ORIGINAL ARTICLE

Association of Toll-like Receptor 9 (TLR9) Alterations in Malaria Susceptibility and Severity: A Tribal Population Based Study in the Malaria Endemic State of Assam, India

Manash P. Kalita[1], Subhash Medhi[2], Sujoy Bose[3], Dipankar Bujarbaruah[4], Tarun K. Basumatary[1], Ramie H. Begum[1]*

[1]Department of Life Sciences and Bio-informatics, Assam University Diphu Campus, Karbi Anglong, Diphu-782462 (Assam) India, [2]Department of Bioengineering and Technology, Institute of Science and Technology, Gauhati University, Gopinath Bardoloi Nagar, Guwahati-781014 (Assam) India, [3]Department of Biotechnology, Gauhati University, Gopinath Bardoloi Nagar, Guwahati-781014, (Assam) India, [4]Department of Zoology, Dimoria College, Khetri-782403, Kamrup (M) (Assam) India

Abstract:

Background: Toll-like receptors (TLRs) are group of trans-membrane proteins that recognizes specific molecular pattern and activates the downstream cytokines for the efficient clearance of pathogens. *Objectives:* To evaluate the role of TLR9 1486T/C polymorphism and its association with the malarial pathogenicity and severity. *Material and Methods*: Present study was a cohort based study and a total number of 150 samples were drawn from three major tribal population group's viz., *Karbi*, *Dimasa* and *Boro-Kachari*. TLR9 (1486T/C) gene polymorphism was assessed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Differential TLR9 m-RNA expression was assessed using Real time PCR (RT-PCR). *Results:* TLR9 1486T/C genotype variant showed increased risk for the susceptibility of malarial infection (p=0.141) compared to controls. The variant genotype showed significant increased risk with susceptibility to severe malaria infection (p=0.047) compared to uncomplicated malaria (UC-M). TLR9 mRNA level expression showed up-regulation in UC-M compared to controls. Decreased TLR9 expression was found in SM cases compared to UC-M (p=0.312). Significant down-regulation of TLR9 gene was found in UC-M with variant C genotype cases compared to wild types (p= 0.039). *Conclusion:* Our finding indicates the association of the TLR9 gene polymorphism and linked differential expression modulation in the development of malarial pathogenicity.

Keywords: Toll–like Receptor 9 (TLR9), Malaria, Nuclear factor-kappa beta (NF-), Polymorphism, Expression

Introduction:

Malaria, caused by the genus *Plasmodium,* is one of the major devastating diseases afflicting nearly 219 million people worldwide and causing around 429,000 deaths annually [1]. In India, malaria is reported from many states, but several deaths of epidemic proportion are reported every year from different parts of Northeast India representing eight states. The region is home to approximately 4% of the country's population with the state of Assam representing 70% of Northeast India [2]. Assam is predominantly inhabited by indigenous ethnic communities of tribal and non-tribal origins and is highly co-endemic to both *Plasmodium falciparum* and *P. vivax* contributing

to 10% of cases and 20% of malaria-attributed deaths in India, *P. falciparum* being dominant and contributing 77% of the cases [3, 4].

Clinical manifestations of malaria vary from individual to individual, ranging from asymptomatic malaria, uncomplicated malaria to severe life threatening forms [5]. This leads to the question why only a small subset of individual develops the severe form of the disease. Understanding the molecular mechanism of how malaria motifs induces differential innate immune system and produces inflammatory cytokines may lead to the probable explanation of the query.

Toll-like Receptors (TLR1-TLR11) are pattern recognition receptors transmembrane proteins present on the immune cells that plays critical role in the early innate immune response to invading pathogens [6, 7]. Among the ten TLRs in humans, TLR2, TLR4, TLR7 and TLR9 have been identified to detect malarial antigens and induce anti-malarial immune response [8]. TLRs 2, 4 and 9 are primarily involved in the recognition of *P. falciparum* ligands and when encountering it with the parasite, mediates a complex cascade of signaling events and activates various transcription factors such as Nuclear Factor (NF)- B, activating protein-1 and interferon regulatory factors [9 -11]. *Plasmodium falciparum* derived malaria pigment Haemozoins (Hz) are recognized by the endosomal TLR9 resulting in activation of innate immune system through production of inflammatory cytokines, chemokines as well as co-stimulatory molecules [12]. Haemozoin acts as a carrier DNA for *Plasmodium* DNA and facilitates the entry into the host cell that stimulates TLR9 and induces inflammation [11, 13]. The TLR9 gene is located on chromosome 3p21.3 and spans around 5kb. It is composed of 2 exons and encodes 1032 amino acids [14]. The TLR9 gene has been found to be associated with malarial pathogenesis in both animal and humans studies. In animal studies, it has been reported that the mice deficient in TLR9 gene survived better than the wild type mice from cerebral malaria [13]. Blockage of the activation of TLR9 gene by its agonist provides protection against cerebral malaria in mice [15]. Association of Single Nucleotide Polymorphisms (SNPs) in TLR2, 4 and 9 genes with the susceptibility to the infectious disease and inflammatory disease has also been demonstrated earlier [16]. Twelve SNPs in TLR9 gene have been characterized, of which 1486 T→C (rs187084) located in the upstream of promoter has been stated to be important [12]. Previous studies have reported that the C genotype of TLR9 gene is associated with reduced TLR9 gene transcription activity and individuals with this genotype are predisposed to diseases related to TLR9 gene [17] of different underlying pathology including sporadic reports on malaria [18, 19]. Presented herein is a study from tribal population of Assam on the associative role of alterations in TLR9 gene in the pathogenesis of *P. falciparum* induced malaria.

Material and Methods:

Study Population

The present study was conducted for a period of four years from 2012 to 2016 in the state of Assam lying Northeast India. The study population (n=150) included samples from tribal origin namely, *Karbi* (N=63), *Dimasa* (N=37) and *Boro-Kachari* (N=50) collected from Civil hospital, Diphu, District- Karbi Anglong and Community Health Centre, Rani, District- Kamrup, Assam (Table 1). These two districts are highly malaria endemic where *P. falciparum* has been known to be the major causative agent of developing malaria (Fig. 1). All blood samples (2ml) were collected

Table 1: Details of Blood Samples Analyzed in the Study

Tribes	Malarial Samples (N)	Healthy controls (N)
Karbi	63	60
Dimasa	37	40
Boro-Kachari	50	50
Total	150	150

N – Represents Number

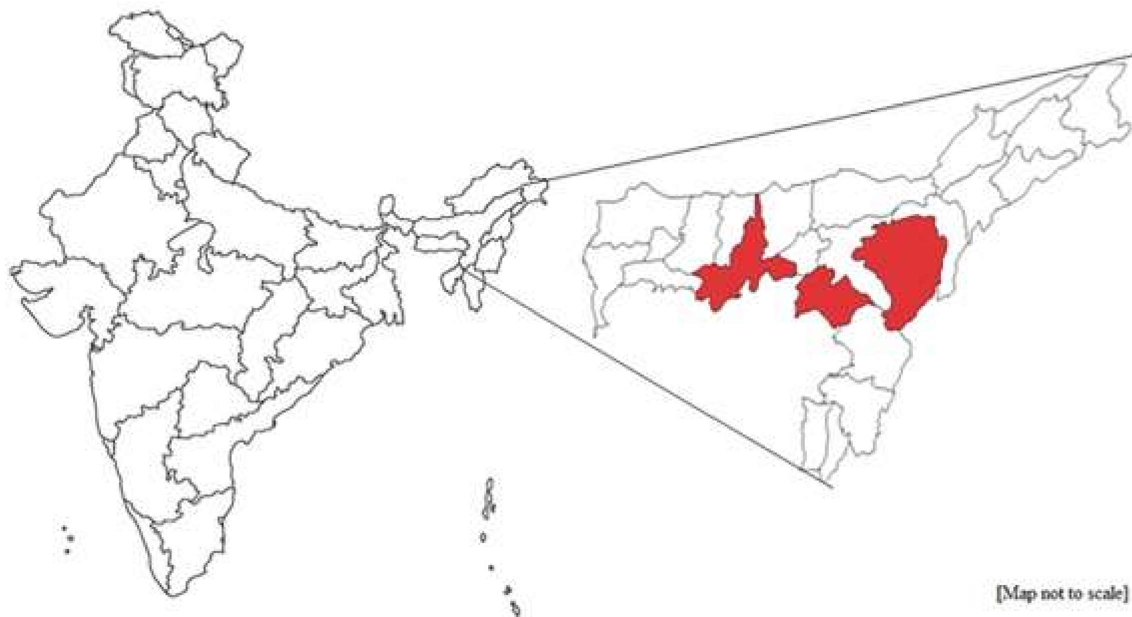

Fig. 1: Showing map of India (Left) and Assam (Inset) with Highlighted Area as the Representative Sites of Sample Collection

from baseline cases showing symptoms of malaria under clinical supervision. The cases that were screened positive for *P. falciparum* infection by RAPID test (CTK Biotech, Cat. No-R0112C, USA) and blood smear examination (N=150) were included in the study. Patients with any other medical complications like inflammatory diseases like encephalitis, jaundice and typhoid were excluded in the present study. Patients below 18 years and above 60 years and pregnant cases were excluded from the present study. The study was approved by the Institutional Ethics Committee, Assam University Diphu Campus (Date-1/09/15). All subjects were enrolled in the study with informed consent. Whole blood (1ml) was also collected from age and sex matched voluntary blood donors (N=150) with no past history of clinically diagnosed malaria.

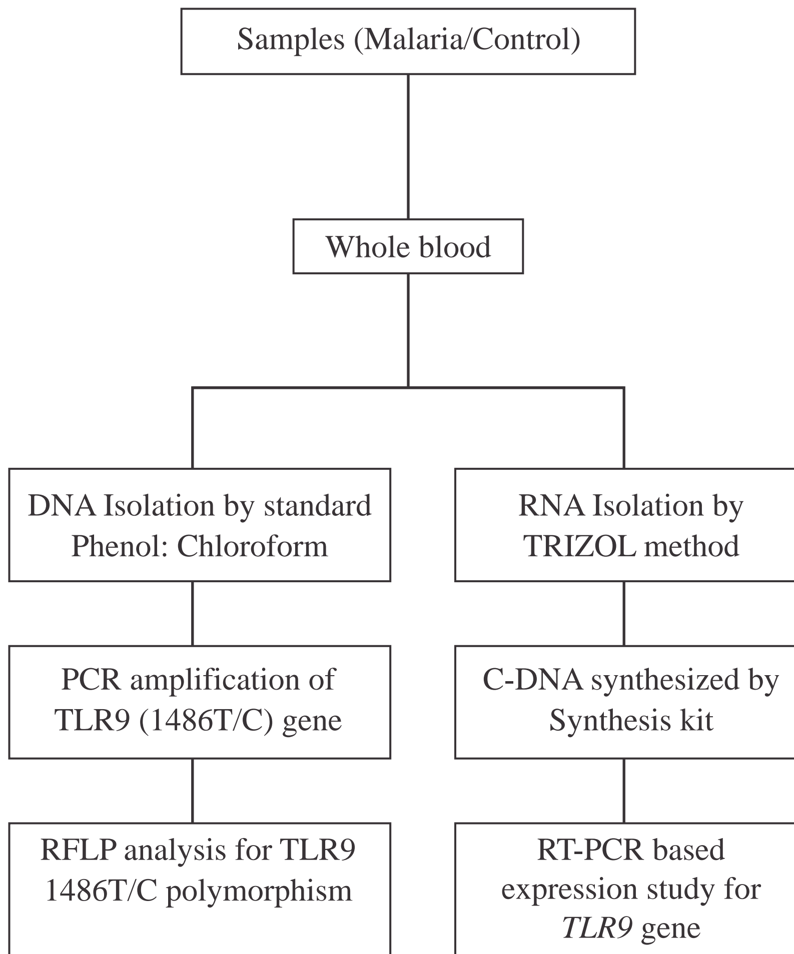

Fig. 2: Flowchart of the Laboratory Procedure

Study Design

The Laboratory procedure was followed according to the flowchart mentioned in Fig. 2.

Screening of distribution of TLR9 1486T/C (rs187084) polymorphism

Detection of TLR9 1486T/C (rs187084) polymorphism in the study cohorts was done by using PCR-RFLP method. Genomic DNA was extracted from 200µl of whole blood by using the standard Phenol-chloroform method [20]. Extracted DNA samples were quantified by Nanodrop (*Nano Vue plus,* Germany) spectrophotometer. The PCR based amplification was performed using primers specific for the TLR9 gene {F: TTCATTCAGCCTTCACTCAG, R: TCAAAGCCACAGTCCACAG} for a total reaction volume of 20µL each. The amplified products (6µL) were screened for the presence of 558bp amplicon by using 1.8% agarose (Cat. no-16500-500, Invitrogen, USA) gel. The PCR products (14µL) were subjected to restriction digestion using the restriction enzyme *Afl*II (Cat. No-R0520S, New England Biolabs, USA), and the RFLP products were analyzed on the basis of band pattern on a 2.5% agarose gel. The wild-type

cases were represented by presence of 413 bp and 145 bp, while the homozygous variant allele was represented by an uncut band of 558 bp. Randomly selected 10% of the samples were re-genotyped in a blinded manner to validate the genotyping results. Statistical analysis was performed by using SPSS software 13.0. The Mann-Whitney U test was performed to correlate the TLR9 SNP at its expressional level. Odds ratios and the corresponding 95% Confidence Intervals (CI) were calculated to determine the associative role between the genetic variation in the given gene and risk of malaria overall and severe malaria. The level of significance for all statistical tests was defined as $P < 0.05$ which was considered to be of significant importance.

Differential TLR9 mRNA expression analysis

Total RNA was isolated from 200µL of whole blood sample using trizol reagent (Cat. No-15596018, Invitrogen, USA). The quality and the quantity of total RNA isolated were checked by Nanodrop. A total of 2µg of total RNA was converted to cDNA using commercially available cDNA synthesis kit (SKU NO- 4368814, Applied Biosystems, US). The differential TLR9 mRNA expression analysis in UC-M and severe malaria cases was compared to healthy controls by Real-time PCR (7500 Real Time PCR, Applied Biosystems, US) analysis using *-actin* as the internal normalization control, and SYBR green chemistry. The fold change was calculated by the 2^{-} C_t method; and difference in expression in studied cohorts was analyzed by SPSS Version 20.0 software.

Results:

Demographical and clinical profile

This study aimed to determine whether the genotype variant of TLR9 gene was associated with malarial severity. An association analysis was performed using case-control cohorts from the different major tribal groups of populations of Assam. Majority of the patients were male (57.77%) (Table 2).

TLR9 1486T/C (rs187084) genotyping analysis

The TLR9 1486T/C (rs187084) polymorphism distribution was studied to analyze malarial susceptibility by PCR-RFLP method. Presence of variant TLR9 genotype (CC) was found to be associated with increased susceptibility to malaria infection [OR=1.717, p=0.141] compared to healthy controls (Table 3).

Table 2: Details of the Demographic and Clinical Profile of the Enrolled Cases

Tribes	Total sample numbers (N)	Average age (in years)	Gender	Clinical manifestations		
			Male	Female	UC-M cases	Severe malaria cases
Karbi	63	37.2±13.96	36[57.14]	27[42.86]	51[80.95]	12[19.95]
Dimasa	37	33.82±12.14	23[62.16]	14[37.84]	34[91.89]	3[8.21]
Boro-Kachari	50	28.06±9.32	27[54.00]	23[46.00]	40[80.00]	10[20.00]

Cases represented as N [%age]

Table 3: TLR9 1486T/C (rs187084) Polymorphism Distribution in Controls and Malaria Cases

Cohort	Numbers (N)	TLR9 (1486T/C)			Variant allele	p value	Odds ratio at 95% CI
		Wild type	Heterozygous	Homozygous			
Healthy Control	150	133[88.66]	15[10.00]	2[1.33]	17[11.33]	Ref	1.717 {0.893-3.304}
Malaria cases	150	123[82.00]	20[13.33]	7[4.66]	27[18.00]	0.103	

Cases represented as N [%]. P<0.05 was considered significant.

Moreover, the presence of the homozygous genotype increased the risk of malaria susceptibility by more than three folds [OR=3.622(0.740-17.731) at 95% CI, p=0.173] compared to controls (p=0.091). When the difference in distribution of the TLR9 variant genotype was studied, it was found that the presence of the TLR9 variant genotype (C-allele) was associated with increased susceptibility among *Karbis* (p=0.393), *Dimasas* (p=0.345) and *Boro-kacharis* (p=0.300) compared to the healthy controls of the respective groups (Table 4).

Table 4: TLR9 1486T/C (rs187084) Polymorphism Distribution in Controls and Malaria Cases in Individual Tribal Population Cohorts

Cohort	Numbers (N)	TLR9 (1486T/C)			Variant allele	p value	Odds ratio at 95%CI
		Wild type	Heterozygous	Homozygous			
Karbi							
Healthy Control	60	52[86.66]	7[11.66]	1[1.66]	8[13.33]	Ref	1.529 {0.577-4.052}
Malaria cases	63	51[80.95]	9[14.28]	3[4.76]	12[19.05]	0.467	
Dimasa							
Healthy Control	40	38[95.00]	2[5.00]	0[0.00]	2[5.00]	Ref	2.303 {0.396-3.390}
Malaria cases	37	33[89.19]	3[8.11]	1[2.70]	4[10.81]	0.419	
Boro-kachari							
Healthy Control	50	43[86.00]	6[12.00]	1[2.00]	7[14.00]	Ref	1.733 {0.611-4.912}
Malaria cases	50	39[78.00]	8[16.00]	3 [6.00]	11[22.00]	0.436	

Cases represented as N [%]. P<0.05 was considered significant

When TLR9 1486T/C (rs187084) polymorphism distribution was analyzed in respect to malaria disease severity by PCR-RFLP method, presence of variant TLR9 genotype was found to be significantly associated with increased susceptibility to severe malaria infection [OR=2.625, p=0.047] compared to uncomplicated malaria (UC-M) (Table 5). Moreover, the presence of the homozygous genotype increased the risk of severe malaria susceptibility by more than four folds [OR=4.125{0.863-19.716) at 95%CI, p=0.091] compared to UC-M (p=0.058). The data therefore shows the associative role of TLR9 1486T/C (rs187084) polymorphism in malaria disease susceptibility and severity is significant with respect to the population under study.

Differential expression of TLR9 gene in malaria pathogenesis

The differential mRNA expression of TLR9 gene was studied by Real-time PCR. The expression of TLR9 was up regulated in the malaria cases compared to healthy controls (1.348 ± 0.895 folds). But with respect to the stratified cohorts, the TLR9 expression was up-regulated in UC-M cases compared to controls, while in the severe malaria cases it was found to be down-regulated when compared to healthy controls (Fig. 3). The expression of TLR9 was found to be sharply down regulated in severe malaria cases as compared to UC-M cases (p=0.312).

Next, the TLR9 gene expression data was correlated with the difference in TLR9 1486T/C (rs187084) polymorphism distribution data. Data indicated that the presence of TLR9 1486 variant C allele was inversely associated with the expression of TLR9 gene in both the stratified malaria disease cohorts (UC-M and severe malaria) compared to controls (Fig. 4). The TLR9 mRNA expression was significantly down regulated in UC-M cases with variant C genotype cases compared to wild type in cases of the same cohort (p=0.039).

Table 5: TLR9 1486T/C (rs187084) Polymorphism Distribution in Malaria Cases

Cohort	Numbers (N)	TLR9 (1486T/C)			Variant allele	p value	Odds ratio at 95%CI
		Wild type	Heterozygous	Homozygous			
UC-M cases	125	106[84.8]	15[12.00]	4[3.2]	19[15.2]	Ref	2.625
Severe malaria cases	25	17[68.00]	5[20.00]	3[12.00]	8[32.00]	0.082	{0.993-6.939}

Cases represented as N [%]. P<0.05 was considered significant.

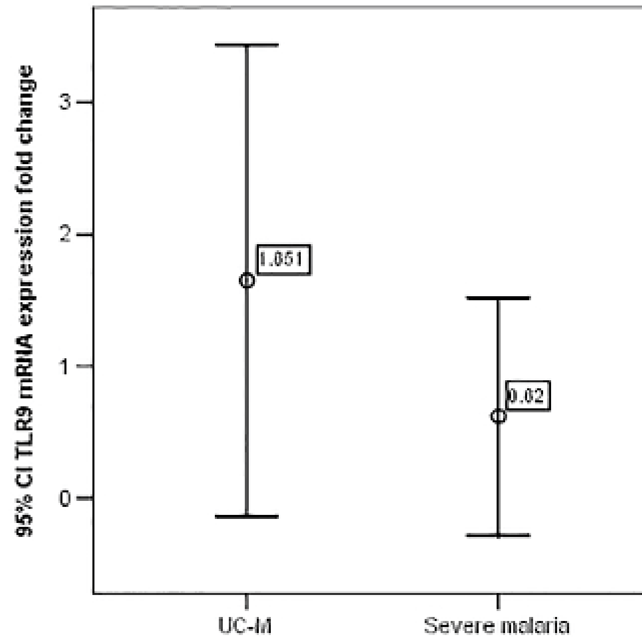

Fig. 3: TLR9 m-RNA Expression in Uncomplicated Malaria (UC-M) and Severe Malaria Compared to Healthy Controls

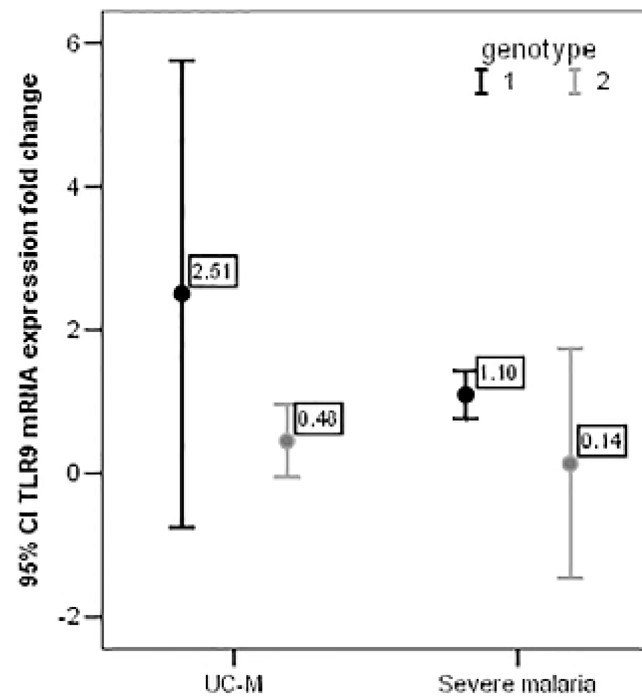

Fig. 4: Difference in TLR9 M-RNA Expression Based on TLR9 1486T/C SNP in UC-M and Severe Malaria

Discussion:

Role of TLRs in the recognition of *P. falciparum* derived pigment and malarial pathogenesis is well established. MyD88, the central mediator of all the TLRs (except TLR3) and IL-1 signaling, was found to be required for the efficient production of IL12 in dendritic cells by of *Plasmodium berghei* parasites [21]. Both TLR-2 and 4 have been established to recognize *P. falciparum* derived Glycosylphosphatidylinositol (GPI) moieties [10]. Moreover, malarial schizont activates the dendritic cells that involve TLR-9, caused by malarial pigment hemozoin, heme polymer formed during degradation of red blood cell hemoglobin by parasites [22, 12].

The TLR9 gene polymorphisms are known to have cis-regulatory effect on TLR9 expression [23] and it modulates the expression of host inflammatory cytokines during severe malarial infection [24]. TLR9 1486 T/C polymorphism has been reported to increase the risk of low birth among infants in malaria infected pregnant women and increase the parasitemia level in adults having mild malaria [18, 19]. In this cohort study, the TLR9 gene polymorphism 1486T/C (rs187084) present within the promoter region of the gene was associated with the increased susceptibility {p=0.141} to malarial infection among the three studied tribal population group's viz. *Karbi*, *Dimasa* and *Boro-Kachari*; contributing more than three folds of higher risk as compared to healthy controls. Previous smaller population based studies in Brazil, Iran, and Ghana reported the association of TLR9 gene polymorphisms with development of the mild malaria [19, 25]. Although, studies with conflicting results where the given SNP did not show any major role in malaria manifestations have also been reported from Iran [5]. No association of any SNPs of the TLR9 gene with the manifestation of severe malaria was also reported from Malawi and Gambia in a family and population based study [23]. Interestingly, the TLR9 1486T/C genotype was found to be negatively associated with complicated malaria and conferring protection against malarial infection in two genetically distinct populations of Assam namely Austro-Asiatic and Tibeto-Burman in the state of Assam where the current study was also undertaken [26]. To date, the role of the given promoter SNP is unclear, but our data supports a major role of this polymorphism in disease progression from uncomplicated malaria to severe form of the disease. In the context of severe malaria, the given variant genotype (CC) was seen significantly associated with increased susceptibility towards severe malaria as compared to uncomplicated malaria with more than four folds higher risk. Whereas, this discrepancy may be explained by the differences in different phenotypes and different population examined. Therefore, the specific role of TLR9 1486T/C SNP on malaria disease progression needs to be further investigated in different ethnic groups. Numerous recent studies have also identified putative regulatory variants and correlated genetic variability with total gene expression [27-30].

Previously, it had been reported that the given allele in combination with G+ 1174A (rs 352139G) allele regulates TLR9 expression whereas, G+ 1174A (rs 352139G) allele combines with rs 187084 allele which poses an up-regulatory effect on TLR9 expression in systemic lupus

erythematosus patients [17]. Induction of the innate immune response via TLR2, TLR9, and MyD88, plays a critical role in the development of cerebral malaria was also previously demonstrated [13]. A study on knockout mice however contradicted the previous findings to and demonstrated that development of cerebral malaria is independent of TLR2, TLR4 and TLR9 gene expression [31]. Association of TLR9 SNPs has also been reported in Japanese ulcerative colitis but no correlation between its expression and the disease progression was observed [32]. We measured the expression of TLR9 gene at m-RNA level on the different study cohorts and found the expression of TLR9 gene enhanced in patients having UC-M as compared to healthy controls which could possibly be an outcome of binding to malarial pigment, Haemozoin as suggested by previous study [33], while a sharp down-regulation of the TLR9 gene was observed in cases of severe malarial as compared to age and sex matched healthy individuals. Upon correlating these findings with the given SNP data, an association of TLR9 1486T/C with the disease susceptibility ($p=0.141$) was observed, indicating that the CC genotype significantly induces the risk of malarial infection as compared to healthy controls whereas the distribution of genotype variants may increase the progression of severe malaria as compared to UC-M. In a previous study involving smaller sample size in the same study area, the association between the SNPs in promoter region of TLR9 gene with the malarial disease was reported but interestingly no difference in mRNA levels between healthy control and the disease cases was observed [26]. The significant down regulation of mRNA expression in UC-M cases as observed in this study with variant C genotype cases against that of wild type suggests that the given TLR9 1486T/C (rs187084) SNP located at the promoter region, may down regulate transcriptional level of TLR9 gene and may play an important role in malaria disease pathogenesis through differential immunomodulation.

Conclusion:

Based on the present study, it can be said that the genetic polymorphism of TLR9 gene differentially modulates its expression in relation to the susceptibility and severity of malaria infection in the studied population. The data is suggestive of the prognostic significance of TLR9 1486T/C genotype and its probability as a therapeutic target for control of severe malaria. However, more elaborate studies with different population groups would be required to establish the role and association of TLRs in malaria pathogenesis especially in the malaria endemic regions where the disease is associated with high mortality.

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*[*]**Author for Correspondence:** Ramie Husneara Begum, Department of Life Sciences and Bio-informatics, Assam University Diphu Campus, Diphu-782462, Karbi Anglong, Assam, India*
Email: ani.ara73@gmail.com, ramie.h.begum@aus.ac.in Cell: +91-9435551442