Amplification of *Plasmodium falciparum* Multidrug Resistance Gene 1 in Isolates from Gabon

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The study of molecular markers of drug resistance is particularly important in surveillance studies of drugs, such as mefloquine, that still retain efficacy in sub-Saharan Africa yet have encountered resistance elsewhere. In a recent study in Thailand, we identified amplification of the *Plasmodium falciparum* multidrug resistance gene 1 (*pfmdr1*) as being the most important predictor of in vitro drug resistance and in vivo failure of mefloquine monotherapy. Here we report amplification of *pfmdr1* in 15% of patient samples from Lambarene, Gabon, collected in 1995. None of the samples collected 7 years later showed *pfmdr1* amplification, suggesting that parasites with increased numbers of *pfmdr1* copies have not substantially spread through the population. Nevertheless, the detection of multicopy *pfmdr1* in African parasites suggests a high potential for rapid selection for resistance, implying that mefloquine use in Africa should be considered only as part of combination therapy.

Malaria due to *Plasmodium falciparum* is increasingly difficult to control and in recent years has caused worsening morbidity and mortality in countries of endemicity. Cheap, conventional antimalarials such as chloroquine have now failed in most of sub-Saharan Africa [1]. Molecular markers of chloroquine resistance confirm that chloroquine (and probably amodiaquine) are ineffective in most places. A K76T mutation in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) is associated with the resistance phenotype both in vitro and in vivo [2–4]. Single-nucleotide point mutations in another transporter, the *P. falciparum* multidrug resistance gene 1 (*pfmdr1*), including N86Y, may have also been selected for by chloroquine pressure [5].

Alternative therapies for multidrug-resistant malaria have been successfully implemented in Southeast Asia, where this problem is even more apparent. Recently, in a study at the Thai-Burmese border, we identified increases in numbers of *pfmdr1* copies as a key molecular marker of treatment failure, after either mefloquine monotherapy alone or combination therapy with mefloquine given with artesunate for 3 days [6]. Amplification of *pfmdr1* was also associated with in vitro resistance to mefloquine, quinine, and halofantrine and elevated artemisinin IC_{50}s. The N86Y, S1034C, and N1042D mutations were almost exclusively detected in parasites with a single copy of *pfmdr1*, and these single-nucleotide point mutations were therefore considered to be surrogate markers of single-copy *pfmdr1* in this geographic region. When present, the N86Y mutation was associated with relatively low mefloquine IC_{50}s.

Surveillance of drug resistance with well-established molecular markers is probably most useful when introduced before and during the clinical use of new chemotherapeutic regimens rather than once resistance is clinically manifest and established. This particularly applies to drugs that retain efficacy in sub-Saharan Africa yet have encountered resistance in Southeast Asia for decades [7, 8].
We therefore examined the pfmdr1 status of malarial parasites from Gabon, where mefloquine is not widely used and where 100% efficacy was earlier documented [9]. This molecular study was also of interest because of prior reports of intrinsic in vitro resistance to mefloquine in western Africa [10, 11].

PATIENTS, MATERIALS, AND METHODS

Study population. Clinical studies were conducted at the Medical Research Unit of the Albert Schweitzer Hospital in Lambarene, Gabon. Lambarene is a small town located in an area of rain forest, where transmission of malaria due to P. falciparum (the predominant species) is hyperendemic, with very little seasonal fluctuation [12]. Samples for molecular analysis were derived from a chemotherapy study conducted between January 1995 and January 1996 [13, 14] and from an in vitro surveillance study conducted between February 2002 to June 2002 [11], as described below.

All clinical and laboratory studies were approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital. Informed consent was obtained from patients or their legal caretakers.

Sample selection and DNA extraction. In January 1995, 252 patients ≤15 years old were enrolled in a malaria chemotherapy trial that extended until January 1996. Of these, 231 could be followed for 28 days or until parasitological recrudescence [13]. Patient histories and urine analyses ruled out consumption of antimalarials in the month before admission to the study. Mefloquine was not locally available, either in shops or by dispensation by local health authorities.

Treatment regimens were randomized and double-blinded and consisted of either sulfadoxine-pyrimethamine (62.5 mg and 3.125 mg, respectively), mefloquine (31.25 mg; approximately one-twelfth of the standard dose), or mefloquine-sulfadoxine-pyrimethamine (31.25 mg, 62.5 mg, and 3.125 mg, respectively) as a single low-dose treatment to investigate the proposed synergistic activity of the triple combination. A stratified randomization scheme was used to maintain equal numbers of participants in 3 different weight groups. Patients were considered to be cured if results of thick blood film became negative within 7 days and remained negative over the 28-day study period. Low-grade resistance was recorded if parasitemia and symptoms cleared during the first week of treatment but reappeared during the 28-day follow-up. Nonresponse was defined as a failure to clear parasites within 1 week, increasing parasitemia, or no clinical improvement by day 2. Blood samples from 29 patients treated with sulfadoxine-pyrimethamine or mefloquine-sulfadoxine-pyrimethamine and from 62 patients treated with mefloquine (collected before and after treatment) were available for DNA extraction [14].

For the in vitro surveillance study, 85 outpatients were recruited [11]. For 44 (52%) of these, drug studies were successfully conducted with cultured parasites, and aliquots of these samples were stored at −70°C. Genomic DNA was extracted from hemolysates (100 μL) with QIAamp blood DNA kits (Qiagen).

Quantitative analysis by TaqMan real-time polymerase chain reaction (PCR). Numbers of pfmdr1 copies were assessed by TaqMan real-time PCR on an ABI 7700 sequence detector (Applied Biosystems), as described elsewhere [6]. In brief, reactions were performed as multiplex PCR in MicroAmp 96-well plates (Applied Biosystems) in 25-μL volumes containing 1× TaqMan buffer, 300 nmol/L each forward and reverse primer, 100 nmol/L each probe, and 5 μL of DNA. Fifty cycles were performed at 95°C for 15 s and 58°C for 1 min. After each cycle, data were collected automatically, and, after completion, real-time data acquisition and analysis were done. Results were analyzed with the comparative ΔCt (cycle threshold) method. Each set of reactions contained 3 samples known to have 1 pfmdr1 copy, as calibrators. All reactions were done in triplicate. Numbers of copies were rounded to the nearest integer unless otherwise stated.

Detection of pfmdr1 and pfcr polymorphisms. The presence of single-nucleotide polymorphisms in pfmdr1 and pfcr was determined by previously described nested PCR–restriction fragment length polymorphism methods [15, 16]. DNA of the laboratory strains 3D7, HB3, 7G8, and Dd2 was included as controls.

Assessment of clonality of infection of field isolates. Three parasite loci (msp-1, msp-2, and glurp) that exhibit polymorphic numbers of tandem repeats were amplified by nested PCR from isolates with ≥1.5 copies, as described elsewhere [17]. PCR products were separated on a 1.7% agarose gel. The appearance of a double band at any of the 3 loci implied the presence of at least 2 clones. Equal band patterns between pre- and post-treatment samples were classified as recrudescent infections.

RESULTS

Low-dose mefloquine study, 1995. To study the prevalence of amplification in pfmdr1 in Lambarene, Gabon, we genotyped 29 samples from patients who were successfully treated with sulfadoxine-pyrimethamine or mefloquine-sulfadoxine-pyrimethamine as control subjects in a chemotherapy trial in 1995 (figure 1). None of these samples revealed increases in numbers of pfmdr1 copies.

We then assessed all available parasite samples from a subgroup of 76 patients from the same study who were treated with a low dose of mefloquine (figure 1). DNA was successfully extracted from blood samples of 62 patients. Nine of these patients were cured by mefloquine treatment. Samples collected on the day of failure were also available for the 24 patients with treatment failure due to low-grade resistance and the 29 patients
who did not respond to treatment. In total, 115 DNA samples derived from the mefloquine treatment group were genotyped for pfmdr1 (figure 1).

In 3 of these 115 samples, we detected duplication of pfmdr1 (table 1). An additional 5 samples reproducibly showed a number of copies between 1 and 2. Given that, in Lambaréné, most cases of malaria in children are due to multiple clones of P. falciparum [18], this strongly suggested polyclonal infections in these samples, because, in polyclonal infections, the assessment of copy number generates a figure that represents a weighted composite of the numbers of copies of genes in individual clones. Three-locus genotyping of these samples confirmed that all samples with a copy number between 1 and 2 were harboring >1 P. falciparum strain (data not shown).

The 8 samples with ≥1.5 copies of pfmdr1 clustered into 5 pairs of patient samples, and these 5 patients had all been clinically classified as experiencing failure due to low-grade resistance. These 5 pairs were further assessed for clonality and pfmdr1 point mutations.

Among parasites with increased numbers of copies, wild-type N86 alone or N86 in combination with mutant Y86 (in multiclonal samples) was highly prevalent (8/10), despite its very low frequency in the overall parasite population [14] (see below). This suggests a link between amplification of pfmdr1 and the wild-type pfmdr1 N86 codon. However, 1 parasite strain (strain 142) carried the N86Y mutation despite harboring amplified pfmdr (table 1).

Changes in 2 other pairs between pre- and posttreatment samples from N86Y or from ≥1.5 to 1 or from 1 to 2 copies were most likely accounted for by infection with new strains, often in addition to nonclearance of the first infection (classified as new and recrudescent infections in table 1). Comparison of glurp-1 and msp-1 and -2 genotyping suggested that the increased numbers of copies were not due to the spread of one single parasite strain through the local population (data not shown).

In vitro surveillance study, 2002. To assess whether increased numbers of copies had spread through the parasite population in and around Lambaréné, we genotyped samples collected 7 years later. Chloroquine, mefloquine, and quinine EC50s were also measured and have been reported elsewhere [11]. Forty-three of 44 samples with known EC50s were available for molecular genotyping. Real-time PCR and single-nucleotide point mutation genotyping were successfully performed for 37 of 43 samples. The drug susceptibilities for this subset of samples and the total number of samples assayed did not differ significantly (table 2), although 3 samples with mefloquine EC50s indicating borderline resistance were, unfortunately, not available for genotyping.

Overall, no amplification of pfmdr1 was detected in any of the samples. This indicated that parasites with increased numbers of pfmdr1 copies had not substantially spread throughout the local population in and around Lambaréné between 1995 and 2002.

Single-nucleotide point mutation genotyping revealed the 86Y mutation to be the predominant allele. This mutation was exclusively detected in 30 samples (plus 3 samples in which it

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**Figure 1.** Samples analyzed in a study of the Plasmodium falciparum multidrug resistance gene 1 (pfmdr1) as a predictor of in vitro drug resistance and in vivo treatment failure of mefloquine (Mfq) monotherapy. MSP, mefloquine-sulfadoxine-pyrimethamine; NR failure, failure due to nonresponse; RI failure, failure due to low-grade resistance; SP, sulfadoxine-pyrimethamine.
was detected along with N86, giving an overall prevalence of 89%). In comparison, only 1 sample was exclusively N86 (plus the 3 mixed samples, giving an overall prevalence of 11%). For codon 184, 6 samples (plus 1 mixed, giving an overall prevalence of 21%) were wild-type Y184, and 26 samples (plus 1 mixed, giving an overall prevalence of 79%) carried the mutant 184F. None of these mutations were associated with different IC50s for any of the drugs tested.

Furthermore, all 34 samples carried the S1034 wild-type and the N1042 wild-type codons. Notably, all but one carried the pfcr K76T mutation. This K76 isolate showed the lowest chloroquine EC50 (2.43 μmol/L of blood) of all of the samples tested in a schizont maturation test, but it was still well above the threshold level for resistance.

**DISCUSSION**

Our data indicate amplification of pfmdr1 in a low proportion of *P. falciparum* isolates from Gabon, some obtained as early as 1995. Furthermore, this amplification occurred despite the presence of the N86Y mutation. Increases in numbers of pfmdr1 copies in multidrug-resistant parasites from Thailand have recently been identified as a major determinant of clinical failure of treatment with mefloquine or mefloquine-artesunate, with gene duplication being the most important independent parameter in multivariable analysis. This gene amplification was inversely related to point mutations at positions 86, 1034, and 1042 [6].

When taking into account the rare administration of mefloquine in Gabon, our observation strongly suggests that drugs (or, speculatively, stressors other than mefloquine) can rapidly select for amplification of pfmdr1. Quinine is another possible selective agent, because quinine IC50s are positively correlated with those of mefloquine and are associated with increased numbers of pfmdr1 copies. However, despite widespread use of quinine, there are no indications of substantial clinical resistance to quinine in Africa (although there is debate about in vitro resistance), and this has clearly not led to a high prevalence of isolates with increased pfmdr1 copies. Potentially, amplification or demultiplication of *P. falciparum* genes is a more frequent event than is the emergence of single-nucleotide point mutations. Already, in early studies, >1 origin of amplification has been demonstrated in a small set of samples from Thailand [19]. This contrasts with the finding that point mutations in antifolate- or chloroquine-resistance determinants were found to have arisen extremely rarely, with just a handful of events worldwide, and then migrated as a result of genetic recombination and drug pressure [20]. Independent of the cause of the observed amplification in Gabon, there was no major population-wide spread or selection, because this amplified locus could not be detected in 37 samples collected 7 years later from the same geographic area.

Only in 2002 was the first isolate resistant in vitro to mefloquine reported in Lambaréné. Unfortunately, it was impossible to recover DNA from this sample, preventing analysis of its pfmdr1 genotype. Very high mefloquine IC50s have been reported from other areas of Gabon [10]; however, the underlying mechanisms remain to be determined. Furthermore, the high failure rate of the low-dose mefloquine treatment tested at that time cannot unambiguously be attributed to the few cases of resistance.
pfmdr1 amplification but perhaps resulted from the low dosage. This low-dosage regimen nevertheless provided sufficient selection pressure for pfmdr1 amplification and has implications for the choice of optimal combination partners for artemisinin in this area.

In Gabon, the prevalent pfmdr1 genotype at position 86 is the N86Y mutation [5, 14, 21], which may be attributed to continuous chloroquine selection pressure. This is in contrast to the situation in Thailand, where the frequency of N86Y is low, possibly because this drug is used relatively infrequently [6]. None of the >600 isolates tested in that region combined the 86Y mutation and an increased number of copies of pfmdr1, but some laboratory isolates—such as Dd2 (derived from W2mef, which originally was obtained from W2 under mefloquine pressure) and FCB (Colombia), as well as 1 isolate originally adapted from Thailand—have been shown to be 86Y while at the same time having amplified pfmdr1 genes. Even though most of the amplified samples from Gabon are N86 or have a combination of N/Y86 (in 4/10 polyclonal samples), 1 isolate clearly carried the N86Y mutant allele as well as an amplified gene. This suggests that parasites carrying the N86Y point mutation can undergo pfmdr1 amplification, but these may not survive under more intense conditions of mefloquine pressure, such as those found in Thailand.

The coexistence of N/Y86 in 1 sample highlights a technical problem in a transmission area such as Gabon, where mixed P. falciparum infections are detected in between 80% and 100% of symptomatic children [18]. Real-time TaqMan PCR is a quantitative technique that does not enable subpopulations of a population to be distinguished within 1 sample. This might explain why repeated assays of some samples (5/10 in this subset; 5/116 overall) gave intermediate results between 1 and 2 copies. These were therefore considered to be composite results [15] and were classified as a copy number of ≥1.5, which tended to underestimate the copy numbers for pfmdr1. Interestingly, no isolates with >2 copies were detected.

Our findings indicate that, in the 1995 study, the mefloquine dose was insufficient to select consistently for subpopulations of parasites with multicopy pfmdr1 in polyclonal infections. Nevertheless, there was evidence for such selection in 1 of the 5 paired samples that we studied. We note that the 5 patients harboring parasites with amplified pfmdr1 all lived in distant areas, making it unlikely that all observed cases were caused by the same parasite strain. This is further supported by the results from the 3-locus genotyping.

If mefloquine is to be used in Gabon, careful consideration must be given to the high potential of rapid selection for resistance to mefloquine and its manifestation, in view of the presence of parasites with increased numbers of pfmdr1 copies. Surveillance of copy numbers and the N86Y mutation at frequent intervals may identify this problem early and help prevent more widespread resistance to antimalarial drugs.

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References


