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Single nucleotide polymorphism (rs1035130C>T) in the interleukin 18 receptor 1 gene and susceptibility to severe *P. falciparum* malaria in Lafia, North-central Nigeria

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Abstract

The interleukin 18 receptor 1 (*IL18R1*) gene encodes a potent cytokine receptor that is critical for IL18 binding and subsequent signal transduction. This gene is a member of the IL1 receptor family that resides in a cluster of genes on human chromosome 2. Several polymorphisms have been shown to exist on this gene locus and some were found to be associated with inflammatory diseases but there is paucity of data on association with malaria. This study therefore, was aimed at determining the possible association of the rs1035130C>T coding polymorphism with severe malaria in Lafia, North-central Nigeria. The rs1035130C>T polymorphism was genotyped in a total of 214 participants including 98 severe malaria cases and 116 asymptomatic controls. DNA was extracted from blood spotted on filter paper using the QIAamp® DNA Mini Kit. The ABI PRISM® 3100 Genetic Analyzer was used to sequence the polymorphic locus. Our data showed a significantly higher frequency ($P=0.021$) of the CT heterozygous genotype in the asymptomatic control group (25.0%) than in the severe malaria group (13.27%). In addition, the frequency of the minor T allele was significantly higher ($P=0.013$) in the asymptomatic controls compared to the severe malaria cases. However, the TT homozygous genotype was not found in the severe malaria group. These results suggest a contributory role of the rs1035130C>T polymorphism in regulating host susceptibility or resistance to severe malaria and consequently have implications for understanding the molecular mechanisms of malaria pathogenesis.

Keywords: Malaria, Interleukin, rs1035130C>T, Polymorphism, Genotype.

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Introduction

Malaria continues to be a major global health problem with devastating impact on the health and livelihood of those affected. Even though progress has been achieved in recent times, following the wide-scale deployment of malaria control interventions (Bhatt et al., 2015), an estimated 212 million cases of malaria and 429 000 deaths were reported to have occurred globally in 2015 (WHO, 2016). Most of these cases were in sub-Saharan Africa; and Nigeria is the

largest single country that is most affected, accounting for about 29% of the total malaria cases was reported to have occurred in 2015 (WHO, 2016).

Five species of apicomplexan parasites of the genus *Plasmodium*, are acknowledged to cause malaria in humans, namely: *Plasmodium falciparum*, *P. malariae*, *P. ovale*, *P. vivax*, and *P. knowlesi*. However, *P. falciparum* is the species responsible for most of the severe malaria cases as well as almost all (99%)

malaria-related deaths and is common in sub-Saharan Africa (Murray et al., 2012; WHO, 2016).

Severe malaria is a clinical manifestation of complicated disease that is associated with vital organ dysfunction (Marsh et al., 1995) but its pathogenesis is not fully understood. However, studies have implicated some host and parasite genetic factors. Specifically, there are data showing that components of the host immune responses, especially the balance between pro-inflammatory and anti-inflammatory cytokines, could contribute to the pathological process of malaria (Angulo and Fresno, 2002). These cytokines are diverse groups of proteins that mediate and regulate many critical aspects of innate and adaptive immune responses. They are essential in immuno-regulation as well as in determining disease outcome (Bakir et al., 2011; Sinha et al., 2010). However, their expression and production is essentially controlled by genetic factors, especially single nucleotide polymorphisms (SNPs) within coding and regulatory regions of cytokine-expressing genes (Bidwell et al., 1999).

Interleukin-18 (IL-18) is a pro-inflammatory cytokine that enhances innate immunity as well as T-helper 1 (Th1) component of adaptive immune responses and can synergize with IL-12 to increase activating interferon-gamma (IFN- γ) production and NK cell cytotoxicity (Akira, 2000; Gracie et al., 2003; Nakanishi et al., 2001; Sims and Smith, 2010; Tominaga et al., 2000). Interleukin-18 has been shown to play a protective role in malaria, especially in early immunity against Plasmodium by enhancing IFN- γ production (Singh et al., 2002) and significant increase in IL-18 concentrations has been observed during the acute and recovery phase of malaria, reflecting a pro-inflammatory role of IL-18 in these patients (Torre et al., 2001).

The functions of IL-18 are mediated through the IL-18 receptor which is composed of two chains: IL-18 receptor alpha (*IL18R1* or IL18Ra), which is the extracellular binding domain and IL-18 receptor beta (IL18R β), which is the signal transducing chain (Smeltz et al., 2001). When IL18R1 binds to IL18, it triggers the recruitment of receptor accessory proteins which initiates signalling (Kato et al., 2003).

IL18R1 is a member of the interleukin 1 receptor family and resides in a cluster of genes on chromosome 2 with other members of the IL1 family, including IL1R2, IL1R1, ILRL2 (IL-1Rrp2), IL1RL1 (T1/ST2) and IL-18 receptor accessory protein (Dale and Nicklin, 1999). The human *IL18R1* gene locus is located on chromosome 2q12.1 (https://www.ncbi.nlm.nih.gov/gene?cmd=retrieve&dopt=full_report&list_uids=8809) with about 42.96Kb on the *Entrez Gene Cytogenetic band*, and is composed of 11 exons and 10 introns.

Several polymorphisms have been reported in the *IL18R1* gene locus amongst which is the rs1035130C>T located on position 753 of the coding sequence. The rs1035130C>T polymorphism is a silent mutation also known as synonymous coding polymorphisms because it does not result in amino acid substitution in the transcribed cDNA. It consists of a C-to-T transition (TTC TTT) that both results in the synthesis of Phenylalanine (Phe251Phe), at the 251st amino acid position (Ovsyannikova et al., 2013). In the NCBI SNP database (dbSNP), the rs1035130 alleles were reported in reverse orientation (The rs1035130A/G) to the genome (https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?searchType=adhoc_search&type=rs&rs=rs1035130#locus).

Few studies have shown association of this SNP with diseases, particularly inflammatory diseases including asthma and atopic dermatitis (Reijmerink et al., 2008; Zhu et al., 2008). There is however, paucity of data on the association of this polymorphism with malaria. Given the role of pro-inflammatory cytokines in protection against protozoan parasites, we hypothesize that polymorphisms within the regulatory region of the *IL18R1* gene, a pro-inflammatory cytokine, will be important in determining susceptibility to malaria. This study therefore, was aimed at investigating the possible association of the *IL18R1* rs1035130 C>T (Phe251Phe) coding polymorphism with severe malaria.

Materials and Methods

Study area and population

This study was conducted in Lafia (Latitude 8° 30' N and Longitude 8° 31' E), the capital city of Nasarawa State, in North-central Nigeria. Lafia lies within the Guinea savannah ecological zone in Nigeria. In this region, malaria is an endemic disease and transmission is stable and intense through most part of the year as described earlier (Oyedeji et al., 2007).

Two hundred and fourteen children were enrolled into one of the following two groups: severe malaria (SM) and asymptomatic infections (AS). Children were enrolled into the severe malaria group at the Dalhatu Araf Specialist Hospital, Lafia, if they had asexual forms of *P. falciparum* in peripheral blood smear and at least one of the following: impaired consciousness assessed introduction.

Malaria continues to be a major global health problem with devastating impact on the health and livelihood of those affected. Even though progress has been achieved in recent times, following the wide-scale deployment of malaria control interventions (Bhatt et

al., 2015), an estimated 212 million cases of malaria and 429 000 deaths were reported to have occurred globally in 2015 (WHO, 2016). Most of these cases were in sub-Saharan Africa; and Nigeria is the largest single country that is most affected, accounting for about 29% of the total malaria cases was reported to have occurred in 2015 (WHO, 2016).

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Severe malaria is a clinical manifestation of complicated disease that is associated with vital organ dysfunction (Marsh et al., 1995) but its pathogenesis is not fully understood. However, studies have implicated some host and parasite genetic factors. Specifically, there are data showing that components of the host immune responses, especially the balance between pro-inflammatory and anti-inflammatory cytokines, could contribute to the pathological process of malaria (Angulo and Fresno, 2002). These cytokines are diverse groups of proteins that mediate and regulate many critical aspects of innate and adaptive immune responses. They are essential in immuno-regulation as well as in determining disease outcome (Bakir et al., 2011; Sinha et al., using the Blantyre coma scale of ≤ 2 (unrousable coma), hyperparasitaemia corresponding to $>5\%$ infected cells ($>250,000/\mu\text{l}$), severe anaemia (haematocrit $<15\%$), hypoglycaemia with serum glucose < 2.2 mmol/L or <40 mg/dL. Children were enrolled into the asymptomatic group if they resided within 3km radius of the hospital, and had asexual forms of *P. falciparum* in peripheral blood smear with no symptoms of malaria, no history of febrile illness or of antimalarial drug use in the preceding 2 weeks and a measured axillary temperature $<37.5^\circ\text{C}$. Informed consent was obtained from parents or guardians of participant before enrolment. The Ethics Review Committees of both the Dalhatu Araf Specialist Hospital and the Nasarawa State Ministry of Health, Lafia approved the study.

Blood sample collection

Venous blood was collected into sterile vacutainers containing ethylenediaminetetra acetic acid (EDTA) for laboratory investigations. Three drops of blood were blotted on 3MM Whatman filter paper from the blood collected for molecular analysis.

Parasitological examination

Thick and thin blood smears were prepared on a clean, grease-free glass from the blood collected

and were stained with 5% Giemsa stain for 20 minutes. They were then examined for malaria parasites on a high-power x100 objective lens. Parasite density was estimated relative to 250 leukocytes on thick blood films and quantified as parasites per microlitre (μl) assuming a mean leukocyte count of 8,000 per μl of blood as described earlier (Oyedeji et al., 2013).

DNA extraction and PCR

The QIAamp[®] DNA Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from the blood blotted on filter paper, in accordance with the manufacturer's instructions. PCR was performed in a reaction volume of 25 μl containing 2.0 μl of DNA template, 2.5 μl x10 reaction buffer, 200 μM of each dNTPs (dATP, dGTP, dTTP, and dCTP), 0.75 units of Taq DNA polymerase and 0.25 μM each of the forward (5'-GCA CCA CGT TTT GCT TTA GG -3') and reverse (5'-ATA CAC ATC AGC CAC CCA GTG-3') primers. Primers were designed by the authors with the aid of two software programs that are freely available on the Internet, for use to the scientific community: Primer3 (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi>) and ExonPrimer (<http://ihg.gsf.de/ihg/ExonPrimer.html>).

Following design of primers, oligonucleotide sequences were sent for synthesis at Operon Biotechnologies GmbH, Cologne, Germany. Amplifications were performed using the following cycling conditions: denaturation at 94°C for 3 minutes followed by 35 cycles at 94°C for 40 seconds, 64°C for 40 seconds and 72°C for 1 minute and a final extension period at 72°C for 3 minutes. The amplified products were separated by electrophoresis on 1.5% agarose gels after staining with SYBR[®] Green, and visualized under ultraviolet light trans-illuminator.

IL18R1 rs1035130 genotyping

Amplified PCR products were purified with an E.Z.N.A.[®] Cycle Pure Kit (Peqlab Biotechnology GmbH, Erlangen, Germany) following the manufacturer's instructions and direct automated sequencing of the PCR products was performed using the Big Dye terminator reaction mix (PE Biosystems, Weiterstadt, Germany). Each amplicon was sequenced in the forward direction using the forward primer stated above. Sequencing amplification was performed in a reaction volume of 10 μl using 40ng of PCR product as template. Sequenced products were then separated by capillary electrophoresis using the ABI PRISM[®] 3100 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). The DNA sequences generated were imported for alignment into BioEdit sequence alignment software for analysis.

Statistical analysis

Data were analyzed using Stata version 9.2 (StataCorp, College Station, Texas). Student's t-test was used to analyze normally distributed variables. Departures from Hardy-Weinberg equilibrium (HWE) were assessed using the Pearson goodness-of-fit test (χ^2 test). This was also used to compare differences in gene and allele frequencies between the severe malaria group and the control group. P value <0.05 was considered to be statistically significant.

Results

Genotyping was successfully performed for the 214 children enrolled into the study which includes 98 severe malaria cases and 116 asymptomatic controls. There was no significant difference between the two groups in terms of sex and ethnic background ($P>0.05$). However, there was statistically significant difference in the mean age of children between the two groups, with the severe malaria group having a mean age of 32.1 (± 16.8) months while those in the asymptomatic group had a mean age of 35.9 (± 14.6) months ($P=0.043$). The severe malaria group had significantly higher parasite densities, with a geometric mean parasite density of 62,941/ μ l compared with the asymptomatic group, with a geometric mean parasite density of 1,026/ μ l ($P=0.001$).

The distribution of the allele and genotype frequencies of the rs1035130C>T polymorphism in the two groups are presented

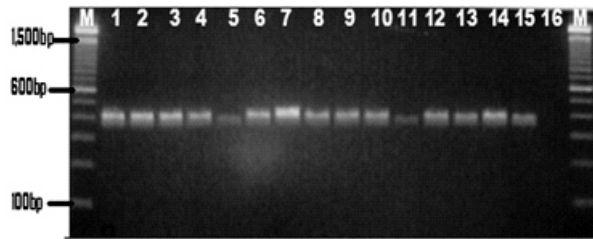


Figure 1: Agarose gel electrophoresis showing ~ 410bp PCR products covering the IL18R1 rs1035130 C>T locus.

Lane M: 100-bp ladder; Lanes 1-15: DNA fragment of study participants; Lane 16: negative control

Locus	SNP ID/	AS (n=116)	SM (n=98)	*P-value
IL18R1	rs1035130C>T			
CC		85(73.28)	85(86.73)	0.562
CT		29(25.0)	13(13.27)	0.021
TT		2(1.72)	0(0)	0.038
C allele		0.858	0.934	0.089
T allele		0.142	0.066	0.013

AS= Asymptomatic Infection

SM= Severe Malaria

SNP ID= Single nucleotide polymorphism identification number

*P-values were calculated from a Chi-squared test.

in Table 1. There was no deviation from the Hardy-Weinberg equilibrium (HWE) in the distributions of the rs1035130C>T genotypes in either the severe malaria group or the asymptomatic control group. The most common genotype in the study population for the rs1035130C>T loci was the homozygote CC, which was marginally higher ($P=0.562$) in the severe malaria group (86.7%) compared to the asymptomatic controls (73.3%). However, the frequency of the CT heterozygous genotype was significantly higher in the asymptomatic control group compared to the severe malaria group ($\chi^2=7.68$; $P=0.021$). Similarly, the frequency of the minor T allele was significantly higher ($P=0.013$) in the asymptomatic control group compared to the severe malaria group (Table 1). The TT homozygous genotype was not found in the severe malaria group. The electrophoretic separation of the 410bp PCR products covering the IL18R1rs1035130C>T locus is shown in Figure 1, while the electropherogram showing the genotype variants of the rs1035130C>T polymorphisms are shown in Figure 2.

Table 1: Genotype and allele frequencies of rs1035130C>T (Phe251Phe)

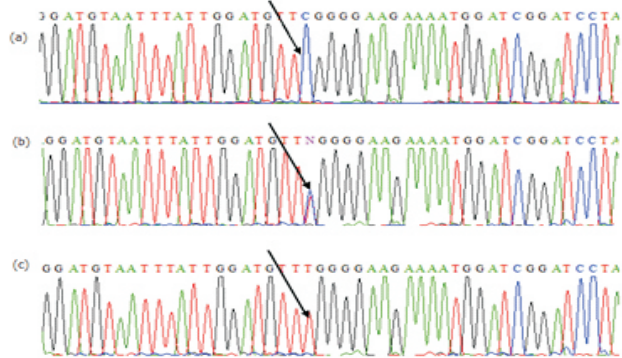


Figure 2: Representative DNA sequence electropherogram from study participants showing the IL18R1 rs1035130C>T polymorphism.

The location of base substitution is highlighted by the arrows: (a) Homozygous IL18R1 rs1035130CC (b) Heterozygous IL18R1 rs1035130CT represented by the ambiguous code N (c) Homozygous IL18R1 rs1035130TT. Sequencing were done using the forward primer.

Discussion

Malaria is a major selective force on the human genome in endemic areas, killing at least half a million people annually before they attain reproductive age. Several immunologically relevant genes have been reported to be associated with the disease but its pathogenesis is still not completely understood. The IL18R1 is a cytokine gene that encodes IL-18 receptor a (IL-18Ra) which forms an IL-

18 signalling complex with IL-18R β and potentially activating IFN- γ , which is crucial for the initial control of parasitaemia (Nasr et al., 2014; Tominaga et al., 2000). The rs1035130 (Phe251Phe) SNP is a synonymous coding polymorphism located on the *IL18R1* gene, that is predicted to have functional effect in splicing regulation during gene expression (Haralambieva et al., 2011).

The rs1035130 is located in the third immunoglobulin-like domain of the extracellular portion of *IL18R1* and is critical for IL-18 binding as well as formation of the ternary (Azam et al., 2003). In this study, we investigated the possible association of rs1035130C>T coding polymorphism in the *IL18R1* gene with severe malaria in North-central Nigeria.

Our data showed that the CC genotype was the most common in the population which is similar to data from the 1000 genomes project 3 on African population from the Ensembl database (http://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=2:102384442-102385442;v=rs1035130;vdb=variation;vf=750076#population_freq_AFR). We however, found a significantly higher frequency of the minor allele genotype (TT) as well as the heterozygous genotype (CT) in the asymptomatic control group compared to the severe malaria group; which probable suggests a protective role of the rs1035130 SNP against severe malaria.

In a previous study, the presence of the homozygous minor allele genotype (TT) or heterozygous genotype (CT) for *IL18R1* rs1035130 polymorphism was significantly associated with an increase in vaccinia virus-specific antibody response (Haralambieva et al., 2011). In addition, the rs1035130 coding polymorphism was found to be significantly associated with a higher IFN- γ enzyme-linked immunospot assay (ELISPOT) and vaccinia-specific neutralizing antibody titers (Ovsyannikova et al., 2013); thus providing evidence to support the regulatory role of *IL18R1* on humoral immune response.

Although there is no available data to show the association of the rs1035130 polymorphism with malaria, there are studies showing association of this SNP with inflammatory diseases including asthma and atopic dermatitis (Reijmerink et al., 2008; Zhu et al., 2008), and inflammatory bowel disease (Zhernakova et al., 2008). In this study, we present data on the association between the *IL18R1* rs1035130 polymorphism and malaria; and we presume that *IL18R1* related pathway may play a role in the pathogenesis of malaria. To our knowledge, this is the first report associating *IL18R1* gene polymorphisms

with malaria and consequently have implications for understanding the molecular mechanisms of malaria pathogenesis. However, further studies, with larger sample sizes and in different geographical regions may be required to confirm our findings.

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