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RESEARCH **Open Access**

Dinucleotide (AC)n Repeat Polymorphism (rs36213840) in the Promoter Region of IL18R1 Gene and Genetic Susceptibility to Severe Malaria

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Abstract

Background: Cytokines are key regulators of human immune response to malaria but polymorphisms within the regulatory or coding regions of their genes may lead to differences in expression levels which may consequently influence disease susceptibility. In this study, we characterized an adenine-cytosine $(AC)_n$ dinucleotide repeat polymorphism (rs36213840) at the promoter region of the Interleukin 18 Receptor 1 (IL18R1) gene and investigated its association with severe malaria.

Methods: We utilised the case-control study design to enrol a total of 207 children including 87 severe malaria cases and 120 asymptomatic controls. DNA was extracted from blood spot on filter paper using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). Genotyping for dinucleotide repeat polymorphisms was done by PCR and capillary electrophoresis of sequenced products on ABI PRISM® 3100 DNA sequencer (PE Applied Biosystems).

Results: The genotype frequencies of the dinucleotide repeats differed significantly between the two groups ($\chi^2 = 8.69$, $P=0.026$). We found a significantly higher frequency of the 14bp $(AC)_7$ allele in severe malaria patients than in asymptomatic controls (odds ratio 1.945, 95% CI: 1.23 - 3.09, $P = 0.005$) while the frequency of the 16bp $(AC)_8$ allele was significant higher in the asymptomatic controls than in severe cases (odds ratio 0.431, 95% CI: 0.244 - 0.761, $P = 0.004$).

Conclusion: Results of this suggest that the 14bp $(AC)_7$ dinucleotide repeats might be a genetic risk factor for susceptibility to severe malaria while the 16bp $(AC)_8$ dinucleotide repeats might be a protective factor against severe malaria

Keywords: Dinucleotide repeat polymorphism, Malaria, Microsatellite, Interleukin 18 receptor, Nigeria

1.0 INTRODUCTION

Malaria is the world's most prevalent parasitic disease and a major public health challenge, particularly in sub-Saharan Africa. Each year, over 200 million new cases of malaria and about half a million deaths occur globally, mostly in children below the age of five [1]. Malaria is caused by infection with apicomplexan parasites of the genus Plasmodium and five species are known to infect humans including: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi.* Of these, *P. falciparum* is the most virulent and the major cause of severe malaria and malaria-associated deaths [1].

The pathogenesis of severe malaria is not completely understood but there is growing evidence that host and parasite genetic factors may be involved [2]. Genetic polymorphisms in host genes, particularly in genes coding for cytokines or cytokine receptors, have been reported to regulate immune responses to malaria [3-6]. Cytokines are key components of the host immune system that mediate, regulate and influence the function of other cells through specific receptor binding and are crucial in the control of malaria [7-9].

Interleukin 18 (IL18) is a pro-inflammatory cytokine involved in the induction and regulation of both T helper type 1 (Th1) and T helper type 2 (Th2) immune responses [10]. IL18 acts by binding to a heterodimeric surface receptor comprising an alpha chain (Interleukin 18 Receptor 1 or IL18R1) which is responsible for extracellular binding and a beta chain (Interleukin 18 Receptor beta or IL18Rβ) which is a nonbinding, signal transducing chain [11-13]. As with proteins generally, the expression of IL18R1 is controlled by a gene, the IL18R1 gene, and the host's ability to produce increased expression levels may be associated with genetic polymorphisms in the IL18R1 gene [14].

The gene that encodes IL18R1 cytokine (Gene ID: 8809) is located on chromosome 2q12.1 (https://www.ncbi.nlm.nih.gov/genome/gdv/browser/gene/?id=8809)[15]. The promoter region of the IL18R1 gene, at approximately 430bp position upstream of the transcription start site (Figure 1), consists of a dinucleotide sequence repeat element, which is the adenine-cytosine (AC)n dinucleotide repeat polymorphism (rs36213840). Such sequence repeats, consisting of two to six base pairs (bp) are known as short tandem repeats (STRs) or microsatellites. They constitute one of the most abundant types of repetitive elements in the human genome and are frequently encountered in promoters, where they frequently induce multiple-base pair variations [16,17].

Although several studies have tested the association of IL18R1 gene variants with various inflammatory diseases, cancer and some infectious diseases [18-24], only scanty data is available on the association of genetic polymorphisms in the IL18R1 gene with malaria [25]. Furthermore, there is no available data on the role of IL18R1 rs36213840 dinucleotide repeats in malaria, although dinucleotide repeat polymorphisms in other genes have previously been suggested to play a role in malaria susceptibility [26,27]. The aim of this study therefore, was to characterize the $(AC)_n$ dinucleotide repeat polymorphism (rs36213840) at the promoter region of IL18R1 in *P. falciparum* infected individuals and to evaluate its association with severe malaria.

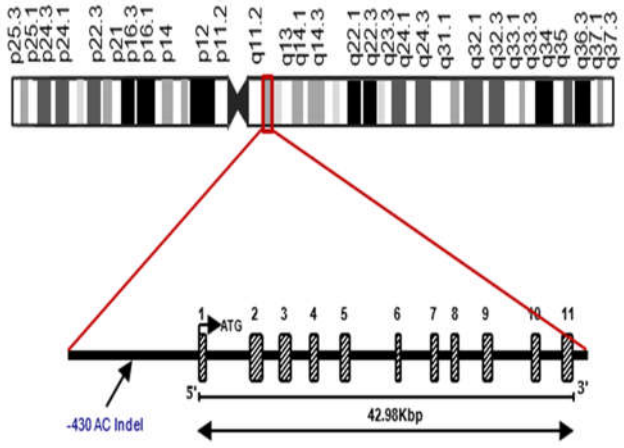

Figure 1. Annotated diagram of IL18R1 gene showing the position of the -430 (AC)n dinucleotide repeat polymorphism (rs36213840). The (AC)n dinucleotide repeat is located in the promoter region of IL18R1 gene at approximately 430 base pair position upstream of the transcription start site, on chromosome 2q12.1 (Genomic coordinate = Chromosome 2:102362229 in the GRCh38.p13 Genome Assembly).

2.0 METHODOLOGY

2.1 Study Site and Participants

This study was conducted in Lafia, a city located about 190km away from Abuja, the capital of Nigeria. Lafia is situated within the middle belt region in north-central Nigeria and lies within Nigeria's Guinea savannah

ecological zone as previously described [28] and malaria transmission is known to be stable and intense in the region [29].

The study participants were paediatric population of both gender, aged 9 months to 154 months. They were enrolled between 2006 and 2011 into the project on "determinants of disease outcome in *P. falciparum*-infected children in Lafia, North-central Nigeria. All the participants were *P. falciparum* infected individuals, categorized into two groups based on clinical presentation: those with severe clinical features (cases) and those without any clinical feature (controls). Severe malaria was defined as presentation with current or recent febrile illness in the presence of asexual forms of *P. falciparum* in peripheral blood smear with no other obvious cause for symptoms, and with one or more of the following: impaired consciousness or cerebral malaria assessed using the Blantyre coma scale of ≤2 (unrousable coma); hyperparasitaemia corresponding to >5% infected cells (>250,000 parasites/µl); severe anaemia (haematocrit <15%); or hypoglycaemia (serum glucose < 2.2 mmol/L or <40 mg/dL). Children who presented with severe malaria were enrolled at the Dalhatu Araf Specialist Hospital, Lafia. Children who lived in the same geographical area and resided within a 3km radius of the hospital were enrolled into the control (asymptomatic group). Asymptomatic infection was defined as infection with microscopically confirmed *P. falciparum* parasites (<5,000 parasites/µl) without symptoms of malaria or any other febrile illness. The study protocol was first explained to the parent or legal guardian of the children before enrolment and then informed consent was obtained. Participation in the study was voluntary and participant's information was kept confidential. The study was approved by the ethics research committees of the Nasarawa State Ministry of Health and Dalhatu Araf Specialist Hospital, Lafia, Nasarawa State, Nigeria.

2.2 Sample Collection and Parasite Identification

Blood samples (1ml) were collected from all participants in EDTA tube for laboratory investigations. Three drops of blood were also spotted on 3MM Whatman filter paper, air dried and sealed in plastic bags for molecular analysis. All samples were coded with alphanumeric identifiers in order to maintain confidentiality of participants. Thick and thin blood smears were prepared and stained with freshly prepared 5% Giemsa stain for 20 minutes. Blood films were examined for malaria parasites under high-power oil immersion objectives at 1000× magnification. Parasitaemia was quantified relative to approximately 250 white blood cells (WBC) on thick films and estimated as parasites per microlitre (µl) assuming a mean WBC of 8,000 per µl of blood as described previously [28]. Blood smears were labelled negative if no parasites were seen after examination of 200 oil immersion fields. Blood haemoglobin levels were estimated by haematocrit measurement of packed cell volume (PCV) using the micro-haematocrit centrifuge.

2.3 DNA Extraction and IL18R1 -430 $(AC)_n$ Dinucleotide Repeat Typing

DNA was extracted from the dried blood spots on filter paper using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The section containing the dinucleotide repeats was amplified by polymerase chain reaction (PCR) using the primer pairs: 5'-AGCCCAGGTTTGTGTGTTTC-3' and 5'-ACATTCTTCCTCAATTACTCATGAA-3' as forward and reverse primers respectively. Amplification reaction was performed in a final volume of 25µl containing 12.5µl of Go Taq® Green Master Mix (Promega Madison, USA) and 2.0µl of each primer (10µM) with the following cycling condition: initial denaturation at 94°C for 3 minutes followed by 35 cycles at 94 °C for 40 seconds, 62 °C for 40 seconds and 72 °C for 60 seconds and a final extension period at 72 °C for 3 minutes.

2.4 IL18R1 -430 AC_n Dinucleotide Sequencing and Sequence Analysis

All samples were sequenced in the forward direction using an internal primer: 5'-CCACTGGGACACAGTCA ATG-3' except for heterozygous sequences that were also sequenced in the reverse direction (i.e. directionally sequenced) using the internal primer 5'-GCCTGGTCTA CTAAATCCTGCT-3'.Sequencing reaction was performed on a Biometra T2 Thermocycler (Biometra, Göttingen, Germany) using the ABI BigDye™ Terminator sequencing kit. The sequenced products were then separated by capillary electrophoresis on an ABI PRISM® 3100 DNA sequencer (PE Applied Biosystems, Weiterstadt, Germany). Sequences generated were analysed using the BioEdit sequence alignment software, available online at http://www.mbio.ncsu.edu/BioEdit/

BioEdit.html. The number of dinucleotide repeats were validated by manual inspection.

2.5 Data Analysis

Data were entered into Microsoft® Excel, 2010 (Microsoft Corporation) and analysed using XLSTAT Version 2019.1.2 [30]. Continuous variables were analysed using the Student's t-test. Numerical data not conforming to normal distribution were log-transformed. Allele and genotype frequencies were estimated by gene counting. Deviation from Hardy-Weinberg equilibrium was assessed by Chi-Square (χ^2) test or Fisher's exact test (if the expected value of any cell count was less than 5). Associations were given as odds ratio (OR) with a confidence interval (CI) established at 95%. P-values were adjusted following Bonferroni correction. The level of statistical significance was defined as $P < 0.05$.

3.0 RESULTS

A total of 207 infected children were enrolled into this study after satisfying the inclusion criteria. They included 87 severe malaria cases and 120 children with asymptomatic infection. They comprise 54.8% males and 45.2% females and were in the age range of 9 months to 154 months with mean age (±SD) of 56.8 (±16.8) months. Parasitaemia was significantly higher among severe malaria cases compared with asymptomatic controls ($P < 0.001$). Details of the comparative demographic and clinical characteristics of the study participants are shown in Table 1.

3.1 Genotype and Allele Frequencies

Three genotypes were observed among the study participants: homozygous $(AC)_{7/7}$, heterozygous $(AC)_{7/8}$ and homozygous (AC)8/8 (Figure 2). Distribution of the genotypes was consistent with Hardy-Weinberg equilibrium (Table 2). There was a significant difference in the distribution of the $(AC)_n$ genotypes between the two groups, which remained significant even after applying the Bonferroni correction ($\chi^2 = 8.69$, P=0.026, Table 2). The homozygous (AC)7/7 genotype was more common in the severe malaria group compared to the asymptomatic group (65.5% vs. 45.0%) while the homozygous $(AC)_{8/8}$ as well as the heterozygous $(AC)_{7/8}$ genotype were more common in the asymptomatic group

Table 1. Demographic and clinical characteristics of study participants (n = 207)

Characteristics	Severe Malaria (n=87)	Asymptomatic Control (n=120)	*P*-Value
Age (months)	35.8 (±12.6)*	68.7 (±19.2)*	0.011
Sex			
Male	46 (52.9%)	62 (51.7%)	NS
Female	41 (47.1%)	58 (48.3%)	NS
Age group			
≤ 5 years	56 (64.4%)	47 (39.2%)	0.023
> 5 years	31 (35.6%)	73 (60.8%)	0.037
Mean axillary temperature (°C)	37.8 (±1.3)*	36.4 (±.06)*	0.049
Mean haemato-crit (%)	21 (±8.2)*	32 (±8.2)*	0.028
§GMPD/µl	96,242 (1,018-1,148,620)	896 (120-4,864)	<0.001

n: number of participants; *±Standard deviation in parentheses ; §GMPD: Geometric mean parasite density (range in parentheses)

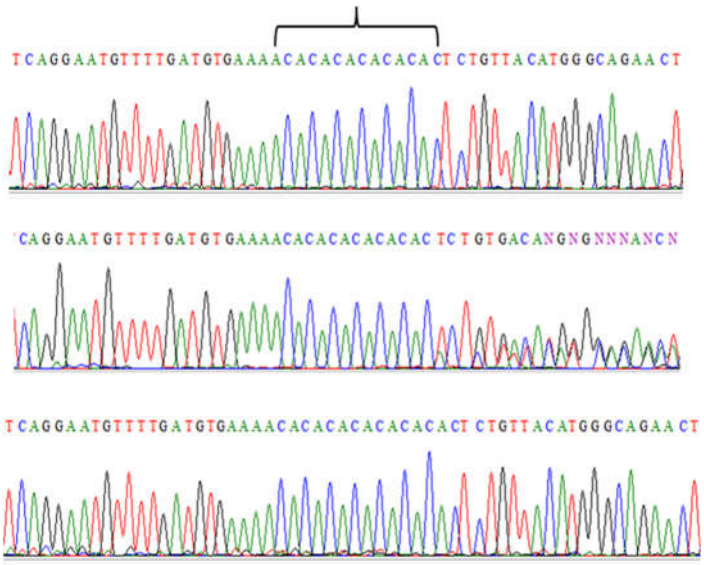

Figure 2. Representative sequence electropherogram of IL18R1 -430 (AC)n dinucleotide repeat polymorphism from this study. The location of dinucleotide repeats is highlighted by the arrow. The upper panel shows homozygous $(AC)_{7/7}$ genotype; the middle panel shows heterozygous $(AC)_{7/8}$ genotype; while the lower panel shows homozygous $(AC)_{8/8}$ genotype. Sequences were viewed using the BioEdit sequence alignment program, with the default nucleotide colour scheme (green for adenine, blue for cytosine, black for guanine, and red for thymine). The ambiguous code N in the middle panel was the default code by the ABI sequencer for unreadable sequences as a result of an additional AC repeat in heterozygous individuals.

Table 2. Distribution of IL18R1 -430(AC)n Dinucleotide Repeat Genotypes in Severe Malaria Patients and Asymptomatic Controls

		Severe Malaria (n=87)				Asymptomatic Control (n=120)			
Gene	SNP(ID)	Genotype	n (%)	MAF	HWE	n (%)	MAF	HWE	*P*-value (corrected)
IL18R1	-430$(AC)_n$ (rs36213840)	$(AC)_{7/7}$	57(65.5)	0.195	0.644	54(45.0)	0.321	0.571	**0.026**
		$(AC)_{7/8}$	26(29.9)			55(45.8)			
		$(AC)_{8/8}$	4(4.6)			11(9.2)			

n, number of participants; **HWE:** Hardy-Weinberg equilibrium; **MAF:** Minor allele frequency

Table 3. Allele Frequencies of $(AC)_n$ Dinucleotide Repeats in Patients with Severe Malaria and in Asymptomatic Controls

	Severe Malaria	Asymptomatic Control		
Allele	n = 174	n = 240	*OR* (95%CI)	*P*-value
$(AC)_7$	140 (80.5%)	163 (67.9%)	1.945 (1.225 – 3.089)	**0.005**
$(AC)_8$	34 (19.5%)	77 (32.1%)		

n, number of alleles; ***OR***: Odds ratio; **CI:** Confidence Interval

(9.2% vs. 4.6%) and (45.8% vs. 29.9%) respectively (Table 2). Two alleles were found in the study population: 14bp $(AC)_7$ allele which is a 14bp sequence consisting of seven $(AC)_7$ dinucleotide repeats and 16bp $(AC)_8$ allele which is a 16bp sequence consisting of eight $(AC)_8$ dinucleotide repeats. The frequency of the 14bp $(AC)_7$ allele was significantly higher in the severe malaria group compared to the asymptomatic group while the frequency of the 16bp $(AC)_8$ allele was significantly higher in the asymptomatic group compared to the severe malaria group (Table 3). For patients with severe malaria, the odds ratio 1.945 (95% CI: 1.225 - 3.089) for the $(AC)_7$ allele vs. $(AC)_8$ allele was significant ($P = 0.005$, Table 3). Meanwhile, the frequency of genotypes containing $(AC)_8$ alleles was also significantly higher in the asymptomatic group compared to the severe malaria group (odds ratio 0.431, 95% CI: 0.244 - 0.761, $P = 0.004$, Table 4).

4.0 DISCUSSION

Interleukin 18 (IL18) is a pro-inflammatory cytokine that enhances the production of interferon-gamma (IFN-γ) which is important for the initial control of malaria parasites [6, 31,32]. Interleukin 18 receptor 1 (IL18R1) gene encodes the alpha chain of IL18 receptor which is critical for IL18 binding and subsequent signal transduction. The IL18R1 gene maps to chromosome 2q12.1 and at approximately 430bp position relative to the transcription start site, it contains an $(AC)_n$ dinucleotide repeat polymorphism (rs36213840) which has not been previously reported in the literature. In this study therefore, we characterized the rs36213840 repeat polymorphism at the promoter region of the IL18R1 gene in P. falciparum infected individuals and investigated its association with severe malaria.

This study found two distinct rs36213840 $(AC)_n$ alleles in the study population: a 14bp $(AC)_7$ allele and a 16bp (AC)

Table 4. Distribution of $(AC)_n$ Genotypes According to the Presence/absence of $(AC)_7$ or $(AC)_8$ Alleles in Patients with Severe Malaria and in Asymptomatic Controls

	Severe Malaria	Asymptomatic Control		
Genotype subgroup	n=87	n=120	OR(95%CI)	*P*-value
Genotypes containing $(AC)_7$ alleles	83 (95.45%)	109 (90.8%)	2.094 (0.64 – 6.81)	0.219
Genotypes without $(AC)_7$ alleles	4 (4.6%)	11 (9.2%)		
Genotypes containing $(AC)_8$ alleles	30 (34.5%)	66 (55.0%)	0.431 (0.244 - 0.761)	**0.004**
Genotypes without $(AC)_8$ alleles	57 (65.5%)	54 (45.0%)		

n, number of participants; ***OR***: Odds ratio; 95% CI, 95% Confidence Interval

$_8$ allele. The results showed that the distribution of the allele and genotype frequencies of the $(AC)_n$ dinucleotide repeats differed significantly between the severe malaria group and the asymptomatic controls. Specifically, our results showed a significantly higher frequency of the 14bp $(AC)_7$ allele in the severe malaria group, suggesting an increased risk of susceptibility to severe malaria (OR = 1.945, $P = 0.005$), while the 16bp $(AC)_8$ allele was the predominant allele in the asymptomatic control group, which suggests that carriage of the 16bp $(AC)_8$ allele probably gives protection against severe malaria (OR = 0.514, $P = 0.004$). In addition, we found a 2.2-fold reduced risk of susceptibility to severe malaria in individuals with heterozygous $(AC)_{7/8}$ repeats.

Although there is no available data on the role of IL18R1 rs36213840 dinucleotide repeat polymorphism in malaria, a previous study has shown that dinucleotide repeat, specifically the (TA) polymorphism on gp91phox gene promoter, does influence the expression of NADPH oxidase and protects against severe malaria [27]. This finding though, was not confirmed to be associated with malaria in a subsequent study elsewhere [33]. Moreover, a dinucleotide (TG) repeat polymorphism in intron 3 of the CD36 gene has been shown to be associated with reduced risk of cerebral malaria by modulating alternative splicing during gene expression [26]. Besides malaria, dinucleotide (GT) repeat polymorphisms has been shown to influence gene expression of Heme oxygenase-1 [34] as well as enhance promoter activity of the FOXP3 gene [35] in patients with acute kidney injury or kidney transplants. In addition, a (TA) dinucleotide repeat polymorphism in the regulatory region of the interleukin-28B gene has been found to be associated with treatment response in patients infected with hepatitis C virus [36].

The mechanism by which the $(AC)_n$ dinucleotide repeat polymorphism could influence susceptibility to severe malaria is not known. It is conceivable however, that variations in the $(AC)_n$ dinucleotide repeats in the promoter region of IL18R1 gene could alter the conformation of binding sites for transcription factors, thereby resulting in differential IL18R1 gene expression. This may modulate IL18 production and consequently influence the clinical phenotypes of malaria. Alternatively, the rs36213840 $(AC)_n$ repeat polymorphism may be in linkage disequilibrium (LD) with yet to be identified locus with modulatory effect on gene expression.

Dinucleotide repeat elements and other microsatellites have been shown to exhibit extensive length polymorphisms by varying the number of times they are repeated and these occur over time either through a process of strand slippage during DNA replication or gene conversion during meiotic recombination [37,38]. The uniform tandem repeats, such as the one in the present study (ACACACACAC) are suggested to even have higher mutation rates than those with compound repeat structures such CACATCACA [39]. These repeat elements are abundant in promoter regions [40] and seem to have regulatory functions since they contribute to variations in gene expression [17] and they could also alter splicing efficiency [41].

The present study has some limitations. First, our study did not measure IL18R1 expression level and consequently, could not establish whether the $(AC)_n$ dinucleotide repeat polymorphism results in enhanced or diminished IL18R1 expression or IL18 cytokine levels. Further studies are therefore required to conduct functional assays in order to elucidate the biological mechanisms underlying the genetic association of the rs36213840 repeat polymorphism with severe malaria. Second, the sample size in this study is relatively small and may limit the statistical power of the study. Although the case-control study design utilised should limit this weakness, further studies with larger sample sizes are recommended to validate our results. Such studies should also investigate whether this rs36213840 polymorphism is associated with specific forms or syndrome of severe malaria, such as cerebral malaria and severe malarial anaemia. On the whole, this study has contributed to evidence of the association of genetic variants in susceptibility to malaria and has bridged an important gap in our knowledge of the role of rs36213840 repeat polymorphism in malaria.

In this study, we analysed the $(AC)_n$ dinucleotide repeat polymorphism in the promoter region of IL18R1 gene. Our$_8$ findings show that the 14bp $(AC)_7$ allele is associated with risk of susceptibility to severe malaria while the 16bp (AC) allele is associated with protection against severe malaria. Our data supports the notion that host genetic factors has a role in the pathogenesis of severe malaria. Further studies are however, required to validate our data and to address the probable mechanism

by which the rs36213840 dinucleotide repeats lead to disease susceptibility.

Conflict of Interest

The authors declare that there is no conflict of interest

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Authors Contribution

SIO conceived and designed the study, performed data collection, analysed data, interpret results and prepare the manuscript; **IMO** contributed to data analysis and results interpretation; **PUB** contributed to data collection; **HOA** contributed to study design and interpretation of results. All authors approved the final version of the manuscript

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