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Copy number variation of plasmepsins 2 and 3 genes in *Plasmodium falciparum* isolates and implication for dihydroartemisinin-piperaquine resistance in Ghana

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Abstract

Background: In 2008, dihydroartemisinin-piperaquine and artemether-lumefantrine were introduced to supplement artesunate-amodiaquine for the treatment of uncomplicated malaria in Ghana. Drug pressure over the years enhances the development of parasite resistance to drugs. The World Health Organization recommends the detection of copy number variations of *plasmepsins 2* (*PfPm2*) *and plasmepsins 3* (*PfPm3*) genes linked to dihydroartemisinin-piperaquine resistance in treatment efficacy studies.

Objective: This study investigated the copy number variations of *PfPm2* and *PfPm3* genes in the malaria parasite population in Ghana.

Methods: Overall, 313 blood samples from children ≤ 9 years presenting with uncomplicated malaria at three sentinel sites used for monitoring antimalarial drug efficacy and resistance in Ghana were used for genetic investigations. The samples were collected in the malaria transmission seasons of 2015 and 2016. Malaria parasite DNA extraction from the blood samples followed by real-time quantitative PCR was used to determine the copy number of the *PfPm2* and *PfPm3* genes. The gene copy number was calculated by the relative expression formula $2^{-\Delta\Delta Ct}$ for quantification, where ΔΔ is the relative delta-delta, and Ct is the cycle threshold. ΔΔCt was calculated as $(Ct_{\beta\text{-}tubulin} - Ct_{pfpm2/3}) - (Ct_{\beta\text{-}tubulin\ cal} - Ct_{pfpm2/3\ cal})$, where cal is the calibration control of genomic 3D7 DNA with one copy of both the *β-tubulin* endogenous control and *pfpm2 and pfpm3*. A change in Ct (ΔCt = Ct *PfPm2/3* - Ct *Pfβ-tubulin*) where is the difference in Ct values for the target gene of interest *PfPm2* and *PfPm3* and the reference gene *Pfβ-tubulin*. Statistical significance was defined as $p < 0.05$.

Results: Of the parasites analyzed, 79.2% (n = 228/288) and 80.5% (n = 227/282) had one gene copy for *PfPm2* and *PfPm3*, respectively. For *PfPm2,* 14.9% (n = 43/288), 3.8% (n = 11/288), and 2.1% (n = 6/288) of the isolates had copy numbers 2, 3 and 4 respectively. For *PfPm3*, gene copies of 2, 3 and 4 were observed in 16.3% (n = 46/282), 2.1% (n = 6/282), and 1.1% (n = 3/282) of isolates. Analysis of the copy number variation across the three study sites in Cape-Coast, Begoro, and the Navrongo areas showed no significant difference for *PfPm2* ($p = 0.93$) *and PfPm3* ($p = 0.94$) genes.

Conclusion: After over a decade of the use of dihydroartemisinin-piperaquine, the mutations associated with resistance to the drug have been observed in Ghanaian *P. falciparum* isolates. This serves as baseline data for further monitoring of this molecular marker extensively as part of ongoing surveillance of antimalarial drug efficacy studies in Ghana.

Keywords: malaria, antimalarial drug resistance, plasmepsins, gene copy number variations

INTRODUCTION

Malaria is still a public health problem globally. The disease causes high morbidity and mortality rates in endemic areas of the world, especially in sub-Saharan Africa [1]. An estimated 241 million cases and 627,000 malaria-related deaths were observed in 2020 amidst the COVID-19 pandemic [1,2]. The strategies employed to reduce morbidity and mortality in endemic areas include the use of chemotherapy, a mainstay control strategy, and vector control. The WHO initiated the use of artemisinin-based combination therapy (ACT) to slow down the development of parasite drug resistance as observed with chloroquine monotherapy [3,4]. Since the implementation of the use of ACT as a first-line treatment regimen in malaria-endemic regions, there have been reports of reduced mortality and morbidity rates [5]. The ACT is a

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combination of a short-acting artemisinin (ART) derivative and a long-acting partner drug. The reports of ART resistance in the Southeast Asia (SEA) region were a great setback for global malaria eradication. Therefore the WHO has initiated the containment of drug resistance in the SEA region with the deployment of a multi-sector strategy which includes surveillance of drug resistance with molecular markers [6]. The molecular markers of antimalarial drug resistance are the genetic mutations occurring at the drug target encoding genes which infer the phenotypic resistance traits exhibited by the parasite. These markers could be single nucleotide polymorphisms or copy number variations of the genes associated with antimalarial drug resistance. The markers provide prompt and relevant information on changes in the parasite's susceptibility to drugs and therefore an important and useful tool for the surveillance of drug resistance.

In 2014, the molecular markers of ART resistance were discovered as mutations in the *Plasmodium falciparum* kelch propeller domain on chromosome 13 (*pfk13*) [7]. Consequently, the WHO validated *pfk13* mutations are *F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L* and *C580Y* [2,7]. Amino acid variants of some of these markers have been observed in the Ghanaian *P. falciparum* population [8]. Regarding resistance of *P. falciparum* to antimalarial combination therapy regimens such as the dihydroartemisinin-piperaquine (DHAP), the copy number variations (CNV) of the genes *pfpm2* and *pfpm3*, which encodes for the proteins *plasmepsins 2* and *plasmepsins 3*, respectively, have been implicated [9]. These genes are recommended by the WHO for surveillance of resistance in malaria-endemic areas [10]. The CNV is a phenomenon in which sections of the genome are repeated causing phenotypic variations in parasite populations [11]. *Plasmepsins* (*PMs*) are gene-encoded enzymes expressed during the erythrocytic stage of the parasite's life cycle for haemoglobin degradation [12]. The PMs are a family of *P. falciparum* aspartic proteases known to be involved in the initiation of haemoglobin degradation and have generated interest as antimalarial drug targets [13]. Ten different *PMs* have been identified in *P. falciparum* known as *PM 1, 2, 4-10* and Histo_Aspartic Proteinase (3/pfhap) [13]. Among all *Plasmodium sp.* only *P. falciparum* has been reported to have multiple food vacuole *PMs* [14]. Studies have shown that increased *pfpms 2* and *pfpms 3* gene copy numbers are associated with DHAP failures in the Great Mekong Region (GMR) of the SEA [9,12,15-18].

Ghana is a malaria-endemic country with 41.5% outpatient department clinical visits [19]. The disease control measures include chemotherapy and vector control. Chemotherapy with the use of ACT regimens for the treatment of uncomplicated malaria started with amodiaquine-artesunate (AA) in 2005 and, artemether-lumefantrine (AL) and DHAP were added in 2008 [20]. In 2018, the cure rate for malaria with AA was 99.6% and that of AL was 97.3% — an indication that there was the development of resistance to antimalarial drugs by *P. falciparum* in Ghana [21]. Investigation on the use and efficacy of DHAP is still ongoing by our group. Since a new marker for DHAP resistance has been discovered, it is appropriate to investigate the presence of the marker in malaria parasites for antimalarial drug resistance surveillance in Ghana. Monitoring molecular markers of drug resistance give firsthand information on the genetic status of the parasite which will portray the phenotypic trait in the long term [22,23]. With the use of DHAP for the past 14 years in Ghana, this study determined the prevalence of increased gene copy number of *pfpm2* and *pfpm3* genes in Ghanaian *P. falciparum* isolates from three ecologically distinct areas of Ghana: Cape-coast, Begoro, and Navorongo. The findings of the work will serve as early warning information of the emergence of DHAP resistance to policymakers in the country.

MATERIALS AND METHODS

Study sites and samples

There are 10 sentinel sites established by the Ghana Malaria Control Programme and the Noguchi Memorial Institute for Medical Research for monitoring antimalarial drug resistance in the country. The sentinel sites are representative of the three ecological zones in Ghana. We used 313 archived filter blood blots obtained in 2015 and 2016 from children ≤ 9 years with uncomplicated malaria presenting to health centres at three study sites [Begoro (forest zone), Cape Coast (coastal savannah zone) and Navrongo (guinea savannah zone) (Figure 1)].

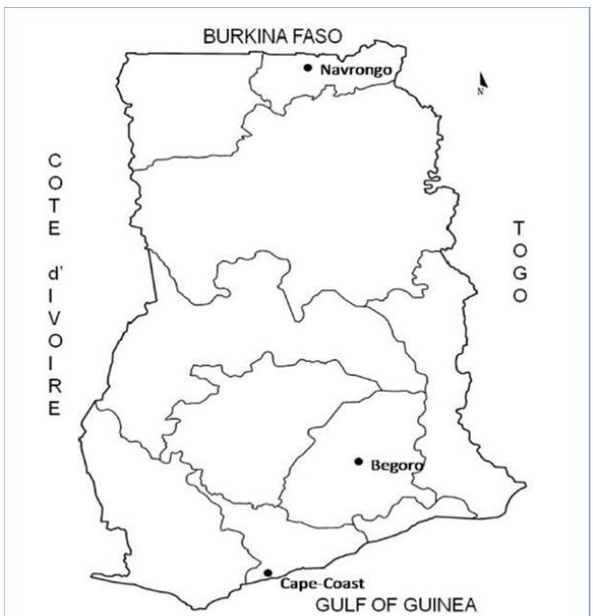

Figure 1: Ghana map showing the sentinel sites for the study

Amplification of *pfpm2* and *pfpm3* genes

Parasite DNA was extracted from 313 filter blood blots using the QIAamp DNA Minikit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The

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pfpm2 and *pfpm3* genes were amplified following protocols described by Witkowski and others [12] with minor modifications for real-time quantitative PCR (rt-qPCR). The *Pfpm2* primers and probe used are forward 5'-GCA ATT CAA CAT TTG ATG GAT TAA C-3'; reverse 5'-CCA CAC ATT ACA CTA CAA AAG AGA AGT ACA-3'; and probe 5'-FAM-CAG AAA GGA TTT CAA ATA C-3' BHQ-1. For *pfpm3*, the primers and probe are forward 5'-AAT CCT TAA CAC GTT TCG AGT AAC TAA-3'; reverse 5'-GCC AAA ACT ATG AAA ACT GTC ACA A-3'; and probe 5'-FAM-AAA AGA TGG AAT GCT AAA AG-3' BHQ-1. For the *β-tubulin* endogenous control, primers and probe are forward 5'-TGA TGT GCG CAA GTG ATC C-3'; reverse 5'-CCT TTG TGG ACA TTC TTC CTC-3'; probe 5'-VIC-TAG CAC ATG CCG TTA AAT ATC TTC CAT GT CT-3' BHQ-1. A master mix for the amplification of *pfpm2* and *pfpm3* genes were made up of 4 µL of nuclease-free water, 10 µL of 1x Luna Commercial Master Mix (New England Biolabs, England), 0.8 µL of 0.3 µM of each primer (for both target DNA and endogenous control), 0.4 µL of 0.3 µM probe (for both target DNA and endogenous control) and 2 µLof DNA was then added to make a final volume of 20µL. All reactions were prepared in triplicates. A MicroAmp Fast Optical 96 well plate (Star Labs, USA) was loaded with 20 µL of the reaction mix in each well.

The DNA from *P. falciparum* cell line 3D7 with a copy number of 1 and DD2 copy number of 2 were used as positive controls. A non-template control and nuclease-free water were used as negative controls. The cycling conditions for the rt-qPCR reaction consisted of initial denaturation at 95 ºC for 15 minutes followed by 45 cycles of denaturation at 95 ºC for 15 seconds and 45 cycles of annealing at 58 ºC for 40 seconds and an extension at 72 ºC for 20 seconds. The detection threshold was set above the mean baseline. Copy number was estimated using the relative expression formula $2^{-\Delta\Delta Ct}$ method for quantification, where ΔΔ is the relative delta-delta, and Ct is the cycle threshold. The threshold was placed to optimize the threshold cycle value for the first standardization reaction, and all subsequent Ct values were obtained by using the specified threshold value. The Efficiency of the *β-tubulin* was assumed to be 2. ΔΔCt was calculated as $(Ct_{\beta\text{-}tubulin} - Ct_{pfpm2/3}) - (Ct_{\beta\text{-}tubulin\ cal} - Ct_{pfpm2/3\ cal})$, where cal is the calibration control of genomic 3D7 DNA with one copy of both the *β-tubulin* endogenous control and *pfpm2 and pfpm3*. A change in Ct (ΔCt = Ct *PfPm2/3* - Ct *Pfβ-tubulin*) where is the difference in Ct values for the target gene of interest *PfPm2* and *PfPm3* and the reference gene *Pfβ-tubulin*.

Statistical analysis

The Chi-square test was used to compare the proportions of the amplification of the two genes in the three sites to determine any significant differences in the prevalence of the mutations among the sites using GraphPad Prism 9 (GraphPad Software Inc, La Jolla, CA, USA). Statistical significance was defined as $p < 0.05$.

RESULTS

Of the 313 blood samples used for the molecular analysis, the number from Cape Coast, Begoro and Navrongo, respectively, were 138, 86 and 89. The variation in the sample size is due to the availability of archived samples. The estimated copy number ($2^{-\Delta\Delta Ct}$ value) for the controls 3D7 (expected one copy of the gene) ranged from 0.7 and 1.0 and that of DD2 (expected 2 and 3 copies of the gene) was 2.3 to 2.8 for 21 rt-qPCR runs. The number of samples with paired data for both genes was 256. A total number of 288 and 282 samples were amplified separately for the *pfpm2* and *pfpm3* genes, respectively.

Single gene copy number variations of *pfpm2* and *pfpm3*

The proportion with single copy of the *pfpm2* and *pfpm3* genes were 79.2% (n = 228/288) and 80.5% (n = 227/282). The distribution of the copy number variation in all the samples analysed is shown in Figure 2. Of the 138 samples analyzed from Cape Coast, the detection of the CNV was done for 121 and 122 for *pfpm2* and *pfpm3*, respectively. Single copies of the genes were observed in 83.4% (n = 101/121) for *pfpm2* and 85.3% (n = 104/122) of the isolates. For Begoro, a total of 86 samples were analysed (86 for *pfpm2* and 81 for *pfpm3*) and 80.2% (n = 69/86) and 72.8% (n = 59/81) respectively had single copies of the two genes. Samples from Navrongo were 89, (81 for *pfpm2* and 79 for *pfpm3*) respectively. Single copy gene were also observed in 71.6% (n = 58/81) and 80.0% (n = 64/79) for *pfpm2* and *pfpm3*, respectively.

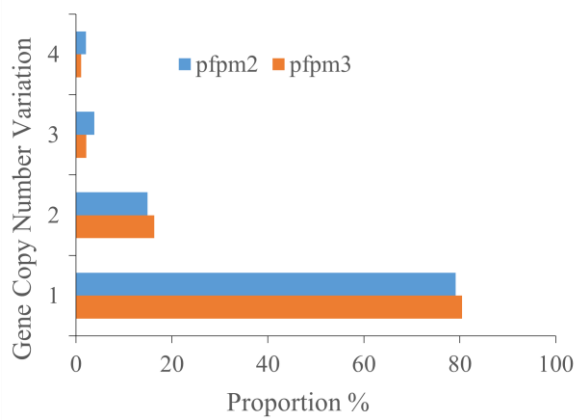

Figure 2: Distribution of gene copy number variations observed from 1 to 4 for both *pfpm2* and *pfpm3*

Increased gene copy number variations

The proportion of samples with increased gene copy numbers from 2 to 4 are shown in Figures 3a and 3b for *pfpm2* and *pfpm3*, respectively. Navrongo isolates had the highest proportion with increased gene copy numbers of 2, 3 and 4 for the *pfpm2* gene whilst Begoro had the highest for the *pfpm3* copy number of 2 and 4 and Cape Coast had the highest for a copy of 3 for *pfpm3*. Increased copy

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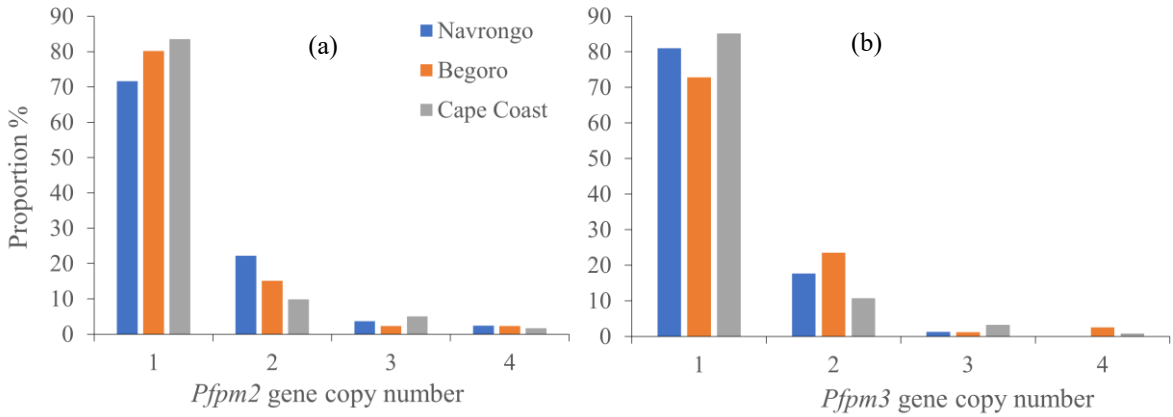

Figure 3: (a) Variation of *pfpm2* copy number in parasites from the 3 sites representing the ecological zones of Ghana. Navrongo represents the guinea savannah zone, Begoro the forest zone and Cape Coast the coastal savannah zone. There was no significant difference in the distribution of CNV in the three ecological zones (p = 0.93) for *pfpm2*. (b) Variation of *pfpm3* copy number in parasites from the 3 sites representing the ecological zones of Ghana There was no significant difference in the distribution of CNV in the three ecological

numbers of 4 for both genes were seen in Begoro and Cape-Coast. For samples with data for both genes, it was observed that 3.1% (n = 8/256) (Cape-Coast: n = 2, Begoro: n = 3, Navrongo: n = 3) had both increased copy number for both genes. There were no significant differences in the proportion of isolates with increased copy number for both genes between the three ecological zones (p = 0.93 for *pfpm2* and p = 0.94 for *pfpm3*).

DISCUSSION

Surveillance of the molecular markers of antimalarial drug resistance is one of the important strategies that the WHO has recommended for the early detection of the development of parasite resistance [2]. The information could be acquired easily by investigating the malaria parasite population from both asymptomatic and clinical cases. We herein report the baseline information on the molecular determinants of DHAP resistance in the Ghanaian *P. falciparum* population. This work was done as part of ongoing surveillance of ACT efficacy/resistance in the country. It is quite interesting to report that after eight years (2008-2016) of DHAP use, the increased copy number of *pfpm2* and *pfpm3* were already present in the genomes of the circulating parasite population in Ghana.

Haemoglobin-digesting proteases are encoded by PMS genes which are on malaria parasite chromosome 14. Gene copy number polymorphisms have been observed on chromosome 14 including *pfpm2* and *pfpm3*. The expression of the genes occurs in the intraerythrocytic cycle of the malaria parasite, and their over-expression has been associated with piperaquine resistance [7]. In laboratory experiments, it has been observed that parasite cell lines 3D7 and DD2 have copy numbers of 1 and 2 respectively [24], and that was also confirmed in this study. Thus, it can be inferred that copy number polymorphism could vary among laboratory strains which may be due to differences in lengths and positions of close repeated sequences [25]. Gene amplification with increased copy number is indicative of high levels of the gene encoding PMS which aids the parasite to evade drug action. Since gene amplification is a more frequent occurrence than point mutations in *P. falciparum* parasites, the resultant observation of the rapid rise and spread of piperaquine resistance is expected based on the presence of these molecular markers [7]. Drug-selected gene amplification is a well-known phenomenon in malaria parasites such as the gene copy number variation of the *P. falciparum* multi-drug resistance gene linked to parasite susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and ART [23, 26]. This study observed that most of the field isolates had a copy number of 1 for *pfpm2* (79%) and *pfpm3* (81%). We also observed an increased copy number of 2, 3 and 4 for both genes. There was minimal variation in the proportion of samples with increased copy numbers from the three ecological zones. It was also observed that most of the isolates with increased copy number had a CNV of 2. Interestingly, a few isolates had both *pfpm2* and *pfpm3* with increased copy numbers. It is not known if more than 2 copies of the two genes would have a phenotypic effect but the increase in samples with 3 or more copies suggests some sort of selective mechanism. There is the possibility that a third copy would "buffer' and prevent the loss of resistance if the duplication is unstable. According to Witkowski and colleagues, an increase in *pfpm2 and pfpm3* copy number amplification is highly predictive of piperaquine resistance [27]. Our result of an increased copy number of 21% is lower than the findings of Leroy et al. indicating that the average increased copy number for *pfpm*2 across African sites was 27%, however, more than 30% were observed for Burkina Faso and Uganda [28]. Piperaquine acts mainly by depleting the ribosomes and causing the swelling of the food vacuole of the parasite. The

drug inhibits the activity of the PMS to prevent haemoglobin degradation, prevents the formation of hemozoin crystals and promotes the accumulation of free heme [29]. The inhibition of haemoglobin degradation will starve the parasite which will result in its death. Therefore, the increased copy of *pfpm2* and *pfpm3* genes indicates that the parasite can produce more of the PMS proteins which increases the ability of the parasite to degrade the host haemoglobin even in the presence of piperaquine. The observed increased *pfpm2* and *pfpm3* copy numbers in Ghanaian isolates may be indicative of parasites with reduced susceptibility to piperaquine. However, no "true" DHAP resistance has been clinically reported. It is worth noting that additional mutations in other genes such as the chloroquine-resistance transporter gene could also contribute to piperaquine resistance, and this is under investigation by our group.

Conclusion

Findings from this study indicate there are parasites with increased copy number of *pfpm2* and *pfpm3* among Ghanaian isolates and this preliminary data could serve as baseline data for future investigations on DHAP resistance surveillance in the country. Since there has been no report of DHAP resistance in the country, these markers would still be monitored in the malaria parasite population in Ghana.

DECLARATIONS

Ethical considerations

The study protocol was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR). The NMIMR-IRB protocol number is 032/05-06a amed.2021

Consent to publish

All authors agreed to the content of the final paper.

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Competing Interests

No potential conflict of interest was reported by the authors.

Author contributions

NODQ, NBQ, BA, and KAK conceived and designed the study. MAA, PH and SB did the laboratory analysis of samples to generate molecular data. Data analysis and drafting of the manuscript were done by NODQ. All authors read, reviewed, and approved the final manuscript.

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Availability of data

Data for this work is available upon reasonable request from the corresponding author.

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