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Tumor Necrosis Factor Receptor II Gene Polymorphism and Association with *Plasmodium falciparum* Malaria in Badagry, Lagos, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author OCO designed the study, wrote the protocol, conducted the experiment, performed statistical analysis and wrote the first draft of the manuscript. Author NAI supervised the work and managed the analyses of the study. Author OOA managed the sample collection study. Author OA co-conducted the experiment and supervised the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Host genetic factors, host-parasite interactions, parasite genetic diversity, and/or environmental influences have been implicated in the molecular basis of susceptibility and resistance to *Plasmodium* parasite infections. Host Tumor Necrosis Factor Receptor II (TNFR II), a 75/80kDa protein encoded by the *tnfr* II gene has been identified as an adhesion protein for *Plasmodium falciparum* parasite. *Tnfr* II gene exhibits functional polymorphism on exon 6 among Caucasians with a potential effect on the ligand-binding function of TNFR II. This study aimed to investigate *tnfr* II gene polymorphisms on exon 6 and its relationship to *Plasmodium falciparum* malaria in Badagry, Lagos Nigeria. Genotyping was done using PCR-Restriction fragment length polymorphism assay. A total of 68 blood samples comprising of 49 controls and 19 malaria cases were genotyped. Result revealed both the wild-type (T587) and a mutant-type (T→587G) genotypes of *tnfr* II. Generally, the analysis showed that the variant-type had a lower prevalence rate (11.76%) than the wild-type

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(88.24%). The frequencies of wild-type were highest in the controls (85.70%) while the frequencies of the mutant-type were lower in the malaria cases (5.30%). However, the Chi-square test showed no statistical significant difference (*p value = 0.30*) between the distribution of the genotypes in malaria cases and controls. This study indicates that polymorphism on exon 6 of *tnfr* II gene was not associated with susceptibility or resistance to malaria infection in Badagry, Nigeria. Further researches with a larger sample size are needed to clarify this subject.

Keywords: Gene polymorphisms; tumor necrosis factor receptors; malaria; tnfr II genes; Plasmodium falciparum; genotypes; parasite antigens; genetic factors.

1. INTRODUCTION

Despite all efforts made at eradicating malaria, it has remained a major public health problem in developing countries of which 93% of the burden is born in Africa [1]. According to the 2018 malaria report, about 219 million malaria cases were recorded worldwide with over 430,000 malaria deaths and the majority (99.7%) were attributed to *Plasmodium falciparum* of which Nigeria had the highest mortality [1]. Worst still is the increasing threat of infection on half of the world's population particularly infants, pregnant women, HIV/AIDS patients, naïve travelers, and non-immune migrants are at risk of malaria [2].

Due to the increasing spread of multidrug-resistant *Plasmodium falciparum* strains and insecticide-resistant strains of the vector, there is an urgent need for additional strategies in the prevention and management of malaria [1,3,4].

Malaria parasites require host receptors for erythrocyte invasion [5]. Once inside the red blood cells, malaria parasites evade the host immune system by binding to adhesion molecules expressed on the erythrocyte surface [6,7].

Certain human receptors, Tumor Necrosis Factor Receptor II (TNFR II) has been identified as an adhesion protein for *Plasmodium falciparum* erythrocyte membrane protein-1 (EMP-1), an invasion ligand for malaria parasites [8]. TNFR II is encoded by the *tnfr II* gene located on chromosome 1p36 [9]. *Tnfr* II gene exhibits functional single nucleotide polymorphism (SNP) at position T587G (dbSNP: rs1061622) on exon 6 among Caucasians [10,11]. This SNP changes the amino acid from Methionine to Arginine (M196R) at the membrane- proximal extracellular domain of TNFRII with a potential effect on the receptor-ligand binding function of TNFRII protein [12]. Researches in other parts of the world have evaluated the association of several known host receptors in susceptibility or resistance to malaria infection [13-17]. However, to date no study has investigated the possible association of *tnfr* II gene polymorphism with susceptibility or resistance to malaria hence the importance of this present study.

This work aimed to investigate the prevalence of polymorphism in *tumor necrosis factor receptor* II gene and its relationship to *Plasmodium falciparum* malaria in Badagry, Lagos, Nigeria. We hypothesized that *tnfr* II gene polymorphisms exist in Badagry population and may influence susceptibility or resistance to malaria infection.

2. MATERIALS AND METHODS

2.1 Study Design and Population

This is a cross-sectional retrospective study involving analysis of archived blood spots for individuals living in Badagry local government in Lagos state (Fig. 1), South western part of Nigeria who gave consent to be screened in the month of April 2017 for a World Malaria day community-based outreach. Badagry is a malaria-endemic area with a peak occurring during the rainy season (April to September). During the field work *Plasmodium falciparum* malaria were screened using Malaria HRP2 (Histidine Rich Protein 2) (Pf) rapid diagnostic test kits (RDT) Access Bio, Somerset NJ, USA) and Malaria microscopy after which their test kits were archived. All individuals who tested positive and confirmed positive by microscopy were treated according to the Federal Ministry of Health guidelines (FMOH, 2005). A total of 68 dried blood samples were included of which 49 were *P. falciparum* malaria negatives and 19 were *P. falciparum* uncomplicated malaria positives. Few malaria positive cases were obtained from the Badagry recruitment site at that time of sampling and there was no budget to sample additional sites.

2.2 Inclusion Criteria

All individuals within the Badagry community who gave approved consent to the collection of their blood and subsequent screening for malaria infection were included in the screening and their blood samples were tested and archived in Malaria HRP2 (Pf) rapid diagnostic test kits. Only archived Malaria HRP2 (Pf) rapid diagnostic test kits with clear red bands at the control lines and/or red bands at the test line were included in the present study for DNA extraction and isolation.

2.3 Exclusion Criteria

Individuals who did not give approved consent to participate were not screened. Blood samples of archived Malaria HRP2 (Pf) rapid diagnostic test kits that showed no defined red bands at the control lines were considered invalid and excluded from this study.

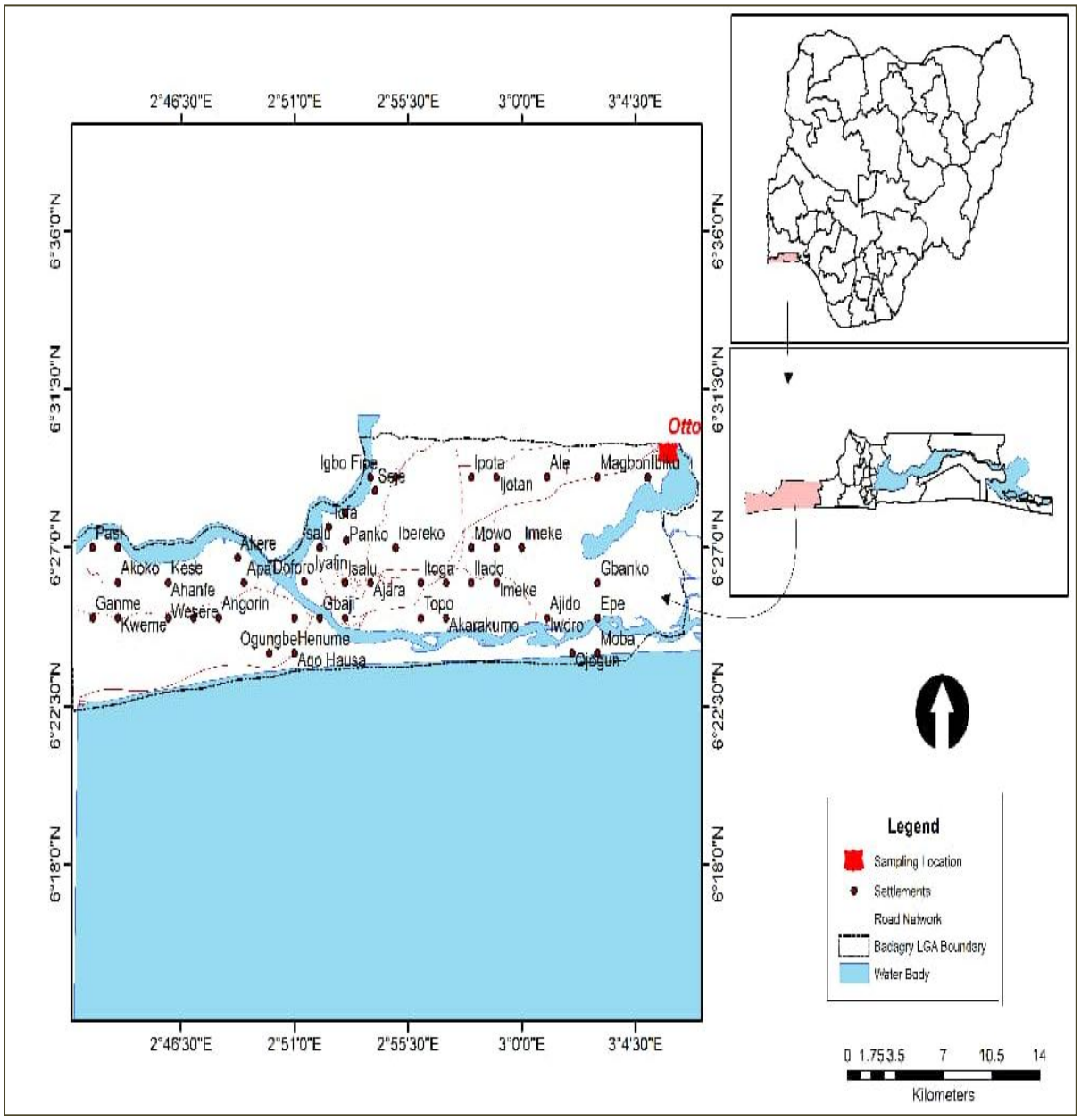

Fig. 1. The map of Nigeria at the top and below is a map of Lagos state showing the sampling location (Otto) in Badagry and the other local government areas in Lagos state; Badagry is a peri-urban area in Lagos State lying in the tropical rainforest belt

2.4 Screening for *Plasmodium falciparum* Malaria

The malaria *Plasmodium falciparum* diagnosis was done using World Health Organization (WHO) recommended Malaria HRP2 (Pf) rapid diagnostics test kits (Access Bio, Inc. U.S.A.) and malaria microscopy in accordance to the WHO guideline of malaria diagnosis [18].

2.4.1 HRP2 *in vitro Plasmodium falciparum* malaria diagnosis

About 5µL of finger pricked blood was collected with a micropipette and immediately transferred to a *Pf* HRP2 (Histidine rich protein-2) malaria rapid diagnostic test kit (Access Bio, Inc. U.S.A.). Then two (2) drops of HRP2 assay buffer were added to the A-well to move the blood across the nitrocellulose membrane and diagnosis was made after 20mins according to the manufacturer's instruction. The formation of a red band at the T-line and C-line (control) indicated a positive *P. falciparum* malaria while the formation of a single red band at the C-line showed a negative result to *P. falciparum* malaria. The formation of a single band only at the T- line indicates an invalid result according to the manufacturer's directives (Access Bio, Inc. U.S.A.).

2.4.2 Malaria microscopy diagnosis of *Plasmodium falciparum* malaria

The thin and thick films of blood smears were prepared by spreading collected blood on a microscopy slide according to WHO malaria microscopy standard procedure [18]. Briefly, the thin blood film (2µL) was fixed with methanol to preserve the morphology of the red blood cells while the thick film (15µL) was not fixed to allow the lysis of the red blood during the staining procedure to aid the concentration, counting and quantification of malaria parasites. The thin and thick blood films were then stained with 10% Giemsa solution, allowed to stand for 10 minutes after which the stain was washed off with water and the slides were air-dried and drops of immersion oil were placed on the slides for examination and counting under the light microscope. The formula below was used for parasitemia density determination:

$$\frac{No.of\ parasites}{No.of\ White\ blood\ cells\ counted} \times 8000\ White\ blood\ cells$$

Formula A1: Parasite density determination

2.5 Dried Blood Samples Recovery from Malaria Rapid Diagnostic Test (RDT) Kit

Dried blood spots were used for DNA extraction as follows: the malaria RDT kits were opened and the blotting nitrocellulose paper inside was gently detached and a small piece (about 3mm + 1mm) was cut out and placed into well labeled tubes and covered immediately [19].

2.6 DNA Extraction and Isolation by Phenol/ Chloroform/Isoamyl DNA Isolation Protocol

The digestion of dried blood spots and extraction of genomic DNA was done using a mixture of phenol-chloroform isoamylalcohol solution supplemented with Proteinase K according to the Phenol/Chloroform/Isoamyl DNA Isolation Protocol of the Harvard Medical School Internal CBDMLab (E. Hyatt 05). Briefly, 250µl of blood digestion buffer was added to dried blood paper (3mm) and incubated overnight at 45^0C after that, 250µl of phenol/chloroform/isoamyl alcohol (25:24:1), was added to the digested blood and shaken for 2mins without vortexing. The resultant mixture was spun at 13,000 revolutions per minute (rpm) for 10minutes. The genomic DNA was precipitated by transferring 200µl of the aqueous phase (supernatant) to a microcentrifuge tube containing 1ml 100% ethanol and spin at 13 000 rpm for 2mins to pellet the DNA. The 100% ethanol was decanted and the DNA pellet was then allowed to dry under room temperature. The DNA pellet was suspended in 100µl of Tris EDTA buffer and stored at 4^oC until used.

2.7 Polymerase Chain Reaction

Genomic DNA isolated was amplified using polymerase chain reaction (PCR). The amplification of a 242bp fragment of the TNFRII exon 6 gene was done using flanking forward (F) and reverse (R) primers (Inqaba Biotec, 2018).

Primer forward (F): 5'-ACT CTC CTA TCC TGC CTG CT-3'
Primer reverse (R): 5'-TTC TGG AGT TGG CTG CGT GT-3' [20]

The PCR amplification was done with One Taq Quick-Load 2X Master mix with standard buffer according to the manufacturer's protocol (Biolabs, New England UK). Briefly, for a single (1X) 25µl reaction, 8.5µL of PCR water was added to a PCR tube, followed by 12.5µl of One

Taq Quick-load 2X master mix with standard buffer, 0.5µl of 10µM forward primer, 0.5µL of 10µM reverse primer and 3µL of purified DNA sample which was mixed by gentle vortexing. The PCR condition for the amplification was done as follows: initial denaturation at 95^0C for 5 min followed by 35 cycles at 95^0C for 1 min, primer annealing at 57^0C for 1 min and gene elongation at 72^0C for 1 min. A final extension step at 72^0 for 5 min. [20].

2.8 TNFRII Polymorphism Genotyping

TNFR II SNP genotyping was done by PCR-Restriction fragment length polymorphism according to the method of Al-Ansari and collaborators [20]. The PCR products were digested with Nla III restriction enzyme (Biolabs, New England UK). The digestion products separated on 1% agarose gel and visualized under UV light.

2.9 DNA Quantification and Purity Determination

The quantification of genomic DNA was done to determine the yield of isolated DNA for a few randomly selected samples while the purity of the isolated genomic DNA was done to determine the quality of the DNA isolated using the nanodrop 1000 spectrophotometer (Thermofisher Scientific, USA).

2.10 Statistical Analysis

Comparisons of TNFR II allele frequency (proportion of chromosomes in which an allele is present) and genotype frequency (proportion of individuals with a genotype) between malaria-infected cases and controls were performed with 2 × 2 tables using Fisher's exact test for significance and Chi-Square test with the aid of STATA statistical Data Analysis software version 13.0.

3. RESULTS

3.1 DNA Yield and Purity

Molecular analysis of the genomic DNA showed that the average yield of the extracted DNA was 3543 ng/µl and the purity level was 1.7 (absorbance 260/280).

3.2 Analysis of Genetic Polymorphism in TNFRII Gene

Restriction digestion of amplified PCR products of TNFRII gene for *Plasmodium falciparum* malaria cases and healthy controls revealed two genotypes (homozygous wild-type and heterozygous variant-type). The variant homozygous genotype (GG) gave two fragments of 133bp and 109bp, the wild type genotype (TT) gave a single fragment of 242bp, while the heterozygous genotype (TG) (242bp, 133bp, 109bp) was not seen. An electrophoregram obtained following the separation of the digested PCR product, staining with ethidium bromide and visualization by ultra violet illuminator (Ultra Violet Products UVP, U.S.A) is shown in Fig. 2.

3.3 Genotypes and Alleles Frequencies in the Badagry Population

The participants included comprises of children and adults of both sex (male and female). The major economic activities of the people in Badagry include fishing, trading and tourism. The distribution of TNFRII genotypes and alleles in healthy controls and *Plasmodium falciparum* malaria cases are shown in Table 1. Generally, a high prevalence of the TNFRSF1B gene wild-type (TT genotype) (88.24%) and low incidence of the variant-type (GG genotype) (11.76%) was observed.

Comparing between controls and malaria cases, the frequencies of TT genotype (wild-type) was higher in the malaria cases (94.70%) than in controls (85.70%). While the variant genotype (GG) frequencies was lower in the malaria cases (5.30%) than in controls (14.30%). A graphical representation of percentage genotype frequencies in malaria cases and controls is shown in Fig. 3. The TG genotype was not observed in both case and control groups (0%).

3.4 Association of TNFR II Genotypes and Alleles with *Plasmodium falciparum* Malaria

There was no association found between TNFR II genotypes and alleles frequencies to risk/protection against *P. falciparum* infection shown in Tables 2 and 3 respectively. *P value > 0.05* was considered not to be statistically significant.

4. DISCUSSION

4.1 Incidence of TNFRII Gene Polymorphism in Badagry, Lagos

TNFRII is a 75/80kDa cytokine protein expressed on endothelial cells, immune cells, neuronal cell

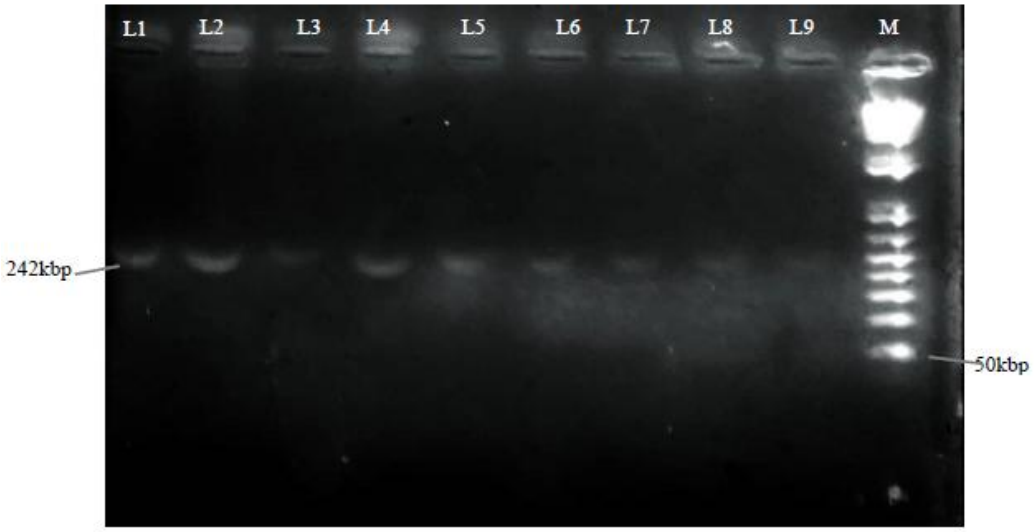

Fig. 2. An electrophoregram of TNFR II gene (242kbp) after restriction digestion with NlaIII enzyme; M = DNA marker, L = lanes

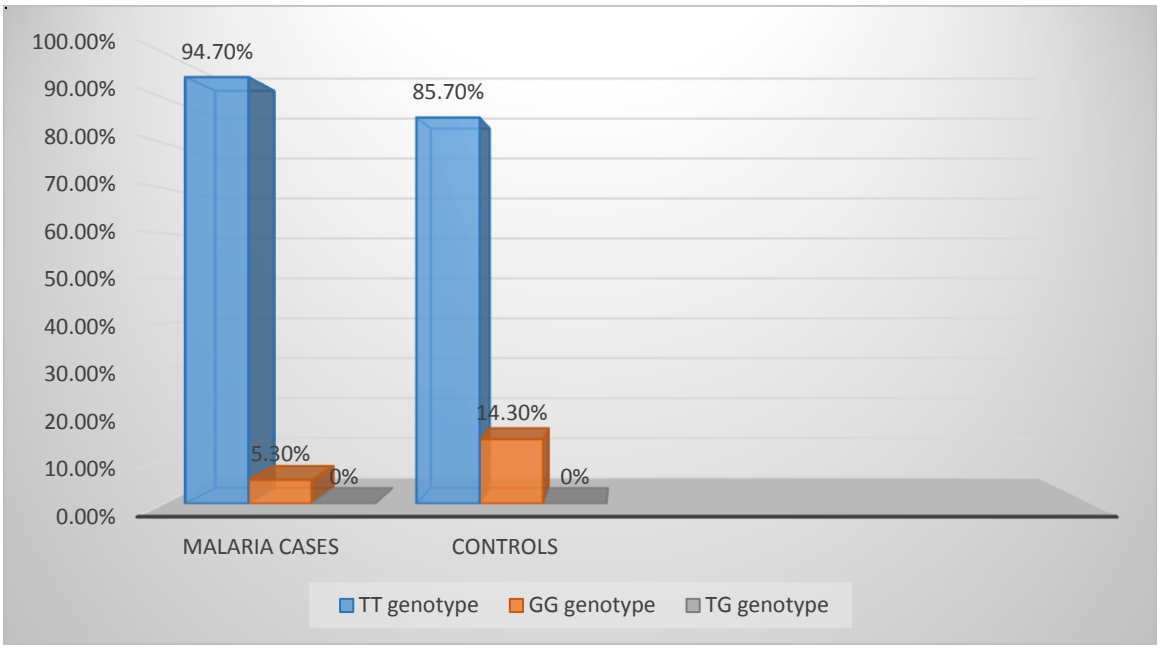

Fig. 3. Percentage Genotype frequencies of Tumor necrosis factor receptor II (*tnfr II*) gene among Malaria cases and controls in Badagry local government in Lagos state, Nigeria

subtypes, cardiac myocytes, and human mesenchymal stem cells [21, 22]. It is released in response to the action of tumor necrosis factor alpha pro-inflammatory cytokine (TNF-α) during infectious disease attacks [23, 24].

Tumor necrosis factor receptors especially the TNFR II receptor to transmembrane TNFα protein have been implicated in the pathology of severe malaria infection, cancer, rheumatoid arthritis, diabetics and so on [8, 25-29]. Tumor necrosis factor receptor II (TNFR II) has been identified as an adhesion molecule for *Plasmodium falciparum* parasite by binding to an evasion ligand of *P. falciparum* known as *Plasmodium falciparum* erythrocyte membrane protein 1 and mediate malaria parasite evasion of the human immune system for effective infection (Pf EMP-1) [8].

Table 1. TNFRII genotype and Allele frequencies in Malaria infected cases and controls and hardy-weinberg equilibrium results

Groups	Genotypes observed			Genotypes expected			Chi-squared (p value) for HWE
	TT	GG	TG	TT	GG	TG	
Malaria cases (n=19)	18	1	0	17.0526	0.0526	1.8947	19 (0.00001)
Controls (n=49)	42	7	0	36	1	12	49 (<0.00001)
	Allele frequencies						
	T	G					
Malaria cases (n=38)	0.947 (36/38)	0.053 (2/38)					
Controls (n=98)	0.857 (84/98)	0.143 (14/98)					

The result is considered significant at p value < 0.05

Table 2. Chi-square test of TNFR II genotypes association with malaria infection

TNFR II gene polymorphism and Association to *Plasmodium falciparum* Malaria			
	TNFR II Genotypes		
	TT	GG	Total
Cases	18	1	19
Controls	42	7	49
	60	8	68

Chi-square = 1.074, df = 1, P value = 0.30

Table 3. Chi-square test of TNFR II gene alleles association with malaria infection

TNFR II gene polymorphism and Association to *Plasmodium falciparum* Malaria			
	TNFR II gene Alleles		
	T	G	Total
Case	36	2	38
Control	84	14	98
	120	16	136

Chi-squared = 2.147, df = 1, P value= 0.14

TNFR II is encoded by the *tnfr II* gene, a member of tumor necrosis factor receptor superfamily 1B gene (TNFRSF1B) located on chromosome 1p36 [9]. The *TNFR*II gene consists of ten (10) exons and nine (9) introns however, exons 4, 6, and 9 exhibit polymorphisms that code for different proteins [9, 11, 22]. TNFR II gene is critically involved in the maintenance of homeostasis of the immune system, control of immunity in malaria, and pathogenicity of malaria infection [24,30]. T*nfr* II gene exhibits polymorphism at T587G (dbSNP: rs1061622) on exon 6 which results in a change in amino acid translation from Methionine to Arginine (M196R) [10,12,31]. This polymorphism occurs at the membrane- proximal extracellular domain of the TNFRII protein and may therefore affect its receptor-ligand binding function [10, 12].

Although, genetic polymorphism in exon 6 of TNFRSF1B gene has been found to be associated with various diseases such as rheumatoid arthritis, cancer, and diabetics among Caucasians [12,32-34] no research study has investigated the consequences of Tumor necrosis factor receptor II gene polymorphism in *Plasmodium falciparum* malaria till to date.

This is the first report to investigate the incidence of Single nucleotide polymorphism in exon 6 of TNFRSF1B gene in the Nigerian population (Badagry ethnic group). Generally, the high prevalence of the TNFRSF1B gene wild type (TT genotype) (88.24%) and low incidence of the variant type (GG genotype) (11.76%) indicate a low genetic polymorphism occurring in the TNFRSF1B gene among this population. There is no other study on TNFRSF1B gene polymorphism investigated among Nigerian populations to compare this result with. However, a comparison of the frequency of the TNFRSF1B variants to the frequencies in other studies conducted among healthy Caucasians, and Japanese show an agreement to our findings. The prevalence of the variants in these groups was also found to be low with Caucasians having 5% distribution [12], and the Japanese had 1% [35].

4.2 Association of *TNFR* II Gene Polymorphism with *Plasmodium falciparum* Malaria in Badagry, Lagos

To the best of our knowledge, this is the first study to investigate the association of the TNFRSF1B gene polymorphism to *Plasmodium falciparum* malaria. In the present study, *tnfr* II variant type had lower frequency (5.30%) in the malaria cases compared to the wild type (94.70%) which could indicate resistance to *Plasmodium falciparum* malaria. This is in line with the finding that TNFR II had decreased level of expression in normal individuals carrying the variant genotype (GG) [36]. The lower level of the TNFR II could lead to lower binding to the parasite ligand (*Plasmodium falciparum* erythrocyte membrane protein-1 (EMP-1)) and reduced infectivity. However, the result of the Chi-square test of association between the TNFR II variant and wild-type genotypes and malaria infection showed no association to susceptibility or resistance to *Plasmodium falciparum* malaria. Hence, neither the genetic variant (GG genotype) nor the wild type genotype (TT genotype) predisposes or protects an individual against *Plasmodium falciparum* infection.

Furthermore, the result for the test of association between the frequencies of G allele and *Plasmodium falciparum* malaria indicate that there is no association between the G allele of TNFRSF1B gene and malaria infection in the studied population.

5. CONCLUSION

This present study revealed the incidence of TNFRII gene polymorphism on exon 6 among Badagry population and the prevalence of the wild-type genotype was the highest while the TNFRII gene variant frequency was low. However, this existing polymorphism was not associated with susceptibility or resistance to *Plasmodium falciparum* malaria within the studied population. New researches with a larger sample size are needed to clarify this association study in malaria-endemic countries.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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CONSENT AND ETHICAL APPROVAL

As per international standard or university standard guideline participant consent & and ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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