ROLES OF SPECIFIC \textit{PLASMODIUM FALCIPARUM} MUTATIONS IN RESISTANCE TO AMODIAQUINE AND SULFADOXINE-PYRIMETHAMINE IN BURKINA FASO

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Abstract. We evaluated associations between key polymorphisms in target genes and responses to treatment with sulfadoxine-pyrimethamine (SP) or amodiaquine (AQ) for uncomplicated \textit{Plasmodium falciparum} malaria in Bobo-Dioulasso, Burkina Faso. Presence of the dihydrofolate reductase (\textit{dhfr}) 108N or 59R mutations (but not \textit{dhfr} 51I or dihydropterotate synthetase [\textit{dhrs}] 437G) and \textit{P. falciparum} chloroquine resistance transporter (\textit{pfcrt}) 76T or \textit{P. falciparum} multidrug resistance 1 (\textit{pfmdr1}) 86Y or 1246Y mutations (but not \textit{pfmdr1} 184F) predicted recrudescence after treatment with SP and AQ, respectively. Treatment led to significant increases in the prevalence of the same mutations (except 1246Y) in new infections that presented after therapy. The \textit{dhrs} 164L and \textit{dhrs} 540E mutations were not seen in any isolates. These results clarify the key roles of a small number of mutations in \textit{P. falciparum} resistance to SP and AQ in west Africa.

INTRODUCTION

The management of \textit{Plasmodium falciparum} malaria in Africa is thwarted by increasing resistance to available antimalarial drugs. Resistance to chloroquine (CQ) mandates the use of other drugs for the treatment of uncomplicated malaria. New artemisinin-based combination therapies (ACTs) are increasingly advocated, but the implementation of these regimens has been slowed by their high cost and limited availability. Two older, less expensive drugs that remain extensively used to treat malaria in Africa are sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ). Despite significant \textit{P. falciparum} resistance to both drugs in many parts of Africa, they generally offer much improved efficacy over that of CQ, and thus may be important stop-gap therapies until more efficacious drugs are readily available. Indeed, the combination of SP and AQ has shown excellent efficacy in a number of recent studies from Africa. Additional continued importance of SP and AQ is highlighted by the inclusion of either drug in some ACT regimens, the recommendation for widespread use of SP in intermittent preventive therapy in pregnant women, and consideration of both drugs for intermittent preventive therapy in infants. With continued use of SP and AQ, an improved understanding of mechanisms of resistance to these drugs is needed.

Mechanisms of resistance of \textit{P. falciparum} to SP are quite well characterized. Mutations in the two target enzymes, dihydrofolate reductase (\textit{dhfr}) and dihydropterotate synthetase (\textit{dhrs}), mediate resistance, and increasing numbers of mutations lead to increasing levels of resistance. Resistance in Africa has spread in part due to sporadic acquisition of resistance-mediating mutations, but expansion of highly resistant clones appears to have played a major role, at least in east and southern Africa. Because a number of resistance-mediating mutations have commonly been seen together in highly resistant strains, the specific roles of particular mutations in mediating resistant phenotypes are incompletely described.

Mechanisms of resistance to amodiaquine have not been well described. Amodiaquine is similar in structure to CQ, and, although resistance to AQ is less common, cross-resistance between the two drugs is seen, suggesting shared resistance mechanisms. Chloroquine resistance is principally mediated by the 76T mutation in the \textit{P. falciparum} chloroquine resistance transporter (\textit{pfcrt}) gene. Additional mutations in \textit{pfcrt} and in another putative transporter gene, \textit{P. falciparum} multidrug resistance 1 (\textit{pfmdr1}), may play additional roles. The roles of these mutations in resistance to amodiaquine have not been studied extensively. To better characterize molecular markers of resistance to SP and AQ, we evaluated key parasite polymorphisms in samples from a recent randomized clinical trial including SP and AQ for the treatment of uncomplicated malaria in Bobo-Dioulasso, Burkina Faso.

MATERIALS AND METHODS

Details of the clinical trial have been published recently. This study was reviewed and approved by the institutional review boards of the University of California, San Francisco and the Center Muraz, Bobo-Dioulasso, Burkina Faso. All research subjects or their parents or guardians approved the use of clinical specimens. Briefly, patients $\geq$ 6 months of age with uncomplicated falciparum malaria were randomized to receive SP, AQ, or AQ plus SP. Patients were followed for 28 days, with follow-up on days 1, 2, 3, 7, 14, 21, and 28, and on any day on which a patient was ill. Treatment outcomes were classified according to World Health Organization guidelines as early treatment failure (ETF), late clinical failure (LCF), late parasitologic failure (LPF), or adequate clinical and parasitologic response (ACPR). For this study, only samples from patients treated with SP or AQ monotherapy were analyzed because failures in the AQ plus SP treatment arm were rare.

For analysis of the clinical trial, failures were classified as due to recrudescence or new infection based on characterization of polymorphisms in the merozoite surface protein 2 gene (\textit{msp} 2), but for this study more rigorous discrimination of recrudescence and new isolates was desired. Therefore, genotyping based on polymorphisms in \textit{msp} 2, \textit{msp} 1, and four microsatellite markers was performed on samples from all patients who developed recurrent parasitemia after day 4 (LCF or LPF) to distinguish recrudescence from new infections. Treatment failure was defined as all ETFs and any LCF.

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or LPF categorized as recrudescence based on genotyping results.

Blood was collected on filter paper on the day of initial diagnosis and for episodes of recurrent parasitemia more than six days after the initiation of therapy. DNA was isolated using the Chelex extraction method. Polymorphisms were assessed in a stepwise fashion at *msp 2*, *msp 1*, and four microsatellites. If there were no alleles in common at any one locus, the infection was classified as new. Otherwise, the next locus was genotyped. If any allele was in common at each of the six loci, the infection was classified as a recrudescence. Details of this genotyping method will be published separately.

Polymorphisms studied were *dhfr* N51I, C59R, S108N, and 108N, and *dhps* A437G and K540E for SP-treated patients and *pfcrt* K76T and *pfmdrl* N86Y, Y184F, and D1246Y for AQ-treated patients. All mutations were identified using a nested polymerase chain reaction followed by restriction enzyme digestion, as previously described. Digestion products were resolved by gel electrophoresis, and results were classified as wild-type, pure mutant, or mixed (both wild-type and mutant alleles in the same sample) based on migration patterns of ethidium bromide-stained fragments. Investigators were blinded to group assignment and outcomes during the molecular analysis.

To evaluate for associations between molecular markers and recrudescence, we compared 200 randomly selected pre-treatment samples resulting in an ACPR with all pre-treatment samples resulting in treatment failure due to recrudescence. To evaluate for the selection of mutations during drug clearance, we compared the prevalence of mutations in the 200 randomly selected pre-treatment samples to that in all samples collected at the time of new infections. All data were entered and verified using SPSS software (SPSS Inc., Chicago, IL) and analyzed using STATA version 8.0 (Stata Corp., College Station, TX). Categorical variables were compared using the chi-square test or Fisher’s exact test as appropriate. A *P* value < 0.05 was considered statistically significant.

RESULTS

Of 264 patients treated with SP with treatment outcomes, 19 had recurrent parasitemia after day 6, 17 of these were successfully genotyped, and recurrent infections were classified as recrudescence in 5 and new in 12. Of 280 patients treated with AQ with treatment outcomes, 48 had recurrent parasitemia after day 6, 44 of these were successfully genotyped, and recurrent infections were classified as recrudescence in 18 and new in 26. To determine whether key polymorphisms predicted treatment failure, we compared the risk of recrudescence when day 0 isolates lacked or included mutations at alleles of interest (Table 1). For this analysis, mixed genotypes were classified with pure mutants because the presence of a mutation that mediates treatment failure would be expected to impact upon treatment response. For patients treated with SP, the sample size was small because recrudescence after treatment was uncommon, but nonetheless two mutations, *dhfr* 108N and *dhfr* 59R, were significantly associated with recrudescence outcomes. These mutations have been previously associated with SP treatment failures. Of interest, two other mutations that have previously been associated with SP treatment failure, *dhfr* 51I and *dhps* 437G, were not associated with failure in our analysis. Two additional polymorphisms that have been associated with SP resistance in other areas, *dhfr* 164L and *dhps* 540E, were not seen in any of our isolates. For patients treated with AQ, mutations significantly associated with recrudescence outcomes were *pfcrt* 76T, *pfmdrl* 86Y, and *pfmdrl* 1246Y. For SP, the combination of the *dhfr* 108N and 59R mutations had a slightly stronger association with recrudescence compared with either mutation alone. For AQ, the combination of the *pfcrt* 76T and *pfmdrl* 86Y mutations had a stronger association with recrudescence compared with either mutation alone, but the addition of the *pfmdrl* 1246Y mutation weakened this association.

We next evaluated the impact of treatment with SP or AQ on the selection of drug resistance–mediating mutations (Figure 1). For SP, the prevalences of the *dhfr* 108N, 51I, and 59R mutations were all significantly higher in subsequent new infections compared with pre-treatment samples. The prevalence of *dhps* 437G was also higher in new infections compared with pre-treatment samples, but this difference was not statistically significant. For AQ, the prevalences of the *pfcrt* 76T and *pfmdrl* 86Y mutations, but not the *pfmdrl* 184F or 1246Y mutations, were higher in subsequent new infections compared with pre-treatment samples.

**Table 1.** Associations between molecular markers and recrudescence

| Treatment group* | Allele† | Wild-type | Mixed or mutant | Wild-type with recrudescence | Mixed or mutant with recrudescence | *P*
<table>
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<tbody>
<tr>
<td>SP (n = 80)</td>
<td><em>dhfr</em> 437G</td>
<td>25</td>
<td>55</td>
<td>3 (12%)</td>
<td>2 (4%)</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td><em>dhfr</em> 51I</td>
<td>46</td>
<td>34</td>
<td>2 (4%)</td>
<td>3 (9%)</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td><em>dhfr</em> 59R</td>
<td>48</td>
<td>32</td>
<td>0 (0%)</td>
<td>5 (16%)</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td><em>dhfr</em> 108N</td>
<td>44</td>
<td>36</td>
<td>0 (0%)</td>
<td>5 (14%)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td><em>dhfr</em> 59R + 108N</td>
<td>49</td>
<td>31</td>
<td>0 (0%)</td>
<td>5 (16%)</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td><em>dhfr</em> 51I + 59R + 108N</td>
<td>58</td>
<td>22</td>
<td>2 (3%)</td>
<td>3 (14%)</td>
<td>0.12</td>
</tr>
<tr>
<td>AQ (n = 110)</td>
<td><em>pfcrt</em> 76T</td>
<td>42</td>
<td>68</td>
<td>2 (5%)</td>
<td>16 (24%)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td><em>pfmdrl</em> 86Y</td>
<td>62</td>
<td>48</td>
<td>4 (6%)</td>
<td>14 (29%)</td>
<td>0.002</td>
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<tr>
<td></td>
<td><em>pfmdrl</em> 184F</td>
<td>44</td>
<td>66</td>
<td>10 (23%)</td>
<td>8 (12%)</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td><em>pfmdrl</em> 1246Y</td>
<td>84</td>
<td>26</td>
<td>10 (12%)</td>
<td>8 (31%)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td><em>pfcrt</em> 76T + <em>pfmdrl</em> 86Y</td>
<td>73</td>
<td>37</td>
<td>4 (5%)</td>
<td>14 (38%) &lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>pfcrt</em> 76T + <em>pfmdrl</em> 86Y + 1246Y</td>
<td>92</td>
<td>188</td>
<td>10 (11%) &lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*SP = sulfadoxine-pyrimethamine; AQ = amodiaquine.
† *dhfr* = dihydrofolate reductase; *dhps* = dihydropteroate synthetase; *pfcrt* = Plasmodium falciparum chloroquine resistance transporter; *pfmdrl* = P. falciparum multidrug resistance 1.
‡ Wild-type at any allele.
§ Mixed or mutant at all alleles.
importance of mutations mediating SP treatment outcomes. Our data show that dhfr 59R is more important than 51I as a marker of SP treatment failure. Furthermore, in the absence of the dhps 540E mutation, the 437G mutation did not predict treatment failure. These studies follow an analysis in Uganda that used statistical techniques to measure the importance of different mutations, and identified dhps 540E as the principal mediator, and dhfr 59R as a secondary mediator of SP resistance in that country. Consistent with those findings, in the absence of the 540E mutation, SP remains quite efficacious in Burkina Faso.

Associations between polymorphisms in pfcr and response to therapy with CQ are clear. The pfcr 76T mutation is the principal mediator of resistance. Additional pfcr mutations probably serve to maintain fitness of parasites with the 76T mutation. Mutations in pfmdr1 have been associated with CQ treatment failure in some, but not other studies. Interestingly, some pfmdr1 mutations that may play secondary roles in mediating CQ resistance lead paradoxically to improved sensitivity to a number of important antimalarials, including mefloquine, artemisinins, and halofantrine. With increased use of AQ, it was of interest to determine if mediators of AQ resistance are identical to those for CQ. Our data suggest that there are important differences. An earlier study in Sudan showed that the pfcr 76T mutation, but not pfmdr1 86Y, was associated with AQ resistance, and a study in Kenya showed that both of these mutations were selected in recurrent isolates by AQ therapy. Our results, which offered larger sample sizes and, unlike the earlier studies, standard 28-day treatment outcomes, showed that both the pfcr 76T and pfmdr1 86Y mutations are strong predictors of AQ treatment failure. Thus, pfmdr1 may play a more important role in mediating responses to AQ than is the case for CQ.

The pfmdr1 mutation encodes a predicted food vacuole transporter homologous to P glycoproteins, which mediate resistance in cancer cells by increasing the efflux of chemotherapeutic agents. Amodiaquine may be more potent than CQ due to increased accumulation of the drug or its metabolites in the food vacuole. Thus, mutations in pfmdr1 may expedite efflux of AQ, but not CQ at clinically relevant levels. This difference takes on particular importance because AQ use is increasingly advocated. As noted above, sensitivity to artemisinins is improved with the pfmdr1 86Y mutation and that to halofantrine, which is closely related to the ACT component lumefantrine, is improved with the 1246Y mutation. Treatment with artemether/lumefantrine selected for wildtype (pfmdr1 N86) parasites. Aminoquinoline ACT components, such as AQ or piperaquine, will likely provide the opposite selective pressure. Thus, different ACTs may select for or against strains that are relatively sensitive to AQ.

Our findings offer important insights into the mechanisms of resistance to SP and AQ. With SP, our data are consistent with the dhps 540E mutation, which is generally absent in Burkina Faso, playing the leading role in mediating high level SP resistance in Africa (albeit not as high as in parasites with the dhfr 164L mutation, which is generally not seen in Africa) and with dhfr 59R principally mediating the relatively low level resistance now seen in the country. With AQ, our data suggest roughly equal roles in resistance for key pfcr and pfmdr1 mutations. This information clarifies the specific molecular determinants of resistance and suggests that simple molecular assays, involving assessment of only a small num-

**Figure 1.** Prevalences of mixed or mutant genotypes in pretreatment samples and samples from subsequent new infections in the sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ) treatment arms. dhps = dihydropyrotoate synthetase; dhfr = dihydrofolate reductase; pfcr = Plasmodium falciparum chloroquine resistance transporter; pfmdr1 = P. falciparum multidrug resistance 1. *P < 0.05.

**DISCUSSION**

A number of polymorphisms were strongly associated with responses to treatment with SP or AQ. For SP, the dhfr 108N and 59R mutations were highly concordant, and this double mutant best predicted recrudescence. For AQ, the pfcr 76T and pfmdr1 86Y and 1246Y mutations were all predictive of recrudescence. For all these mutations except pfmdr1 1246Y, the importance of these associations was supported by the additional demonstration that treatment with SP or AQ selected for the respective mutations in subsequently infected isolates. For SP, other mutations that have previously been associated with outcomes, dhfr 51I and dhps 437G, were not predictive of treatment failure. For AQ, our findings suggest that treatment outcomes may be more dependent than those with CQ on pfmdr1 genotypes, with important implications for new combination regimens, as will be discussed below.

Despite many prior studies of associations between SP treatment outcomes and genetic polymorphisms, the roles of certain mutations in mediating resistance remain uncertain. Since most studies have been in east Africa, where SP resistance has apparently been driven by rapid expansion of a highly resistant clone, it is difficult to determine the importance of some single mutations. In particular, in east Africa dhfr 51I is commonly linked with 59R, and dhps 437G is tightly linked with 540E. In contrast, as was seen in a nearby country, in isolates circulating in Burkina Faso, dhfr 51I and 59R are less tightly linked, and dhps 437G is common despite absence of the 540E genotype. The different genotypes of west African isolates allowed discrimination of the relative levels resistance now seen in the country.
ber of alleles, can reliably gauge the level of resistance to SP and AQ in clinical isolates.

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