Synergism between Pfcrt and Pfmdr1 genes could account for the slow recovery of chloroquine sensitive Plasmodium falciparum strains in Ghana after chloroquine withdrawal

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Received 3 December 2015; received in revised form 4 February 2016; accepted 20 February 2016

Summary Unlike other countries, the chloroquine resistant marker Pfcrt T76 mutant has remained fairly stable in Ghana several years after official disuse of chloroquine. Certain mutations in Pfmdr1 may potentiate Pfcrt T76, offering a possible explanation for this observation. To understand the phenomenon, the coexistence of mutations in Pfmdr1 with Pfcrt T76 in Ghanaian Plasmodium falciparum
Synergism between Pfcr and Pfmdr1 genes in the recovery of Plasmodium falciparum strains

Introduction

Several years after the replacement of chloroquine as a first line antimalarial drug in Malawi, Ethiopia and Tanzania, among others, chloroquine (CQ) sensitive Plasmodium falciparum has recovered [1–4]. The re-emergence of CQ-sensitive *P. falciparum* resulted from an increased dominance of wild-type parasites that have lysine at codon 76 of the Pfcr gene [5–8]. Interestingly, the period of this recovery coincided with a reduction in point mutations of the Pfmdr1 genes, such as Asn86Tyr, Asn1042Asp and Asp1246Tyr [9]. This observation strongly suggests a possible link of Pfcr with Pfmdr1 mutations in a modulation of the sensitivity of *P. falciparum* to CQ.

In our previous report, we showed the main CQ resistant marker K76T of the Pfcr gene has remained fairly stable with a gradual decrease in certain parts of Ghana compared to Malawi [10,11]. Persistence of chloroquine resistant parasites in Ghana after the changes in anti-malarial drug policies has also been observed in other countries [12–14]. Though the prevalence of mutations in the Pfcr gene on chromosome 7 and the Pfmdr1 gene on chromosome 5 has been determined individually in Ghana, the interplay between them has yet to be assessed and discussed in detail. Study is needed due to reports that indicate both Pfmdr1 gene duplication and mutations at codons 86, 184, 1034, 1042 and 1246 are associated with resistance to chloroquine, mefloquine, quinine and artemisinin derivatives [12,15,16]. Of particular interest is how different haplotypes of Pfmdr1 contribute to specific antimalarial drug resistance. Specifically, the combined effect of Y184F, N1042D and D1246Y (FDY) haplotype isolates from Africa, Asia and South America have been reported to be associated with CQ resistance phenotypes [17–19]. Additionally, study is needed for the S1034C, N1042D and D1246Y (CDY) isolates from South America that are associated with quinine resistance [20], as well as for the N86, F184 and D1246 (NFD) isolates from Tanzania and Mozambique that are associated with artemether lumefantrine recrudescence [21,22]. This study investigated the impact of the combined mutations in the Pfmdr1 and Pfcr mutant genes on the delayed restoration of sensitivity of malarial parasites to CQ in Ghana. Additionally, the study sought an explanation for the reports of increasing levels of *P. falciparum* resistance to quinine and amodiaquine.

Methods

Study sites

This report describes a cross-sectional study conducted in Ghana health facilities located in areas
representing two distinct ecological zones, both forest and coastal. The facilities are the Twifo Praso district and St. Francis Xavier hospitals located in the forest zone, and the Cape Coast Metropolitan hospital and Elmina health center which are located in the coastal zone (Fig. 1). Apart from ecology, other considerations for selection of these sites were based on malaria endemicity, prevalence of Pfcr7T mutations and the level of chloroquine usage as determined in our previous study [10].

As in other areas in Ghana, treatment of febrile illness with chloroquine was the mainstay of malaria control until 2005 when field-based evidence indicated the presence of P. falciparum isolates resistant to the drug. Based on this evidence, and upon the recommendation of the WHO and other organizations, in 2005 Ghana officially changed from the use of chloroquine to an artemisinin-based combination therapy (ACT) as the first choice antimalarial drug for treatment of uncomplicated malaria. An artesunate—amodiaquine combination is the antimalarial drug most used in the selected study sites. The use of long lasting insecticide-treated nets (LLINs), indoor residual spraying (IRS) and intermittent preventive treatment among pregnant women (IPTp) remains the major malaria intervention measures in these areas.

**Ethical considerations**

All patients presenting with symptoms of malaria to the outpatient department of the selected health facility on the day of recruitment were screened for study inclusion. Only those with P. falciparum infection detected by microscopy were recruited to participate. The purpose of the study was explained to the adults. For children, both the children and their parents or guardian were encouraged to ask questions if clarity was needed. Patients were only recruited after giving consent.
Table 1  Prevalence of mutations in Pfmdr1 and Pfcrt genes in Plasmodium falciparum isolates.

<table>
<thead>
<tr>
<th>Gene/codon position</th>
<th>Polymorphisms (%)</th>
<th>Wild-type</th>
<th>Mutant type</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfmdr1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86</td>
<td></td>
<td>137 (64.0)</td>
<td>77 (36.0)</td>
<td>None</td>
</tr>
<tr>
<td>184</td>
<td></td>
<td>26 (12.1)</td>
<td>188 (87.9)</td>
<td>None</td>
</tr>
<tr>
<td>1034</td>
<td></td>
<td>62 (29.0)</td>
<td>152 (71.0)</td>
<td>None</td>
</tr>
<tr>
<td>1042</td>
<td></td>
<td>18 (8.4)</td>
<td>196 (91.6)</td>
<td>None</td>
</tr>
<tr>
<td>1246</td>
<td></td>
<td>179 (83.6)</td>
<td>18 (8.4)</td>
<td>17 (7.9)</td>
</tr>
<tr>
<td>Pfcrt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>76</td>
<td></td>
<td>60 (28.0)</td>
<td>115 (53.7)</td>
<td>39 (18.3)</td>
</tr>
</tbody>
</table>

Sample collection

A total of 618 patients were screened, out of which 217 were qualified to participate in the study. Basic biodata of all participants was collected and a 1 ml blood sample was collected and stored in tubes containing EDTA for parasitological analysis. A filter paper blood blot was prepared for each selected patient and later air-dried and stored at −20 °C in zip-locked plastic bags containing silica gel for molecular analysis.

Malaria microscopy

Thick and thin blood films were prepared from the patient’s blood samples and stained with 10% Giemsa. The slides were examined under oil immersion with the light microscope for the presence of P. falciparum.

Detection of Pfcrt and Pfmdr1 polymorphisms

Nested PCR for the amplification of Pfcrt and Pfmdr1 genes, followed by restriction fragment length polymorphism (PCR-RFLP), was used to detect mutations in the genes following published protocols [23,24]. Presence of mutations in Pfcrt at position 76 and Pfmdr1 at positions 86, 184, 1034, 1042 and 1246 were determined.

Data analysis

Data were organized using Microsoft Office Excel 2007 (Microsoft Corporation) and analyzed with SPSS Statistical Software version 16 (SPSS Inc.). Simple proportion was used to estimate the prevalence of the mutation. Statistical association between polymorphisms in Pfmdr1 and Pfcrt at codon 76 was determined with Pearson chi-square test. A p-value ≤ 0.05 was considered statistically significant. Additionally, synergism between Pfcrt and Pfmdr1 gene was assessed using the Jonckheere–Terpstra test and statistical significance was determined with a Monte Carlo simulation. The accuracy of synergism between Pfcrt and Pfmdr1 mutant genes was also tested using the Receiver Operating Characteristic (ROC) curve.

Results

Prevalence of point mutations in Pfmdr1 and Pfcrt gene of the P. falciparum isolates

Two hundred and fourteen P. falciparum isolates were analyzed for detection of mutations at N86Y, Y184F, S1034C, D1042N, and D1246Y of the Pfmdr1 gene and at codon 76 (K76T) of the Pfcrt gene. The prevalence of Y86, F184, C1034, Y1246 mutations of the Pfmdr1 gene determined were 36.0%, 87.9%, 71.0%, 91.6% and 8.4%, respectively (Table 1). Around half (53.7%) of the examined isolates had the T76 mutation of the Pfcrt gene (Table 1). The prevalence of Pfmdr1 haplotypes in the P. falciparum isolates were mainly NFCDD (n = 93, 43.46%), YFCDD (n = 59, 27.57%), NFSDD (n = 16, 7.48%), NYSNY (n = 11, 5.14%) and YFSD (n = 10, 4.67%; Tables S1 and S2).

Co-existence of Pfcrt and Pfmdr1 genes

Table 2 shows the association of Pfcrt alleles at codon 76 with alleles of Pfmdr1 at codon 86, 184, 1034, 1042 and 1246. One hundred thirty-seven of the total number of isolates examined had the wild-type allele at codon 86 of the Pfmdr1 gene together with the Pfcrt gene, while 29.2% had K76, 55.5% had T76 and 15.3% had both the K76 and T76 alleles. There was no significant difference between the codon 76 polymorphisms of the Pfcrt gene and the wild-type allele at codon 86 of the
<table>
<thead>
<tr>
<th>Codon</th>
<th>Pfmdr1 86</th>
<th>Pfcr 76</th>
<th>Fcrt 184</th>
<th>Fcrt 1034</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>T</td>
<td>K</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>1034</td>
<td>D</td>
<td>C</td>
<td>N</td>
<td>F</td>
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<tr>
<td>1246</td>
<td>Y</td>
<td>D</td>
<td>Y</td>
<td>N</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Codon</th>
<th>Single nucleotide polymorphisms N (%)</th>
<th>Total</th>
<th>Pearson $\chi^2$</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>T</td>
<td>24</td>
<td>13.770</td>
<td>0.032</td>
</tr>
<tr>
<td>1034</td>
<td>D</td>
<td>180</td>
<td>16.487</td>
<td>0.006</td>
</tr>
<tr>
<td>1246</td>
<td>Y</td>
<td>160</td>
<td>8.130</td>
<td>0.011</td>
</tr>
</tbody>
</table>

**Table 3** Association of Pfcr76 T76 mutation with the double mutation polymorphisms of the Pfmdr1 gene.

<table>
<thead>
<tr>
<th>Codon polymorphisms</th>
<th>Pearson $\chi^2$</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfmdr1 86 and 184</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>NY</td>
<td>T</td>
<td>4.083</td>
</tr>
<tr>
<td>YY</td>
<td>T</td>
<td>6.667</td>
</tr>
<tr>
<td>YY</td>
<td>K</td>
<td>6.667</td>
</tr>
<tr>
<td>NF</td>
<td>T</td>
<td>18.045</td>
</tr>
<tr>
<td>NF</td>
<td>K</td>
<td>18.045</td>
</tr>
<tr>
<td>YF</td>
<td>T</td>
<td>18.045</td>
</tr>
<tr>
<td>YF</td>
<td>K</td>
<td>18.045</td>
</tr>
</tbody>
</table>

*NB: The bold and underlined amino acids are mutant alleles.

Pfmdr1 gene ($\chi^2 = 10.961, p = 0.090$). Seventy-seven (77/214) of the isolates had the mutant allele at codon 86 of Pfmdr1. A statistically significant difference was observed between the isolates with a mutation at codon 86 of Pfmdr1 co-existing with that at codon 76 of Pfcr (mutant allele) compared to isolates with a mutant allele at codon 86 of Pfmdr1 co-existing with wild-type allele at codon 76 of the Pfcr gene ($\chi^2 = 19.506, p = 0.003$). Similar trends were observed between the mutations at codon 184, 1034 and 1042 of the Pfmdr1 and the mutant Pfcr gene. Observations made in this study indicated an association between a Pfcr 76T mutation with multiple mutations at 86, 184, 86, 184 and 1034 codons of the Pfmdr1 gene. The association between Pfcr T76 and double and triple mutations at 86, 184 and 1034 codon positions of the Pfmdr1 gene was also discovered. The results shown in Table 3 indicate a significant association between Pfcr T76 and Y86-F184 haplotype of Pfmdr1 ($\chi^2 = 18.045, p = 0.006$) but not the NY, YY or NF haplotypes of the Pfmdr1 isolates when only polymorphisms at codon 86 and 184 of Pfmdr1 were considered. The test of association between Pfcr T76 and the triple codon haplotype mutations of Pfmdr1 showed a similar trend to that observed for the double codon polymorphisms. Two levels of association were observed for the triple haplotype at 86, 184 and 1034 of Pfmdr1 and Pfcr T76, NFC ($\chi^2 = 13.770, p = 0.032$) and YFC ($\chi^2 = 16.489, p = 0.011$), but not with YFS ($\chi^2 = 5.511, p = 0.480$; Table 4). The pattern of association did not change when haplotypes for 86, 184, 1034 and 1042 of Pfmdr1 were determined. Similarly, haplotypes for 86, 184, 1034, 1042 and 1246 of Pfmdr1 showed an association with Pfcr T76 as did the triple haplotypes of Pfmdr1: NFCDD ($\chi^2 = 13.770, p = 0.032$) and YFCDD ($\chi^2 = 16.487$,
Table 4 Association of Pfcr76T mutation with the triple mutations polymorphism of the Pfmdr1 gene.

<table>
<thead>
<tr>
<th>Codon polymorphisms</th>
<th>Pearson $\chi^2$</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfmdr1 86, 184 and 1034</td>
<td>Pfcr76</td>
<td></td>
</tr>
<tr>
<td>NYS</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>NYS</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td>YYS</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>NFS</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td>YFS</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td>NFC</td>
<td>T</td>
<td></td>
</tr>
</tbody>
</table>

*NB: The bold and underlined amino acids are mutant alleles.

$p = 0.011$). No association was observed for YYSNY ($\chi^2 = 5.000$, $p = 0.287$; Tables S1 and S2).

**Synergism between Pfcr7 and Pfmdr1 gene**

The co-existence between the T76 mutation of Pfcr7 gene and mutations at codon positions 86, 184, and 1034 of Pfmdr1 were further tested for stochastic prevalence using the Jonckheere-Terpstra test (J–T). The standard J–T statistic between the Pfcr7 at codon 76 polymorphism and Pfmdr1 at codon positions 86, 184, 1034, 1042 and 1246 were 6.830, 8.695, 14.264, 7.086 and 4.028, respectively, with $p < 0.0001$. A significant synergism ($p < 0.0001$) between Pfcr7 mutation and the five point mutations of Pfmdr1 were also observed using Monte Carlo simulation at a 99% confidence interval using the Pfcr7 76 polymorphism as a group variable (Table 5). Accuracy of the synergism between the Pfcr7 and Pfmdr1 genes was tested again using the Receiver Operating characteristic (ROC) curve. The analysis indicated synergism between the Pfcr7 76 mutation and Pfmdr1-1034 mutation perfect (area under ROC curve = 0.994 ± 0.005, $p < 0.0001$) while that between Pfcr7 76 and Pfmdr1-1034 mutation at codon positions 86, 184, 1042 and 1246 were also significant, with a ROC area of 0.750 ± 0.032, 0.717 ± 0.045, 0.650 ± 0.046 and 0.614±0.039, respectively. However, synergism between Pfcr7 76 and Pfmdr1-1042 ($p = 0.001$) and Pfmdr1-1246 ($p = 0.01$) were observed to be comparatively weaker (Fig. 2 and Table 6).

**Discussion**

The association between chloroquine treatment failure and K76T mutation in Pfcr7 is well-known [25,26]. The contribution of N86Y and D1246Y mutations of Pfmdr1 to the fitness of CQ resistance P. falciparum strains has also been documented [27,28]. The withdrawal of CQ from Malawi, Mozambique and Kenya led to a rapid recovery of CQ sensitive P. falciparum, which increased the limited fitness costs that accompany CQ resistance [29,30]. However, a rapid recovery of CQ sensitive strains was not observed within the same timeframe in other disease endemic countries, such as Ghana and Uganda after CQ withdrawal [10–12,14,31]. Thus, the principle objective for this study became ascertaining reasons for the slow recovery of CQ sensitive P. falciparum with an increasing presence of amodiaquine and quinine resistance in Ghana. We have previously shown that the CQ resistance marker Pfcr7 T76 remains very high in Ghana even after several years of CQ withdrawal [10,11]. In the current study we demonstrated an association of the chloroquine resistance marker with all the point mutations in Pfmdr1; at codon 86, 184, 1034 and 1042, except at codon 1246. This finding may explain the continuous presence of significant levels of CQ resistant parasites in Ghana.

The mutations of Pfmdr1 gene are complex and the mechanism for modulating P. falciparum multiple antimalarial drug resistance is not well-understood [32,33]. The effect of this mutation changes unpredictably, as different mutations
can increase the parasite’s susceptibility toward one antimalarial drug or simultaneously confer resistance to another [34,35]. For instance, the N86Y-Y184F-D1246Y mutation of Pfmdr1 polymorphism causes *P. falciparum* to be susceptible to artemisinin-based combination therapy, whereas NFCDY or NFSDD haplotypes at codon 86, 184, 1034, 1042, and 1246 are associated with AQ failure in South America [36–39].

To understand the complexities of mutant Pfmdr1 genes and their relationship with the mutant Pfcr1 gene at codon 76, we tested for synergism between them using statistical methods. The results indicated a strong synergic interaction between Pfcr1 76 polymorphism and that of Pfmdr1 at 86, 184, 1034, 1042 and 1246. When the accuracy of the synergy was verified using ROC, the outcome showed a stronger synergy between Pfcr1 76 polymorphism and that of Pfmdr1 at codon 1034, 86, and 184 but was comparatively weaker with that at codon 1042 and 1246.

Amodiaquine and quinine are important antimalarial drugs in Ghana. The former is a co-partner in the first line of treatment of uncomplicated malaria using ACT, and the latter is the drug of choice for the management of complicated malaria. However, recent reports indicate some degree of parasite resistance to these drugs. In an attempt to understand the mechanism of resistance to these drugs in Ghana, we examined the association between Pfcr1 K76T and double mutational haplotypes at 86 and 184 of the Pfmdr1 gene. The results showed that Pfcr1 T76 was strongly associated with the double mutation YF haplotype but not in NY, YY or NF. This unique association has not been previously reported, although individually the 86Y of Pfmdr1 has been reported to be associated with CQ or amodiaquine resistance, whereas 184F and 1042D have been shown to have less sensitivity to QN [40,41]. Double mutation at 184 and 1042 of Pfmdr1 gene has been reported to reduce *P. falciparum* sensitivity to quinine whereas mutation at 86 and 1042 increases the parasite’s susceptibility to quinine treatment [42,43]. This means that modulation of quinine resistance could depend on specific combinations of mutations of Pfmdr1. For instance, a single mutation at codon 86 has been shown to increase *P. falciparum* susceptibility to quinine.
However, a N1042D mutation of Pfmdr1 can either increase or reduce the parasite’s susceptibility to quinine [42]. Furthermore, the insertion or deletion of haplotype mutations 1034C, 1042D and 1246Y of Pfmdr1 resulted in either quinine-resistance or quinine sensitive \textit{P. falciparum} strains [43]. This tends to suggest that the PfcrtaT76 & YFC haplotype of Pfmdr1 could contribute to the emergence of quinine resistance in Ghana. Although quinine remains the antimalarial drug of choice for the management of complicated malaria in Ghana, the recent report of an elevated IC$_{50}$ value [13,14] for the drug, in addition to the observations made in this study, should be a matter of concern to stakeholders.

From the data gathered in this study, it is reasonable to assert the association of PfcrT776 with a N86-F184-C1034 mutation of Pfmdr1 may be responsible for the presence of amodiaquine resistance in Ghana. This assertion is justified by field-base report of strains of \textit{P. falciparum} carrying either CVIET or SVMNT at codon 72–76 of the Pfcr gene and NFCDY or NFSDA at codon 86, 184, 1034, 1042, 1246 of the Pfmdr1 gene [42], as well as an association with high in vitro IC$_{50}$ values for monodesethylamodiaquine.

\textit{P. falciparum} strains with the N86, F184 and D1246 (NFD) haplotype have been reported to have a fitness advantage under artemether-lumefantrine (AL) pressure [39]. Thus, the observed increase in prevalence of NFD in Tanzania and Mozambique after the introduction of AL could involve NFD under AL pressure. Based on observations made in this study, is possible artemether-lumefantrine resistant \textit{P. falciparum} may emerge in Ghana in the near future. The reported high variation in patient response to treatment with artemether-lumefantrine in different ecological zones [41] could signify an onset of resistance to the combination antimalarial drug.

Several reports have shown that integrated vector control strategies, such as the use of long lasting insecticide-treated nets (LLIN) and indoor residual spraying (IRS), have resulted in reduced malaria morbidity and mortality especially among children [44–46]. In Ghana, vector control programs have been in place since 2005 but have not had any significant impact on disease prevalence until a recent refocus of efforts. New efforts involve not only free distribution of LLIN, but a door-to-door campaign to hang these nets by community-based volunteers to enhance usage. Additionally, indoor residual spraying in communities, including the study areas, has been ongoing. These interventional measures appear to have positive impact, as latest reports from the NMCP indicate the malaria prevalence rate is declining in Ghana [47]. Despite the clear public health benefit of the vector control measures, the impact of these measures on the spread of drug resistance \textit{P. falciparum} is not well understood. In 2011, Shi et al. reported there is no evidence to suggest sustained transmission reduction by LLIN usage reduces the prevalence of the genes associated with malaria drug resistance [48]. The influence of these vector control measures on the study findings could not be immediately determined.

Observations made in this study strongly suggest the slow recovery of chloroquine sensitive \textit{P. falciparum} strains and the presence of amodiaquine and quinine resistance in Ghana could be due to synergism between PfcrT and Pfmdr1 mutant genes. It must be emphasized that this could be caused by indiscriminate use of CQ with the resultant selection for resistant strains of the parasite. Chloroquine is a safe and affordable drug, and with gains in efficacy, could be adapted for use as one of the partners in a drug combination therapy. It is therefore imperative to put measures in place to ensure the fast restoration of chloroquine use in Ghana.

**Conclusions**

Findings from this study suggest synergism between mutant PfcrT and Pfmdr1 genes in \textit{P. falciparum} could account for the slow recovery of chloroquine sensitive parasites long after the disuse of chloroquine in Ghana. This phenomenon could also be responsible for the presence of quinine and amodiaquine resistance in the country. From the gathered data, if the right preventive measures are not put in place, artemether-lumefantrine resistance could emerge in Ghana sooner rather than later.

**Funding**

No funding sources.

**Competing interests**

None declared.

**Ethical approval**

The study proposal and protocol was approved by the Ghana Health Service Ethics Committee
Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiph.2016.02.004.

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