Genomewide Scan Reveals Amplification of *mdr1* as a Common Denominator of Resistance to Mefloquine, Lumefantrine, and Artemisinin in *Plasmodium chabaudi* Malaria Parasites^{▽†‡}

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Multidrug-resistant *Plasmodium falciparum* malaria parasites pose a threat to effective drug control, even to artemisinin-based combination therapies (ACTs). Here we used linkage group selection and Solexa whole-genome resequencing to investigate the genetic basis of resistance to component drugs of ACTs. Using the rodent malaria parasite *P. chabaudi*, we analyzed the uncloned progeny of a genetic backcross between the mefloquine-, lumefantrine-, and artemisinin-resistant mutant AS-15MF and a genetically distinct sensitive clone, AJ, following drug treatment. Genomewide scans of selection showed that parasites surviving each drug treatment bore a duplication of a segment of chromosome 12 (translocated to chromosome 04) present in AS-15MF. Whole-genome resequencing identified the size of the duplicated segment and its position on chromosome 4. The duplicated fragment extends for ~393 kbp and contains over 100 genes, including *mdr1*, encoding the multidrug resistance P-glycoprotein homologue 1. We therefore show that resistance to chemically distinct components of ACTs is mediated by the same genetic mutation, highlighting a possible limitation of these therapies.

In the absence of an effective malaria vaccine, malaria control is still heavily dependent upon the use of drugs. However, because compounds such as chloroquine are no longer effective, malaria treatment now depends upon new approaches based on the use of artemisinin-based combination therapies (ACTs) (41). If ACTs lose their efficacy, severe setbacks in efforts to control malaria will be experienced because there are few alternative drugs in development. Alarming, recent reports have documented the first signs of resistance to artemisinin derivatives (ARTs) in *Plasmodium falciparum* on the Thai-Cambodian border, an area where drug-resistant parasites have emerged in the past (8, 15, 19, 31).

It has been suggested that some components of ACTs may share similar underlying mechanisms of resistance (40), possibly compromising one important advantage of combination therapy. A comprehensive knowledge of the genes underlying resistance to these drugs is therefore imperative because the generation of molecular markers for drug surveillance will not only allow for timely detection of potential drug resistance foci

but also provide information for future choices of ACT components.

Research to identify the genetic modulators of responses to ACT component drugs has relied largely upon a “single candidate gene” approach, leading to examination of the *P. falciparum* homologue of P-glycoprotein (Pgh-1), an ATP-binding cassette (ABC) membrane transporter whose amplification was first described to be responsible for multidrug resistance in cancer cells (29).

The *P. falciparum* Pgh-1 protein, encoded by the *multidrug resistance 1* gene (*pfmdr1*), is localized on the membrane of the parasite's digestive vacuole and may directly mediate the transport of several antimalarial drugs and reduce drug efficacy (9, 32). This gene was originally found to be amplified and overexpressed in parasites selected *in vitro* for mefloquine (MF) resistance (5). Also, different alleles of the *pfmdr1* gene harboring specific single nucleotide polymorphisms (SNPs) have been implicated in modulating the response of the malaria parasite to different quinolines and ARTs (10). Additionally, field studies (15, 24–26, 39), drug selection experiments (3, 5, 21, 23), genetic manipulation (27, 34), and the identification of selective sweeps around the *mdr1* locus (17) have provided a large body of evidence to support the role of *pfmdr1* gene amplification in resistance to arylaminoalcohols, such as MF and lumefantrine (LM), as well as to the chemically unrelated ARTs. Recently, Uhlemann et al. (36) reported increased recrudescence, reinfection, or intrahost selection of *P. falciparum* parasites harboring an increased *pfmdr1* copy number in humans treated with MF.

For the rodent malaria parasite *P. chabaudi*, the MF-resis-

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FIG. 1. AS lineage of drug-resistant *P. chabaudi* parasites. The AS-sens clone is sensitive to pyrimethamine (PYR), chloroquine (CQ), and mefloquine (MF). Passage in mice in the presence of PYR (single dose) and multiple passages with increasing sublethal doses of CQ and MF gave rise to AS-PYR (38), AS-3CQ (33), AS-15CQ (20), and AS-15MF (6). Note that AS-15CQ was not cloned, and therefore AS-3CQ was used as a control.

tant mutant clone AS-15MF (6) has an ~400-kbp duplicated segment of chromosome 12 (chr12) that contains the *P. chabaudi* orthologue *pcmdr1* (PCHAS_123820 gene), among other genes. This large segment has translocated to chr04 (denoted chr04/12). This duplication/translocation event was shown to be associated with MF resistance by genetic linkage analysis of progeny clones of a genetic cross between AS-15MF and a genetically distinct drug-sensitive strain, AJ (6). However, it was suggested that additional gene loci could be involved.

This study attempted to clarify whether *mdr1* duplication is the dominant determinant of resistance and if other genetic loci are involved. Experimental genomewide scans of selection are possible using linkage group selection (LGS) (7), which analyzes quantitative genomewide markers in populations of uncloned progeny of genetic crosses before and after drug treatment. Genetic loci showing markedly reduced proportions of alleles from the sensitive parent (selection valleys) indicate the positions of genes conferring the drug resistance phenotype. Also, an inventory of all mutations may be constructed using whole-genome resequencing of the mutant parasite and its sensitive progenitor (13). Together, these approaches will identify the precise mutations and major genetic determinants of drug resistance.

In this study, we characterized the responses of the MF-resistant rodent malaria parasite AS-15MF to MF, LM, and ART. The genetic basis of these responses was then investigated in a genetic cross by mapping genomewide signatures of drug selection. Finally, genome resequencing of the AS-15MF genome was used to specify the critical mutations.

MATERIALS AND METHODS

Parasites and hosts. The parasites used were cloned lines of *P. chabaudi chabaudi* derived from isolates AJ and AS, originally isolated from *Thomomys rutilans* thick-tailed rats from the Central African Republic (2). The AS parasite has subsequently been used to derive a lineage (AS lineage) of clones resistant to antimalarial drugs. This lineage includes AS-3CQ (33), which presents resistance to chloroquine (CQ), and AS-15MF (6), which is resistant to both CQ and MF (Fig. 1).

Parasite clones were available as deep-frozen samples kept in liquid nitrogen. Four- to 6-week-old laboratory CBA female mice were used for parasite infections, and 6- to 8-week-old C57BL/6 mice were used for parasite transmission through *Anopheles stephensi* mosquitoes, as described by Walliker et al. (38). Mouse drinking water was supplemented with 0.05% *para*-aminobenzoic acid to enhance parasite growth (14). Parasite growth was monitored from day 4 post-inoculation (p.i.) onwards (up to day 20 p.i.) by examination of blood smears stained with Giemsa stain. The percentage of parasitemia was ascertained by optical microscopy at a magnification of $\times 100$.

All animal procedures were conducted under license in compliance with the United Kingdom Animals (Scientific Procedures) Act 1986.

Tests of drug responses. MF hydrochloride powder was purchased from Roche Basel. LM was a gift from Stephen Ward (Liverpool School of Tropical Medicine). ART was acquired as a gift from Dafra Pharma. Drugs were made up as solutions in dimethyl sulfoxide (DMSO) and were administered orally at various doses to mice by gavage in 100 μ l/20 g of body weight.

In order to characterize drug responses, *P. chabaudi* clones (AS-15MF, AS-3CQ, and AJ) (Fig. 1) were exposed to MF, LM, and ART treatment. Thus, for each clone, groups of mice were inoculated intraperitoneally with 10^6 parasites (day 0). MF (4 mg kg⁻¹ day⁻¹ or 6 mg kg⁻¹ day⁻¹), LM (3 mg kg⁻¹ day⁻¹), and ART (50 mg kg⁻¹ day⁻¹) were given to treated groups daily, starting at day 1 p.i., for a total of 3 days. An untreated control group was included and was given DMSO only by gavage. Daily blood smears were taken from day 4 p.i. onwards (up to day 20 p.i.), and percentages of parasitemia were determined.

Genetic backcross. The uncloned progeny of a genetic cross previously obtained between AS-15MF and the genetically unrelated MF-sensitive clone AJ (termed AS-15MF $\times$ AJ) (6) were initially grown in mice and selected with 4 mg kg⁻¹ day⁻¹ of MF for 3 days. The resulting surviving parasites were backcrossed with AJ by a method previously described by Hunt et al. (13), with minor modifications. Briefly, in order to increase gametocyte production, we stimulated red blood cell production by administration of erythropoietin (EPO). A volume of 100 μ l of EPO (1 μ g/ml) was injected intraperitoneally into each mouse for 5 days, starting 2 days prior to parasite inoculation. The parasite inoculum contained 10^6 parasites of both AJ and the selected parasites from the AS-15MF $\times$ AJ cross, in approximately equal proportions. Mosquitoes were allowed to feed on day 6 p.i. After 4 days, mosquitoes were further fed on uninfected mice (booster feed) to maximize oocyst maturation. Oocysts were monitored at day 8 postfeed. After completion of the sporogonic cycle, between days 15 and 16 post-blood feed, sporozoites were collected from salivary glands of infected mosquitoes and injected intraperitoneally into uninfected mice. When the sporozoite-induced infections reached percentages of parasitemia of ~20%, parasites were harvested, pooled, and used for two independent LGS experiments, herein termed selections 1 and 2.

LGS. A total of 10^6 parasites were inoculated into individual mice (groups of 5) (day 0) that were either left untreated or treated with drug daily for 3 days, starting on day 1 postinfection, as follows: MF (4 mg kg⁻¹ day⁻¹) or ART (50 mg kg⁻¹ day⁻¹) was used in selection 1, and MF (6 mg kg⁻¹ day⁻¹), LM (3 mg kg⁻¹ day⁻¹), or ART (100 mg kg⁻¹ day⁻¹) was used in selection 2.

Both the drug-treated and untreated blood-stage cross progeny were harvested when percentages of parasitemia reached 30% to 40% (untreated groups) or 15% to 20% (treated groups). Blood samples from all mice in each treatment group were harvested into citrate saline (pH 7.2) and pooled.

Parasite harvesting and DNA extraction. Pooled blood samples were passed twice through columns of powdered cellulose (CF11; Sigma) and twice through Plasmidipur filters (Euro-Diagnostica) in order to remove mouse leukocytes. After erythrocyte lysis with 0.15% saponin, the parasite pellet was washed twice in phosphate-buffered saline (PBS) and stored at -70°C . Parasite DNA was extracted by incubation at 37°C overnight in lysis buffer (150 mM NaCl, 25 mM EDTA, 0.25% sodium dodecyl sulfate [SDS], 0.125 mg/ml proteinase K). After phenol-chloroform extraction, DNA in 300 mM sodium acetate (pH 5.2) was precipitated with 2 volumes of ethanol, further washed in 70% ethanol, and dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). DNA concentrations were measured, and samples were stored at -20°C until further use.

Pyrosequencing. DNA samples from treated and untreated groups were analyzed using genomewide quantitative SNP markers for pyrosequencing (4). A genomewide library of more than 100 pyrosequencing SNP assays distinguishing AS and AJ alleles was previously developed using Pyrosequencing Assay Design software, version 1.0.6 (Biotage AB). This defined two oligonucleotides as PCR primers (one is biotinylated) and a sequencing primer near the SNP. Pyrosequencing assays were conducted according to the manufacturer's protocols (Biotage), using a PSQ HS-96A instrument, as described by Cheesman et al. (4). For this study, approximately 80 SNP markers were used, and comparative indices (CI) were calculated by dividing the

FIG. 2. Chromosomes 04 and 12 in mefloquine-resistant parasite AS-15MF. AS-15MF shows duplication of a 393-kbp segment (striped rectangles) of chr12 containing the *mdr1* gene and its translocation to chr04. This new hybrid chromosome is called chr04/12. The precise translocation site was mapped by PCR using primers targeting chr12 (12F and 12R1) and chr04 (04F), as shown, and by sequencing of the fragments (see the text for further details).

proportions of markers from the sensitive parent (AJ) in drug-selected progeny by those from the unselected progeny (7).

Identification and fine mapping of a translocation event involving chr12 and chr04 of AS-15MF. Whole-genome sequencing data for clones AS-15MF and AS-sens were generated using Solexa short-read sequencing as described by Hunt et al. (13). Single-end 36-base reads were mapped onto the *P. chabaudi* AS genome assembly of the Wellcome Trust Sanger Institute (AS-WTSI) (http://ftp.sanger.ac.uk/pub/pathogens/P_chabaudi/Archive/September_2009_assembly/). The local and genomewide fold coverages were calculated for both clones.

In order to confirm the predicted translocation event on chr04, oligonucleotide

primers were designed (Fig. 2) as follows: a chr12 forward primer (12F [5'-TTC GTT GGT GAT TTT ATT TCT TC-3']) maps upstream (left) of the predicted translocation point in chr12 (nucleotides [nt] 1313813 to 1313835), a chr12 reverse primer (12R1 [5'-GAT GAA CTG ATG AAT CTA GAG-3']) maps downstream (right) of the predicted translocation point (nt 1314181 to 1314201), and a chr04 forward primer (04F [5'-GTG TTT TAT GGG TTA TTT CTC G-3']) maps to the right-hand end of chr04 (nt 783833 to 783854), upstream (left) of the proposed deletion/translocation point.

PCRs were carried out by pairing each forward primer (12F and 04F) with the reverse primer (12R1), using DNAs from the AS-15MF and AS-3CQ clones. Briefly, template DNA was used in a 50- μ l PCR mixture containing 0.2 μ M (each) primers, 1 $\times$ PCR buffer (Promega), 1.5 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, and 25 mU/ μ l of *Taq* DNA polymerase. Amplification was carried out under the following conditions: 95°C for 3 min, 48°C for 1 min, and 65°C for 1 min, followed by 40 cycles of 94°C for 1 min, 50°C for 30 s, and 65°C for 30 s, with a final extension of 65°C for 2 min.

In cases where amplification occurred, PCR products were purified using a GFX PCR DNA purification kit from GE Healthcare and were sequenced using the terminal primers.

RESULTS

AS-15MF shows cross-resistance between mefloquine, lumefantrine, and artemisinin. In order to characterize drug responses and infer potential patterns of cross-resistance in the *P. chabaudi* lineage, the MF-resistant clone AS-15MF, its sensitive progenitor AS-3CQ (Fig. 1), and AJ were exposed to MF, LM, and ART *in vivo* (Fig. 3A to D). AJ was the sensitive clone subsequently used in genetic crosses.

In the absence of drug, all clones grew normally, with peak parasitemia occurring on day 7 p.i. (Fig. 3A to D). Following MF treatment (Fig. 3A and B), both AJ and AS-3CQ showed only a slight recrudescence on day 13 or 14 p.i. at 4 mg kg^{-1} and 6 mg kg^{-1} of MF, never exceeding 3% parasitemia throughout the infection. In contrast, AS-15MF reached peak percentages of parasitemia of about 35% (day 7 p.i.) and 10% (day 9 p.i.) at low (4 mg kg^{-1}) and high (6 mg kg^{-1}) drug concentrations, respectively. These observations thus con-

FIG. 3. Mean % parasitemia of AS-15MF, AS-3CQ, and AJ infections under 3-day drug treatment ($n = 4$ mice). MF, mefloquine; LM, lumefantrine; ART, artemisinin. (A) MF at 4 mg kg^{-1} day $^{-1}$; (B) MF at 6 mg kg^{-1} day $^{-1}$; (C) LM at 3 mg kg^{-1} day $^{-1}$; (D) ART at 50 mg kg^{-1} day $^{-1}$.

firmed the MF resistance phenotype of AS-15MF relative to that of AS-3CQ and AJ.

Under LM treatment (Fig. 3C), peak parasitemia (10%) was reached at day 10 p.i. for AS-15MF, in contrast with the case for AJ and AS-3CQ, which showed only a low-level recrudescence (<3%) after day 14.

For ART at the lower dose of 50 mg kg⁻¹ (Fig. 3D), peak parasitemia (30%) was reached by day 7 p.i. for AS-15MF, while AJ and AS-3CQ peaked at 10% parasitemia by days 11 and 9, respectively. In response to a higher dose of ART (100 mg kg⁻¹), previous work has shown that AS-15MF showed recrudescence on day 8, reaching peak parasitemia on day 15, while AJ and AS-3CQ were totally eliminated (13).

These data demonstrate that the MF-resistant clone AS-15MF also shows decreased sensitivity to LM and ART, despite never having been exposed to these two drugs previously. Therefore, we postulated that MF resistance in *P. chabaudi* AS-15MF arose along with the selection of one or more genetic mutations that also function to protect the parasite against the effects of LM and the chemically unrelated compound ART.

LGS defines MF, LM, and ART selection signatures. In order to map the genetic loci underlying MF, LM, and ART resistance, we used LGS, a strategy for identifying signatures of drug selection in the uncloned progeny of a genetic cross, in this case between AS-15MF and the genetically unrelated MF-sensitive clone AJ (6). To further increase the resolution of selection, a backcross was performed between the uncloned progeny of that cross (treated with MF) and AJ. The resulting recombinant progeny from this cross were either treated with MF, LM, or ART or left untreated. This procedure was repeated in two independent experiments (selections 1 and 2) with various drug doses, as described in Materials and Methods. The proportions of parental alleles (AS and AJ) in parasites surviving drug treatment were compared with those in the untreated parasites. Genomewide libraries of 77 and 84 pyrosequencing SNP markers, for selection 1 and selection 2, respectively, were used to quantify the proportions of the sensitive AJ parental alleles found in the progeny at loci distributed throughout the genome. Results obtained for selections 1 and 2 are shown in Fig. 4A and B, respectively.

Under MF treatment (4 mg kg⁻¹) (Fig. 4A), two main selection valleys were identified, on chr12 and chr04, consistent with the locations of the *pcmdr1* gene and its duplicated copy, respectively. The selection on chr04 appears at the right-hand end of the chromosome. With a higher dose of MF (6 mg kg⁻¹) (Fig. 4B), the depths of these valleys were more pronounced. We interpret these data as confirming that MF selects for parasites which contain the duplicated fragment on chr04/12 and hence have two copies of *mdr1*. Selection for AS alleles is stronger on chr04 than on chr12. This pattern of selection is consistent with a model where (i) the duplicated segment is to the extreme right of chr04/12, (ii) meiotic recombination may occur to the left of this segment, and (iii) under drug selection, the duplicated portion (on chr04) is necessarily inherited from the AS parent, while the original segment on chr12 can bear either AS or AJ alleles.

After selecting the AS-15MF × AJ backcross with 3 mg kg⁻¹ of lumefantrine (Fig. 4B), two major selection valleys were also identified, again on chr12 and chr04, demonstrating that par-

asites harboring a duplicated *mdr1* gene were also selected by this drug.

ART selection in both LGS experiments, at 50 mg kg⁻¹ and 100 mg kg⁻¹, (Fig. 4A and B, respectively), generated selective signatures on chr12 and chr04, showing that this drug also selects for parasites containing a duplicated chromosomal segment containing the *mdr1* gene.

In summary, MF, LM, and ART each selected parasites with a duplicated chromosomal region containing the *mdr1* gene.

In selection 2, indications of a distinct selection valley profile for chr12 (Fig. 4B) raised the possibility that there may be selection for AS alleles of *mdr1* (or a linked gene) relative to AJ alleles, superimposed upon the (apparent) selection of AS alleles which arises as a necessary consequence of selection for a gene duplication. However, previous data (6) showed that there was not a distinct difference in MF resistance in resistant clones carrying two AS alleles of *mdr1* and those containing one AS and one AJ allele. Instead, we believe that the finer structure of the valley profile may arise not because of genetic selection processes but because of local mapping artifacts, such as differences between the AS-WTSI genome assembly used and the genomes of the parental clones used in our study (data not shown).

There was some evidence of selection at other loci. For instance, a high dose of MF (Fig. 3B) or ART (Fig. 4B) was found to select for AS alleles on chr02. This locus was also selected by ART in two additional independent genetic crosses (12, 13) and contained a sole mutation (V2728F; formerly annotated V770F [12]) in the *ubp1* gene (PCHAS_020720 gene), encoding a deubiquitinating enzyme (12). We therefore suggest that this mutation contributes to the survival of parasites at higher doses of MF as well as being a principal determinant of ART resistance (13).

Furthermore, at the higher dose of MF or ART (Fig. 4B), possible selection signatures were indicated toward the right-hand end of chr01. This locus requires further investigation in independent experiments. This result could occur, for example, if a segment in this region had translocated to a new chromosomal location on chr02 or if some chromosomal segments currently assembled on chr01 were actually physically linked with those on chr02.

Fine mapping reveals the exact point of translocation involving the segment containing the *mdr1* gene. A previous study demonstrated that AS-15MF bears an ~400-kbp duplication event on chr12 (containing *mdr1*) which translocated to chr04 (6). We investigated this event further by using Illumina (Solexa) whole-genome sequencing of clones AS-15MF and AS-sens as described by Hunt et al. (13). Analysis of the number of reads obtained at each nucleotide (13) (see Materials and Methods) revealed an extended region on chr12 where the comparative coverage was ~2-fold (i.e., the relative coverage for AS-15MF was approximately twice that for AS-sens). This region extends from approximately base 1,315,000 to the end of the chromosome and totals ~368 kbp (Fig. 5A). It contains *pcmdr1* and many other linked genes (see Table S1 in the supplemental material). We therefore suggest that these data confirm the previous gene amplification analysis, which predicted a duplicated region of ~400 kbp (6). Fold coverage analysis also revealed a region at the right end of chr04 with fold coverage of ~0 for AS-15MF but not for AS-sens (Fig.

FIG. 4. LGS of AS-15MF $\times$ AJ backcross under 3-day drug treatment (selection 1 and selection 2). The figure shows the CI calculated for each of the ~ 80 SNP markers specific to the sensitive parent (AJ) and their physical locations in the *P. chabaudi* genome (kbp). Data points are means for 3 independent measurements. (A) Selection 1, using 4 mg kg⁻¹ of MF and 50 mg kg⁻¹ of ART. (B) Selection 2, using 6 mg kg⁻¹ of MF, 3 mg kg⁻¹ of LM, and 100 mg kg⁻¹ of ART for 3 days. *ubp1* indicates the position of the sole point mutation (V2728F in *ubp1* [see the text for further details]) on chr02, as determined by Solexa analysis (13). *mdr1* indicates the position of the multidrug resistance locus and the position of the duplicated fragment on chr04/12, as determined by Solexa fold coverage analysis (see the text).

5B). This region was mapped to between approximately nucleotide 784,140 and the end of the chromosome and was ~ 9.8 kbp long. We predicted that this region is deleted in AS-15MF. It spans two annotated genes, namely, the PCHAS_042070 and PCHAS_042080 genes, both predicted to encode *P. chabaudi*-specific interspersed repeat proteins (*cir*).

We believe that this fold coverage analysis was possible in the *P. chabaudi* AS lineage for three reasons. First, comparisons between sensitive (AS-sens) and resistant (AS-15MF) clones are more likely to be informative and valid because the clones are almost isogenic. Second, the variations in relative

coverage across the genomes are remarkably conserved between the two clones. Third, the reference sequence used for mapping reads (AS-WTSI) is identical or nearly identical to those of both clones used. These similarities allow a large proportion of reads to be mapped accurately and similarly for both clones.

We hypothesized that in AS-15MF, the duplication and translocation event involving part of chr12 was coupled with the deletion event at the right-hand end of chr04, specifically, that the duplicated segment from chr12 replaced the deleted right-hand end of chr04. On this basis, we defined oligonucle-

FIG. 5. Analysis of fold coverage (Illumina genome resequencing) for clones AS-15MF and AS-sens in regions of chromosome 12 (A) and chromosome 4 (B) and in unassigned contigs (C). Mean fold coverages (or read depths) are shown for windows of 1,000 bases (A and C) or 50 bp (B). Variations in fold coverage are highly conserved between AS-sens and AS-15MF, except for regions (blue boxes) of ~368 kbp on chr12 (A), ~9.8 kbp on chr04 (B), and ~25 kbp in unassigned contigs (corresponding to contig 1257 in the previous assembly) (C). These regions indicate duplication of a large fragment of chr12 containing *mdr1*, a predicted deletion in chr04, and a contig predicted to map to the duplicated part of chr12, respectively.

otide primers 04F and 12R1 to amplify DNA across the putative translocation point on chr04/12 in AS-15MF (Fig. 2). A PCR product (510 bp) was amplified from AS-15MF but not from AS-3CQ, and the product was sequenced. Nucleotide sequences of opposite ends of this fragment aligned with the predicted sequences from chr04 and chr12 (Fig. 6). The alignments revealed that an 11-bp sequence string (ATTTTGTTT) common to both chr04 (nt 784143 to 784153) and chr12 (nt 1314002 to 1314012) separated the chr04 and chr12 alignments, suggesting that it mediated homologous recombination during the translocation event. Additionally, a PCR product of

FIG. 6. A partial fragment amplified from AS-15MF (using primers 04 and 12R1 [see Materials and Methods]) aligns with contig sequences mapping to chr04 and chr12. This reveals an 11-bp sequence string (ATTTTGTTT) (shaded) present in both chr04 and chr12 that forms the translocation site.

389 bp was obtained from both AS-15MF and AS-3CQ by using primers 12F and 12R1, as expected, showing that the chr12 sequence was conserved during duplication and translocation.

Further inspection of the fold coverage data indicated that contig 1257 (25 kbp; not mapped to a specific chromosome) also displayed an ~2-fold increase in relative coverage in AS-15MF compared to that for AS-sens (Fig. 5C). Although there are no syntenic genes present in this contig, we identified sequence similarity between sequences on this fragment and rRNA sequences from *P. falciparum* chr05 (MAL5_18S, MAL5_ITS1, MAL5_5.8S, MAL5_ITS2, and MAL5_28S). Therefore, we suggest that this contig maps to the right-hand end of chr12 in AS-sens and that this region was also duplicated (and translocated) along with the (contiguous) ~368-kbp segment.

Thus, Solexa genome resequencing mapped an amplified region of ~393 kbp on chr04, the precise range of the duplicated region (including *mdr1*) on chr12 in AS-15MF, and its translocation to the right-hand end of chr04.

The duplicated segment extends from within the PCHAS_123610 gene (the orthologue of the *P. falciparum* PFE1045c gene) on chr12 up to the known end of the chromosome, including the last annotated gene, PCHAS_124620 (the orthologue of the *P. falciparum* PFE1570c gene), for a total of 105 genes (see Table S1 in the supplemental material). Additionally, the six *P. chabaudi*-specific genes (no orthologues in *P. falciparum*) contained within contig 1257 (PCHAS_000600 to PCHAS_000650 genes) (see Table S1) are also duplicated.

DISCUSSION

The parasite *P. chabaudi* AS-15MF presents cross-resistance to the chemically related drugs MF and LM. More surprisingly, AS-15MF also has resistance to ART, despite the fact that this parasite has not previously been exposed to ARTs.

Whereas the involvement of *mdr1* in parasite resistance to a number of drugs has been documented (5, 21, 23, 25, 34), the possible influence of other mutations throughout the parasite genome has not been ruled out. Therefore, we scanned the whole genome for additional signatures of selection and for corresponding mutations therein. Using this strategy, we observed major selection signatures from MF, LM, and ART on chr12 and chr04, consistent with the known locations of the *pcmdr1* gene and its translocated copy, respectively. This provides conclusive and unambiguous genomewide evidence of a predominant role for the large duplicated chromosomal region containing the *mdr1* gene (among others) in modulating resistance to different components of ACT.

In cases where resistance to each component drug requires mutations in a different genetic pathway, combination therapies are thought to reduce the probability that parasites resistant to both of the component drugs will survive treatment. Here we show that this may not be the case for MF-artesunate combinations and that this specific combination, widely used in Southeast Asia, may not be designed optimally as previously suggested. Actually, despite the fact that mefloquine monotherapy has selected parasites with *mdr1* amplification in Southeast Asia (25), the MF-artesunate combination has

proved clinically effective. This may reflect the potency of artemisinins and the combination of the fast-acting but rapidly cleared artesunate drug with the slower kinetics of mefloquine. Also, *mdr1* amplification may not be the sole determinant of resistance to the different ACT components. Indeed, we recently showed in a different report (30) and also in the present study that although *mdr1* amplification plays a dominant role, contributions of other determinants are indicated as well, particularly at higher doses of mefloquine: e.g., *ubp1* (13) or other genes linked to *mdr1* and contained within the chr12 translocated fragment may have a role.

Artemether-LM mixtures (Coartem) are recommended in many African countries (41). Our results suggest that the effectiveness of these mixtures may be compromised similarly by the emergence and spread of parasites bearing *mdr1* amplifications. However, at present, *pfmdr1* amplification is not a frequently observed event in sub-Saharan African parasites. Our data support the hypothesis that the use of artemether-LM or MF-artesunate might increase the prevalence of such parasites, that these parasites would challenge the future use of some ACTs, and therefore that the prevalence of these genotypes should be monitored closely.

Is *mdr1* the critical gene in the duplicated region? The large body of evidence from field and laboratory studies that implicates *mdr1* strongly suggests that this is the case. For instance, identification of copy number variations (CNVs) in both laboratory strains and clinical isolates of *P. falciparum* identified duplications of chromosome 05 that contained several different genes, among which two, PFE1150w (*pfmdr1*) and PFE1145w, were always duplicated (28). The chromosomal fragment investigated here was found to contain more than 100 genes, from the PCHAS_123610 gene to the orthologue of the *P. falciparum* MAL5_28S gene. Could any of these genes (other than *mdr1*) also be involved in responses to the drugs under investigation? Zinc finger proteins that are capable of binding DNA and acting as transcriptional regulators, e.g., PCHAS_123810 and PCHAS_124020, orthologues of PFE1145w (as described above) and PFE1245w, respectively, may be implicated in pleiotropic drug resistance through *trans*-regulation of drug resistance genes both in yeasts and in cancer systems (16, 35). Also, drug resistance phenotypes have been shown to be mediated by membrane transporters such as those encoded by *mdr1* and *crt* (37). Within the translocated fragment, genes other than *mdr1* encode proteins with predicted transmembrane domains. For instance, PCHAS_123900 contains a motif characteristic of Nramps (natural resistance-associated macrophage proteins), a family of divalent cation transporters.

We noted additional signatures of selection at higher drug doses, including a signature in chr02, where a mutation in the *ubp1* gene, encoding a deubiquitinating enzyme (12), is known to be the major determinant of ART resistance in *P. chabaudi* parasites lacking a duplication of *mdr1* (13). Because both *mdr1* and *ubp1* mutations have pleiotropic (multidrug) effects, it is possible that the encoded proteins interact (directly or indirectly). For example, the *ubp1* gene product may exert posttranscriptional control on *mdr1* expression or subcellular localization.

In conclusion, we have investigated the genetic determinants of drug resistance by using two rapid genetic and genomic tools, with no prior knowledge regarding the modes of action

or resistance of the drugs. We have clearly and directly demonstrated, on a genomewide scale, that the duplication of a chromosomal segment containing *mdr1* is selected by MF, LM, and ART from a complex *P. chabaudi* genetic mixture. In this respect, the *P. chabaudi* model yields insights which can be investigated more fully in *P. falciparum*. The observed phenotype of cross-resistance between the above components of ACTs and the common underlying mechanisms of resistance indicate that the effectiveness of current treatments for human malaria may be compromised by preexisting resistance or because the different component drugs may share the same underlying genes. Consequently, these findings emphasize the critical importance of choosing therapies that combine drug components having antagonistic signatures of selection at both the phenotypic and genetic levels in order to minimize the evolution of drug-resistant parasites.

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