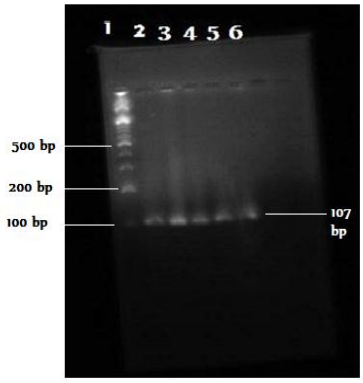

Fig. 1. Agarose gel electrophoresis of 107 bp PCR products. Lane1= 100bpDNA ladder, lane 2-6=107bp PCR products.

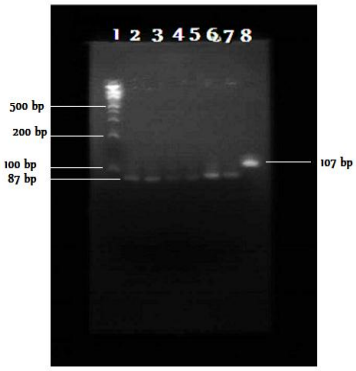

Fig. 2. *Nco*I digested restriction products. Lane 1=100 bpDNA ladder, lane 2-7= 87bp restriction products (*TNF-α-308*G allele), lane 8=107bp uncut DNA (*TNF-α-308*A allele).

The result of this study showed no significant difference between falciparum positive cases and control groups for the distribution of *TNF-α-308* genotypes and alleles. Based on the binary logistic regression result, there was no statistically significant association between *TNF-α-308* genotypes and risk of malaria infection (*P*=0.616)

Table 3. The frequency of *TNF-α-308* genotypes and allele frequency in cases and controls.

Groups	**Genotype frequency**			**Allele frequency**		***P*-value**
	TNF-1/ TNF- 1	*TNF-1/ TNF- 2*	*TNF-2/TNF- 2*	*TNF-1*	*TNF-2*	
Cases	86 (84.64%)	12 (14.72%)	2(0.64%)	0.92	0.08	0.065
Controls	92 (92.16%)	8 (7.68%)	0(0.16%)	0.96	0.04	0.677

DISCUSSION

Several studies with different populations in the world described the presence of genetic alterations in the *TNF-α* gene. In different populations the effects of these genetic alterations were contradictory (Vitor *et al.,* 2012). Besides geographical location, environmental condition and different socio-demographic characteristics, genetic behaviors of individuals have roles for malaria infection and severity (Alexandra *et al.,* 1999).

The presence of *TNF-α-308* G>A polymorphism was determined through PCR-RFLP techniques by using specific primers and a specific restriction enzyme. In this study, among the 100 *P. falciparum* positive study subjects, only 2 individuals (2%) were homozygote for *TNF2* and no individual were homozygote for *TNF-2* from the 100 control groups. However, there was no statistically significant difference between *P. falciparum* malaria patients and controls (*P*> 0.05) for the distribution of *TNF-α -308* genotypes. In Sudan, from 109 cerebral malaria infected children only 1.8% of them were homozygote for *TNF-2* and no individual were homozygote for *TNF-2* from the control groups (Mergani *et al.,* 2010). So, the distribution of *TNF-2* allele was consistent with the result of the present study.

The distribution of *TNF-α-308* G>A genotypes in the *P. falciparum* malaria patients and control groups were consistent with the Hardy-Weinberg equilibrium. The *TNF-2* allele frequency was found to be higher in the *P. falciparum* malaria patients (0.08) than in control subjects (0.04), however, the difference was not statistically significant. The result of this study indicated no association between *P. falciparum* malaria infection and *TNF-α-308* G>A polymorphism (*P*>0.616). Similar results were reported in the following studies that proved lack of association between mild *P. falciparum* malaria infection and *TNF-α-308* gene polymorphism. These studies showed the effect of *TNF-α-308*Aallele with cerebral malaria rather than mild malaria infection. In Gambia, the *TNF-α-308*A was associated with an increased risk of cerebral malaria (McGuire *et al.,* 2012). In the same way a study in Sri Lanka indicated association of *TNF-α-308*G>A point mutation with sever malaria not with mild malaria (Wattavidanage *et al.,* 1999). Different *TNF-α* polymorphisms analyzed in the Fulani ethnic group in Mali including *TNF-α-308*Aallele associated with the severity of malaria infection (Israelsson *et al.,* 2011). All those studies described association of *TNF-α-308*Aalleleand other *TNF-α* promoter polymorphisms specifically to severe but not mild malaria, which is consistent with the result of the present study which showed lack of association between *TNF-α-308*G>A polymorphism and malaria infection. On the contrary, a study in Gabon's School children associated the *TNF-α-308*A (*TNF-2*) with a shorter interval to malaria reinfection and mild disease (Mombo *et al*., 2003). The disagreement of this result with the present study may be associated with the difference in ages of study subjects. In a family based study which was carried out in Burkina Faso, several *TNF* variants determined as part of the genetic determinants for maximum

parasitaemia or mild malaria attack or for both. Among those *TNF* variants, *TNF-α-308*A (*TNF-2*) was moderately associated with mild malaria attack. This discrepancy may be explained by the difference in age, genetic back ground, sample size and study design employed in the two studies.

CONCLUSION

In this study, there was no statistically significant difference between cases and control groups in the frequency of *TNF-α-308* gene polymorphism. The distribution of *TNF-α-308* genotypes in cases and control groups were consistent with the Hardy-Weinberg equilibrium. From this study *TNF-α-308* gene variants were not found to be associated with risk of malaria disease.

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