

High prevalence of *Plasmodium falciparum* *Pfmdr1* 86Y mutant gene in sickle cell disease in Nigeria

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Background and Purpose: In areas where malaria is endemic, drug prophylaxis is required for people with sickle cell disease. Chloroquine resistance has been associated with the *Plasmodium falciparum* multidrug resistance 1 (*Pfmdr1*) mutant gene. This study tested for the *Pfmdr1* 86Y mutation in *P. falciparum* isolates from individuals with sickle cell disease and sickle cell trait, who also underwent hemoglobin genotyping.

Methods: Blood samples were collected from patients presenting with symptoms of malaria in an endemic region. The subjects were screened for hemoglobin genotype using hemoglobin electrophoresis and *P. falciparum* *Pfmdr1* genotyping was carried out using polymerase chain reaction-restriction fragment length polymorphism.

Results: 229 subjects, comprising 144 with hemoglobin AA genotype, 57 with hemoglobin AS genotype and 28 with hemoglobin SS genotype, were enrolled in this study. There was no significant difference in the infective rate of malaria in the 3 groups ($p>0.05$). However, the prevalence of *Pfmdr1* 86Y was higher in those with hemoglobin SS genotype than in hemoglobin AA and AS subjects ($p<0.05$).

Conclusions: Uncontrolled use of chloroquine is a major cause of chloroquine resistance in Nigeria. Chloroquine prophylaxis may be the underlying cause of the high prevalence of *Pfmdr1* 86Y mutant gene in individuals with hemoglobin SS genotype.

Key words: Anemia, sickle cell; Chloroquine; Mutation; Nigeria; *Plasmodium falciparum*; Sickle cell trait

Introduction

Malaria is the most common cause of crises in sickle cell disease in malaria-endemic countries [1]. Health professionals often recommend lifelong malaria chemoprophylaxis for people with homozygous sickle cell disease living in these areas [2]. In malarious areas, the high frequency of hemoglobinopathies, such as sickle cell disease, is consistent with their protective role against *Plasmodium falciparum* malaria [3].

The precise mechanism by which sickle cell trait imparts resistance to malaria has not been determined. It is likely that a number of factors are involved and

contribute in varying degrees to the modification of infected erythrocytes and host immune response against malaria [4]. Infected erythrocytes from people with sickle cell trait do not cause significant sickling at normal venous oxygen tension. However, sickling occurs in the situation of low oxygen tension. A study found that exercise and hypoxia increase sickling in venous blood samples from people with sickle cell trait [5]. An erythrocyte with sickle trait infected with the *P. falciparum* parasite tends to sickle, especially in the deep vasculature where the oxygen tension is low [6].

This study tested for the *P. falciparum* multidrug resistance 1 (*Pfmdr1*) 86Y mutation in *P. falciparum* isolates from individuals with sickle cell disease and sickle cell trait, who also underwent hemoglobin genotyping.

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Methods

Study design

A total of 229 capillary blood samples were collected for this study. These samples were collected from individuals who presented with symptoms of uncomplicated malaria between the months of April and June 2006 in medical centers in the Benin metropolis. Benin City is a cosmopolitan town where malaria infection is endemic. The months of April through June in Benin City are characterized by high malaria transmission rates, as these months mark the beginning of the rainy season.

Hemoglobin genotyping

An aliquot of washed blood cells was transferred into a clean test tube and lysed by adding a few drops of water. Each sample, along with a control, was spotted on a cellulose acetate paper with an applicator stick. The cellulose acetate paper was then placed in an electrophoretic tank. Electrophoresis was run at 60 volts for 10 min. The hemoglobin types were determined by comparing the distance traveled with that of a standard preparation [7].

DNA extraction and PCR-RFLP of *Pfmdr1*

Fresh venous or capillary blood was blotted onto a piece of filter paper. DNA of *P. falciparum* was extracted from the dried blood spots by a modified Saponin/Chelex[®] method (Sigma-Aldrich Corp, St Louis, MO, USA) [8].

Genotyping of the resistance marker *Pfmdr1* was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to distinguish between types of Asn-86 and Tyr-86 mutant, as described by Frean et al [9]. A seminested PCR was used to increase the sensitivity of the assay. In the round of PCR, primers A*-(5'ATgggTAAAg-AgCAGAAA-gA3') and A/-(5'TATTACTATg-ACACCAAA3') were used to amplify the region flanking codon 86. A nested PCR was then carried out with primers An-(5'TATC-ATTTgTATgTgCTgTA3') and A/. Cycling conditions for the seminested PCR were, 45 cycles: 95°C for 3 min, 95°C for 30 sec, 45°C for 30 sec, 65°C for 45 sec, 72°C for 15 min, while those of nested PCR were, 35 cycles: 95°C for 3 min, 95°C for 30 sec, 45°C for 30 sec, 65°C for 45 sec, and finally 72°C for 15 min.

Following nested amplification for *Pfmdr1*, an aliquot was subjected to restriction digestion with the antifreeze-like III (*Af*/III) restriction enzyme (New

England Biolabs, Ipswich, MA, USA). *Af*/III cuts the PCR product and generates fragments of 190 and 110 base pairs if a mutation is present at codon 86 Tyr (TAT). The digestion products resulting from restriction digests were checked with 2% agarose gel on a horizontal electrophoretic apparatus (Edvotek M12), together with a marker. Gels were stained with ethidium bromide and visualized under ultraviolet transillumination [8].

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows (SPSS Chicago, IL, USA). The statistical parameters used included the chi-squared test, analysis of variance and Student's *t* test.

Results

Among 229 subjects investigated, 144 (62.9%) had hemoglobin genotype AA, 57 (24.9%) had genotype AS and 28 (12.2%) had genotype SS. The malaria parasite was detected in 116 of the AA subjects (80.6%), 49 of the AS subjects (85.9%), and 22 of the SS subjects (78.6%). Malaria prevalence rate did not vary significantly among the 3 hemoglobin genotypes ($p>0.05$). Individuals with hemoglobin AA were found to have higher rates of parasitemia (>1000 parasites/ μ L) than those with AS and SS genotypes (Table 1).

The prevalence of the wild type and mutant genes for each genotype is shown in Table 2. Of 187 positive samples, 17.5% of patients harbored the mutant gene of *P. falciparum* malaria. The mutant *Pfmdr1* gene was present in 6 of 22 individuals with hemoglobin SS genotype (27.2%), a significantly higher prevalence ($p<0.05$) than that of individuals with AA and AS genotypes. The gel pattern results of PCR-RFLP are shown in Fig. 1.

Discussion

This study showed that no statistical differences existed in the prevalence of malaria infection among subjects of the 3 genotypes. Of the 144 AA malaria-infected subjects studied, 116 (80.5%) were infected, while 49 AS genotypes (85.9%) were infected and 28 SS genotype subjects (78.5%) developed the infection. Although the infective rate was similar among the 3 hemoglobin types, higher levels of parasitemia were found in the AA and SS individuals than in those with

Table 1. Infective rate of malaria according to hemoglobin genotype determined by examination of blood film^a

Parasite density (/μL)	Genotype No. (%)			Total (n = 229)
	AA (n = 144)	AS (n = 57)	SS (n = 28)	
1-1000	49 (34.0)	26 (45.6)	6 (21.4)	81 (35.3)
1001-2000	52 (36.1)	17 (29.8)	7 (25.0)	76 (33.1)
2001-3000	5 (3.4)	- (0.0)	3 (10.7)	8 (3.4)
3001-4000	6 (4.1)	4 (7.0)	4 (14.2)	14 (6.1)
4001-5000	2 (1.3)	2 (3.5)	2 (7.1)	6 (2.6)
>5000	2 (1.3)	- (0.0)	- (0.0)	2 (0.8)
Total	116 (80.5)	49 (85.9)	22 (78.5)	187 (81.6)

^a $\chi^2 = 19.821$; df = 2; asymp sig (2-sided) = 0.071; $p > 0.05$.

Table 2. Prevalence of *Pfmdr1* mutant gene according to hemoglobin genotype^a

<i>Pfmdr1</i>	Genotype No. (%)			Total (n = 187)
	AA (n = 116)	AS (n = 49)	SS (n = 22)	
Wild type	97 (83.7)	43 (85.8)	16 (76.8)	155 (82.5)
Mutant	19 (16.3)	7 (14.2)	6 (27.2)	32 (17.5)

^a $\chi^2 = 64.221$; df = 2; $p < 0.05$.

the AS type. This may indicate a form of resistance to proliferation of parasites in the hemoglobin AS individuals. Genes that select against severe falciparum malaria include the hemoglobin S gene, thalassemia genes, glucose-6-phosphate dehydrogenase deficiency genes, ovalocytosis and Duffy blood groups [10]. Individuals with AA genotype are believed to suffer more severe malaria complications compared to AS genotyped individuals, although the SS genotyped individuals are also known to develop severe malaria infection [10].

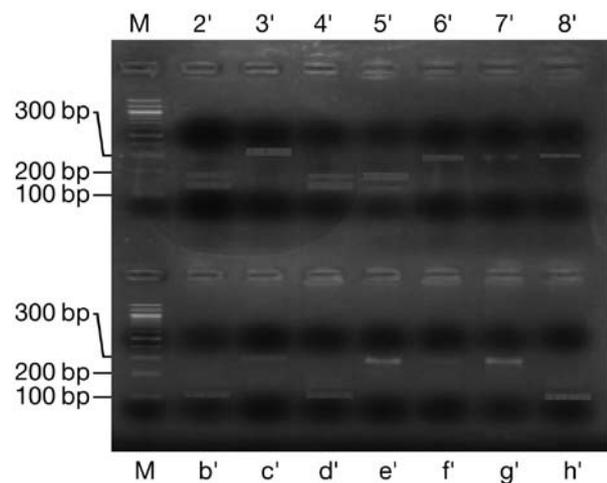


Fig. 1. Agarose gel depicting the 86Y (wild type) and mutant types after restriction fragment length polymorphism digestion with *Afl*III. Lane M = marker; lanes 2', 4', 5', b', d' and h' = mutant type bands; lanes 3, 6-8, c', e', f' and g' = 86Y bands.

Protection against severe malaria is afforded only to heterozygous individuals (hemoglobin AS) [10]. The protective mechanism is not fully understood. Erythrocytes containing hemoglobin AS will sickle more rapidly when they are infected with parasites, due to lower pH. The parasitized cells are also easily recognized and eliminated by phagocytes, and the parasites are therefore destroyed before they develop into schizonts. In persons with the SS genotype, the phagocytic activity of the spleen is frequently inadequate to remove parasitized and non-parasitized but sickle cells simultaneously. Therefore, severe malaria infection is usually fatal in these individuals.

Higher prevalence of *Pfmdr1* mutant gene was found in the SS genotyped individuals compared to the AA and AS individuals. Several factors have been proposed as contributing to the emergence of the resistant gene. A major selective pressure in this group of individuals might be constant and regular exposure to antimalarial drugs in preventive regimens.

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