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Complex Polymorphisms in the *Plasmodium falciparum* Multidrug Resistance Protein 2 Gene and Its Contribution to Antimalarial Response

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***Plasmodium falciparum* has the capacity to escape the actions of essentially all antimalarial drugs. ATP-binding cassette (ABC) transporter proteins are known to cause multidrug resistance in a large range of organisms, including the *Apicomplexa* parasites. *P. falciparum* genome analysis has revealed two genes coding for the multidrug resistance protein (MRP) type of ABC transporters: *Pfmrp1*, previously associated with decreased parasite drug susceptibility, and the poorly studied *Pfmrp2*. The role of *Pfmrp2* polymorphisms in modulating sensitivity to antimalarial drugs has not been established. We herein report a comprehensive account of the *Pfmrp2* genetic variability in 46 isolates from Thailand. A notably high frequency of 2.8 single nucleotide polymorphisms (SNPs)/kb was identified for this gene, including some novel SNPs. Additionally, we found that *Pfmrp2* harbors a significant number of microindels, some previously not reported. We also investigated the potential association of the identified *Pfmrp2* polymorphisms with altered *in vitro* susceptibility to several antimalarials used in artemisinin-based combination therapy and with parasite clearance time. Association analysis suggested *Pfmrp2* polymorphisms modulate the parasite's *in vitro* response to quinoline antimalarials, including chloroquine, piperaquine, and mefloquine, and association with *in vivo* parasite clearance. In conclusion, our study reveals that the *Pfmrp2* gene is the most diverse ABC transporter known in *P. falciparum* with a potential role in antimalarial drug resistance.**

Plasmodium falciparum, the lethal pathogen of human malaria, is notorious for its capacity to develop resistance to chemotherapy. From the first observations involving quinine (1) to the latest reports on emerging artemisinin (ART) resistance (2–4), the parasite has shown a resilient ability to evade the action of essentially every launched antimalarial drug, independently of the chemical structure involved. Such an evasive capacity raises the hypothesis that the parasite is presently developing broad-range resistance phenotypes, possibly akin to the extensive drug resistance witnessed in other infectious diseases, namely, tuberculosis (5). This is a legitimate concern, particularly because of the recent reports of decreased ART sensitivity of the parasite in Southeast Asia.

Conventional *in vitro* drug response and the parasite clearance rate (2, 3) are the main measurements to assess the resistance phenotype of the parasite, but these procedures involve demanding and time-consuming protocols. A practical alternative is the use of molecular sentinel tools, i.e., molecular markers based on genetic variations with a valuable predictive capacity in the identification of the resistance status of the analyzed infection. The development of such tools is dependent on the understanding of the drug resistance mechanisms and their associations with variants of particular genes. Such information can also provide key clues for the development of new evidence-based resistance-refractory antimalarials. Moreover, this type of information is of particular relevance at a moment when technology for point-of-care genetic analysis is starting to emerge, allowing a first glimpse of the possibility of personalized medicine in the future (6).

Transporter proteins belonging to the ATP-binding cassette (ABC) superfamily are well known to be involved in drug efflux, as they are associated with resistance in a large variety of phylogenetically different biological systems (7). These proteins are able to transport substrates across cell membranes against a concentration gradient, an action driven by ATP hydrolysis. In particular, the multidrug resistance-associated protein (MRP)-like subclass of ABC transporters is well known for transport of drugs out of cells, contributing to resistance as well as to the redox metabolism pathway (8). Since the antimalarial chloroquine (CQ), and potentially mefloquine (MQ), was reported to be transported by the human MRP1 and MRP4 proteins, it has been speculated that the putative *P. falciparum* MRP could have the same capacity and thus contribute to drug resistance (9, 10). Indeed, *P. falciparum* is pres-

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ently known to contain in its genome two genes coding for MRP-like proteins, namely, *Pfmrp1* (11) and *Pfmrp2* (12). Both PfMRP1 (PlasmoDB gene ID PF3D7_0112200) and PfMRP2 (PlasmoDB gene ID PF3D7_1229100) proteins are localized in the cytoplasmic membrane of the parasite in the asexual stages (13). Additionally, it was observed that the loss of PfMRP1 drug transport capability resulted in an increased accumulation of antimalarials that was paralleled by an enhanced *in vitro* susceptibility to several antimalarial drugs, including CQ, quinine, and ART (14). A number of studies on *Pfmrp1* diversity have associated single nucleotide polymorphisms (SNPs) in this gene to the parasite *in vivo* drug responses, manifested by the selection of specific SNPs upon treatment (15–17).

In contrast, our knowledge on *Pfmrp2* is limited, and its biodiversity and possible involvement in antimalarial drug resistance are still to be disclosed. The localization of PfMRP2 in the plasma membrane suggests that this ABC transporter may be of relevance in the efflux of xenobiotics from the parasite cytoplasm, as previously reported for the structurally related PfMRP1 (14). Also, we recently showed that upon MQ exposure *in vitro*, different levels of *Pfmrp2* transcription induction were observed between sensitive and less susceptible strains (18). Additionally, *Pfmrp1* and *Pfmrp2* genes have essentially opposite transcriptional patterns throughout the *P. falciparum* asexual intraerythrocytic cell cycle, so that each protein is expressed during different morphological stages (18, 19). This pattern further suggests a potential functional complementation between the two proteins that might be of relevance in complex phenotypes of the cell cycle stage-specific drug response. Lastly, recent microarray-based approaches have identified a *P. falciparum* 3D7 subvariant which carries a ca. 4-kb deletion at the *Pfmrp2* 5′ putative promoter that is associated with decreased susceptibility to CQ and MQ (20).

We focused here on the study of *Pfmrp2* complete open reading frame (ORF) sequence diversity in a set of adapted parasites from an ART resistance focus at the Thai-Burma border, disclosing a previously unknown natural variation of this gene. Taking advantage of available data on *in vitro* antimalarial 50% inhibitory concentrations (IC_{50}s) and parasite clearance times (PCTs) for this set of samples (21, 22), we explored the possible association of the polymorphisms found with modulation of drug sensitivity.

MATERIALS AND METHODS

***P. falciparum* field isolates.** Forty-six culture-adapted *P. falciparum* field isolates, originally adapted at Karolinska Institutet (21), were enrolled in this study. All were isolated from clinical cases of uncomplicated malaria diagnosed and managed at the Shoklo Malaria Unit in the Mae Sot District, Tak Province, Thailand, where parasite clearance time data were collected. The chosen parasite set of infections represented a relatively homogeneous and specific population, in line with the objectives of obtaining a better understanding of the specific influence of the *Pfmrp2* variation in drug responses.

The study was ethically cleared by the relevant institutions, and the blood samples were obtained upon informed consent provided in the local language (21).

Pfmrp2 molecular analysis. The *Pfmrp2* gene (6,327 bp on the 3D7 laboratory strain) was PCR amplified from genomic DNA, extracted from culture-adapted Thai strains (21) in 8 fragments, in order to cover the entire ORF. Amplification and sequencing primers are described in Table S1 in the supplemental material. The PCR amplifications were performed in a total volume of 50 μl with 0.2 μM each forward and reverse primer, 0.2 mM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, and 2 U GoTaq DNA polymerase (Promega Biotech AB). The thermal cycle program was performed with a starting DNA denaturation temperature of 94°C for 2 min, followed by three touchdown cycles: 10 cycles of 93°C for 15 s, 57°C for 30 s, and 72°C for 90 s; 25 cycles of 93°C for 15 s, 55°C for 30 s, and 72°C for 90 s; followed by 10 cycles of 93°C for 15 s, 53°C for 30 s, and 72°C for 90 s. Fragments that did not amplify were subjected to a nested amplification with the primers described in Table S1 in the supplemental material and the same thermal cycle program. The resulting PCR products were confirmed through agarose gel electrophoresis for specificity and a low presence of unspecific amplification. The direct sequencing of the amplicons was outsourced to Macrogen Inc. (Seoul, South Korea).

Analysis of the sequence chromatograms. Due to the complexity of the gene, sequence chromatogram files were analyzed by using two bioinformatic approaches. Sequence chromatogram files were analyzed by base calls using phred version 0.020425.c (23) and aligned to the *P. falciparum* 3D7 genome reference sequence (PlasmoDB gene ID PF3D7_1229100) by using the alignment program ssaha2 version 2.5.1 (24). The program was configured to report only the best alignment for each match. A custom Perl script was used to call single nucleotide polymorphisms and polymorphic microindels (MIs) from the alignments. A position was called polymorphic if either the position had more than one read to support the nucleotide difference or the quality score of the position was 20 or higher. The Tablet program was used to visualize alignments (25). The other approach to analyze the sequence chromatogram files was with the Staden package (26). To identify sequence polymorphisms, the consensus sequence for each strain was compared with the corresponding gene sequence of the *Plasmodium falciparum* 3D7 genome reference sequence by using MUSCLE (27) and manually inspected for alignment errors.

In addition to the 3D7 genome reference sequence, five other reference and published parasite lines sequences were downloaded: Dd2 (http://www.ncbi.nlm.nih.gov/nuccore/AASM00000000), Hb3 (http://www.ncbi.nlm.nih.gov/nuccore/AANS00000000), UGT5.1 (http://www.ncbi.nlm.nih.gov/nuccore/AMYP00000000), RAJ116 (http://www.ncbi.nlm.nih.gov/nuccore/ACBR00000000), and IGH-CR14 (http://www.ncbi.nlm.nih.gov/nuccore/ACBS00000000). The *Pfmrp2* gene sequence was extracted and used in the alignments for sequence comparisons with our set of samples.

Determined antimalarial *in vitro* 50% inhibitory concentrations and parasite clearance times. The 46 Thai isolates were previously phenotyped for the *in vitro* IC_{50}s of ART, dihydroartemisinin (DHA), MQ, lumefantrine (LUM), piperaquine (PPQ), and CQ. Briefly, the IC_{50}s were determined by culturing the parasite (synchronized at ring stage with 0.05% parasitemia and 1.5% hematocrit) in the presence of serial dilutions of the aforementioned antimalarial drugs. After 72 h of incubation at 37°C under restricted O_2 conditions, the cells were lysed and analyzed by the histidine-rich protein 2-based double-site sandwich enzyme-linked immunosorbent assay (28). Further detailed information can be found in the original reports (21, 22).

In this set of Thai isolates, the patient parasite clearance times ranged from 6 to 132 h. A high parasite clearance rate was detected in 19 patients, with PCTs from 6 to 36 h, and 27 patients had a low parasite clearance rate, with PCTs between 90 and 132 h. *In vitro* drug susceptibility and PCT phenotype data and *Pfmrp2* genotype associations were tested by using a one-way analysis of variance (ANOVA) with SPSS statistics software.

Topology of the PfMRP2 protein. To predict the transmembranes (TM) in the ABC transporter PfMRP2, we used two different software programs. The most-used program was TMHMM server v. 2.0 (transMembrane hidden Markov model; http://www.cbs.dtu.dk/services/TMHMM/). The other program used was the OCTOPUS software (29), which uses a combination of hidden Markov models and artificial neural networks. The topology predictor program OCTOPUS, in particular, is the first to integrate modeling of reentrant, membrane dip, and TM hairpin regions in the topological grammar. It performs a homology search by using BLAST to create a sequence profile used as the input to a set of neural networks that predict both the preference for each residue to be located in a TM, interface, close loop, or globular loop environment and

TABLE 1 Membrane-spanning domains and transmembrane predictions for PfMRP2

MSD	TM	Predicted TM domain[a] (aa span) based on: Previously published (13)	OCTOPUS	TMHMM	O/T merged
MSD1	1	133–155	133–157	132–155	134–155
	2	180–202	178–199	180–203	180–199
	3	402–424	401–422	396–418	401–418
	4	434–456	425–449	428–454	428–448
	5	517–539	511–531	511–533	512–531
	6	554–575	544–564	548–565	551–562
		—	**787–807**	—	—
MSD2	7	—	1397–1419	1398–1426	1400–1417
	8	1430–1452	1445–1472	1446–1471	1448–1470
		1473–1495	—	1485–1506	—
	9	1510–1532	1524–1545	1526–1546	1528–1545
	10	1553–1570	1547–1564	1547–1565	1547–1564
	11	**1574–1593**	1629–1658	1629–1651	1631–1651
	12	1654–1676	1667–1689	1671–1693	1671–1689

[a] The TM segments shown are those reported from a previous study (13) or predicted in this study by using the OCTOPUS and TMHMM software. To obtain a reliable model, we supported the information for the TM segments obtained by merging the OCTOPUS/TMHMM probabilities (i.e., data in the O/T merged column). TM domain amino acid ranges shown in bold had the greatest differences from the data obtained from reference 13 or via analysis using the OCTOPUS and TMHMM software programs. TM column represents the transmembrane number for the O/T merged model. Dashes (—) represent TM segments.

to be inside or outside the membrane. These predictions are used as input for a two-track hidden Markov model, which uses them to calculate the most likely topology. To get a reliable model, we merged the data of the two transmembrane predictive software programs. Table 1 and Fig. S1 in the supplemental material show the membrane-spanning domains and *trans*-membrane prediction of PfMRP2, and Fig. 1 shows a PfMRP2 two-dimensional (2D) representation with the localization of the analyzed SNPs and microindels.

Statistical analysis. To evaluate associations between the genetic polymorphisms found in *Pfmrp2* and *in vitro* drug resistance, a one-way ANOVA was performed to compare the mean IC_{50}s of ART, DHA, MQ, LUM, PPQ, and CQ. This analysis was complemented with chi-square analysis to verify the presence of differences in the parasite clearance rates that were high (<36 h) and low (>90 h). The SPSS package (IBM SPSS Statistics v. 19) was used to conduct all statistical analyses, and results were considered significant when P was <0.05.

Nucleotide sequence accession numbers. The *Pfmrp2* consensus sequences from the 46 Thai parasites were submitted to GenBank (accession numbers KM213123 to KM213168).

RESULTS

Analysis of the primary sequence organization of PfMRP2. The predicted two-dimensional structure of PfMRP2 suggested a classical ABC transporter protein, with 12 TM domains, distributed in two membrane-spanning domains (MSDs), and two nucleotide-binding domains (NBDs). The data support previously proposed models (13) of PfMRP2 as a transporter with 2 MSDs of 6 units each (prediction probability range, 0.9 to 1). However, according

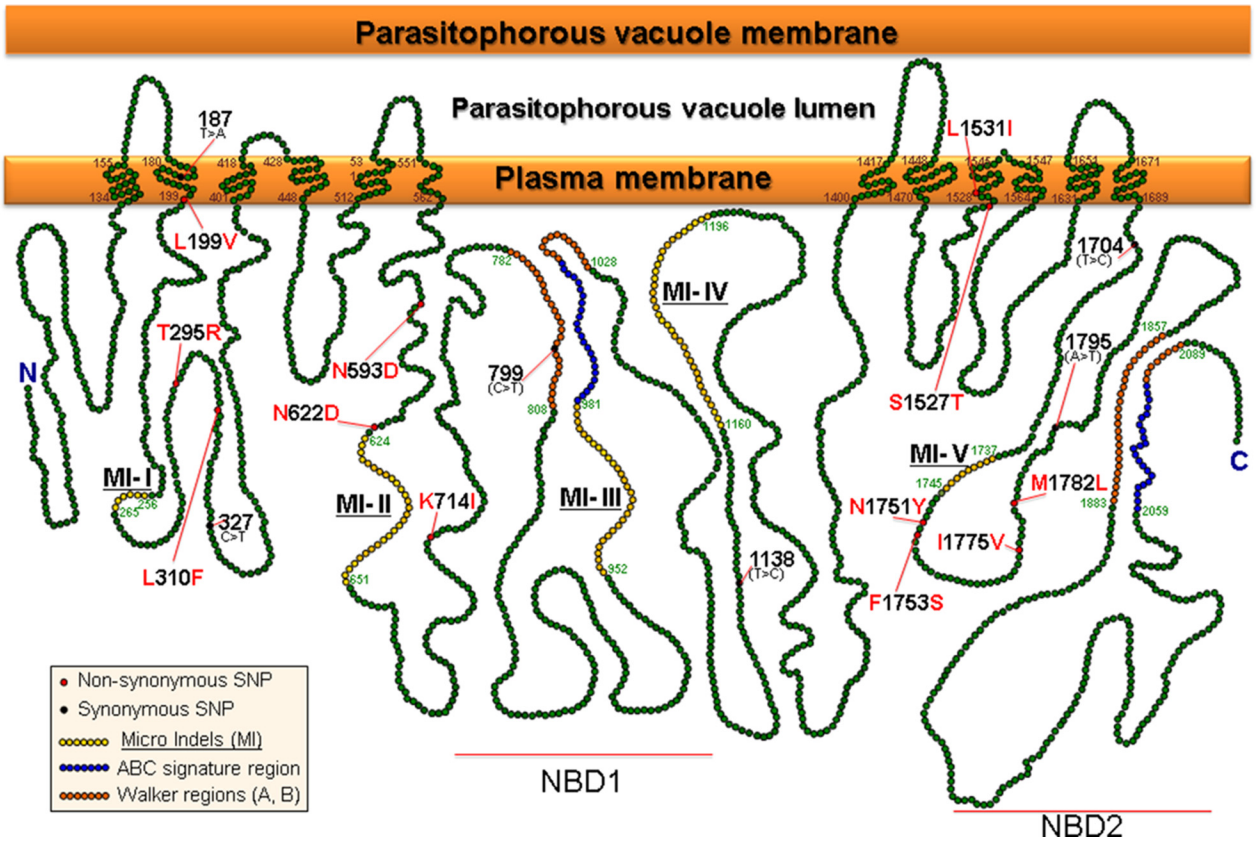

FIG 1 2D representation of PfMRP2, with the location of the analyzed SNPs and microindels. The structure depicted refers to the reference *P. falciparum* 3D7 clone sequence, comprising 2,108 aa. Note that the numbering of amino acids follows the 3D7 reference genomic data, independently of the microindel variation observed in the studied Thai set of parasites.

TABLE 2 SNPs identified in *Pfmrp2* by sequencing the Thai isolates

Chromosome position[a]	Nucleotide position	Nucleotide change	Amino acid position	Amino acid change	Frequency (proportion)	Location
1198644	561	T → A	187	Synonymous	0.02 (1/46)	TM2
1198610*#	595	C → G	199	L → V	1.00 (46/46)	TM2
1198321	884	C → G	295	T → R	0.02 (1/46)	loop
1198275	930	G → T	310	L → F	0.02 (1/46)	loop
1198224	981	C → T	327	Synonymous	0.02 (1/46)	loop
1197428*§	1777	A → G	593	N → D	0.07 (3/46)	NBD1
1197341*	1864	A → G	622	N → D	0.22 (10/46)	NBD1
1197064*§#	2141	A → T	714	K → I	1.00 (46/46)	NBD1
1196808*§	2397	C → T	799	Synonymous	0.39 (16/41)	NBD1
1195791*§	3414	T → C	1138	Synonymous	0.20 (9/44)	NBD1
1194626*#	4579	T → A	1527	S → T	1.00 (46/46)	TM9
1194614*#	4591	C → A	1531	L → I	1.00 (46/46)	TM9
1194093	5112	T → C	1704	Synonymous	0.02 (1/46)	NBD2
1193954	5251	A → T	1751	N → Y	0.09 (3/34)	NBD2
1193947§	5258	T → C	1753	F → S	0.07 (3/46)	NBD2
1193882	5323	A → G	1775	I → V	0.14 (5/36)	NBD2
1193861	5344	A → T	1782	M → L	0.02 (1/46)	NBD2
1193820	5385	A → T	1795	Synonymous	0.08 (3/37)	NBD2

[a] Symbols: *, SNP previously registered with PlasmoDB; §, SNP previously found by deep sequencing of 227 samples from Africa, Asia, and Oceania (30); #, SNP previously described in reference 31.

to our analysis, we detected some differences in TM7 and TM11 predictions when we used the merged data from TMHMM and OCTOPUS (Table 1; see also Fig. S1 in the supplemental material). In our work, we identified TM7 between amino acids (aa) 1400 and 1417 which were not detected in previous analysis (13), while T11 was repositioned. These observed divergences did not interfere with the analysis or interpretation of our data, since none of the subsequently identified SNPs were located in these regions.

In spite of its large size, PfMRP2 is predicted to represent a short MRP-type protein, as it did not show the extra 5 TM N-terminal domains present in the long MRPs, such as the human MRPs 1, 2, 3, 6, and 7. This was largely compensated by a large (~850-aa) NBD1-containing cytoplasmic loop. Also notable was the unusually distal localization of the NBD2 ABC signature plus Walker B motif, which was only 17 aa from the protein C-terminal.

Pfmrp2 polymorphisms. The *Pfmrp2* ORF was sequenced for 46 *P. falciparum* strains that originated from the Thai-Burma border. The full sequence was determined for 37/46 isolates, and on average 93% of the gene was covered for the remaining 9 isolates. A total of 12 nonsynonymous and 6 synonymous SNPs were identified, in comparison with the 3D7 reference sequence. Among these SNPs, we found novel SNPs not previously annotated within PlasmoDB (http://plasmodb.org) nor observed in recently performed genome surveys (30) or in other available genome sequencing data (31) (Table 2). The proportions of the analyzed SNPs in this set of isolates varied from a single event (1/46) to situations of monomorphism (46/46).

The only three nonsynonymous SNPs located in the predicted TM domains of the transporter, L199V (TM2), S1527T (TM9), and L1531I (TM9), were found to be monomorphic among the studied isolates, suggesting that inheritance was from a common ancestor (Table 2).

We also found a significant number of MIs, based on the presence of tandem repeats (Fig. 1 and 2), relative to the 3D7 reference sequence. This is an unusual observation among *P. falciparum* ABC transporters. The identified MIs comprise 5 regions of the gene (herein referred as MI-I to MI-V) and lead to a notable length variation of the coded protein (2,109 to 2,135 aa). Within the MI regions, we also found variations in single nucleotides, troubling a forthright sequence alignment. Three of these variations (D976N, D1180N, and D1188N) are annotated as SNPs within PlasmoDB. Due to the changeability of possible alignments, allocating an amino acid position becomes biased, and therefore we decided not to include them as definitive SNPs but instead as residues of variability within the MIs (Fig. 2).

To additionally support the observation of such complex *Pfmrp2* MI regions, publicly available sequence data from 5 distinct parasite lines (Dd2, Hb3, UGT5.1, RAJ116, and IGH-CR14) were aligned with the set of Thai isolates under study. The resulting analysis confirmed the presence of the 5 MIs determined in our set of samples, further increasing the number of variants in each MI region (Fig. 2). All MI regions were located outside the TM domains.

Genotype/phenotype associations. The isolates under study were previously shown to have a relatively large range of IC values for the tested antimalarial drugs, with median IC_{50}s (maximum to minimum) of 7.4 nM (1.2 to 19.5 nM) for ART, 1.2 nM (0.3 to 5.3 nM) for DHA, 92.5 nM (16.5 to 270.8 nM) for MQ, 11.9 nM (2.0 to 40.8 nM) for LUM, and 39.4 nM (13.8 to 108.2 nM) for PPQ; all strains were highly resistant to CQ, with IC_{50}s of >450 nM (454 to 1,027 nM) (21, 22).

One-way analysis of variance revealed an association between the synonymous SNP coding for an asparagine at position 1138 (codon change of GA**T** → GA**C** at nucleotide position 3414) and the parasite response to CQ ($P = 0.040$), with an average IC_{50} for the t3414 variant of 660 nM and for the c3414 variant of 564.3 nM (Fig. 3). No other significant association was found within the identified SNPs.

The 5 MIs identified were subcategorized into MI variants that were type a, such as the 3D7 reference genomic sequence (Fig. 2). With this classification approach, associative trends were revealed

FIG 2 Microindels identified in the *Pfmrp2* by sequencing the Thai isolates and aligning the sequences with publicly available sequencing data. The 5 microindels identified were subcategorized as MI variants of type a (the 3D7 reference genomic sequence type); the other types are designated b to l for the variants found among the Thai isolates (N) and the publicly available genomes. Symbols for publicly available genomes: £, Dd2; &, Hb3; #, UGT5.1; §, RAJ116; +, IGH-CR14. Highlighted black boxes denote amino acid positions that are described in the PlasmoDB database (http://www.plasmodb.org) as single nucleotide polymorphisms.

				Artemisinin			Dihydroartemisinin			Mefloquine			Lumefantrine			Piperaquine			Chloroquine			Parasite Clearance		
			# N	mean	stdev	sig	mean	stdev	sig	mean	stdev	sig	mean	stdev	sig	mean	stdev	sig	mean	stdev	sig	High	Low	chi2-sig
		Total	46	7.82	5.41		1.4	1.15		106.79	74.63		14.27	10.44		40	20.98		639.94	123.56		27	19	
SNPs	N622D	absent	36	7.90	5.46	0.857	1.42	1.12	0.821	106.12	78.03	0.910	14.19	10.76	0.924	41.76	21.05	0.283	645.24	120.17	0.587	20	16	0.328
		present	10	7.55	5.51		1.33	1.33		109.20	64.49		14.55	9.73		33.63	20.53		620.86	140.19		7	3	
	syn c2397t	absent	25	8.46	5.67	0.461	1.66	1.37	0.115	113.16	81.02	0.597	14.97	11.39	0.795	43.98	22.50	0.305	655.78	133.22	0.388	14	11	0.315
		present	16	7.17	4.90		1.08	0.61		100.58	60.09		14.07	9.37		36.94	18.86		620.54	113.93		11	5	
	syn t3414c	absent	35	7.40	4.94	0.637	1.39	1.05	0.820	98.71	73.88	0.366	13.27	9.53	0.441	42.06	21.98	0.185	660.06	131.73	0.04*	21	14	0.514
		present	9	8.36	7.09		1.29	1.54		124.39	80.50		16.34	14.04		31.36	17.71		564.30	52.69		6	3	
	I1775V	absent	31	7.92	5.21	0.833	1.49	1.21	0.429	114.55	73.83	0.867	14.67	10.62	0.757	38.98	21.75	0.935	646.70	127.34	0.684	17	14	0.445
		present	5	8.43	3.17		1.97	1.53		108.64	60.95		13.14	5.34		39.84	21.94		622.57	70.77		2	3	
Microindel type	I-c	absent	15	7.32	5.31	0.666	1.3	1.23	0.691	109.16	85.15	0.883	15.23	11.84	0.670	37.12	17.82	0.523	614.88	114.41	0.344	7	8	0.202
		present	31	8.07	5.53		1.45	1.14		105.64	70.47		13.81	9.87		41.39	22.5		652.07	127.77		20	11	
	I-e	absent	40	7.86	5.35	0.902	1.35	1.06	0.439	104.37	71.3	0.576	13.47	9.96	0.183	39.57	21.54	0.725	632.73	121.43	0.312	25	15	0.182
		present	6	7.57	6.32		1.75	1.75		122.92	100.55		19.6	12.97		42.85	18.26		687.99	138.54		2	4	
	I-d	absent	41	7.74	5.4	0.756	1.42	1.2	0.773	104.85	75.68	0.620	13.87	10.31	0.461	40.44	21.38	0.683	646.2	127.2	0.330	24	17	0.667
		present	5	8.55	6.12		1.26	0.81		122.64	70.93		17.56	12.2		36.32	19.11		588.56	79.01		3	2	
	II-d	absent	33	7.72	5.32	0.843	1.43	1.13	0.758	103.84	78.69	0.674	13.58	10.41	0.481	42.56	21	0.189	645.33	122.53	0.643	16	17	0.025*
		present	13	8.08	5.85		1.32	1.25		114.28	65.48		16.02	10.75		33.48	20.28		626.26	130.13		11	2	
	II-e	absent	39	7.56	5.16	0.443	1.34	1.02	0.441	104.71	73.56	0.661	13.67	9.83	0.364	40.99	21.95	0.456	650.6	129.44	0.170	24	15	0.303
		present	7	9.29	6.93		1.72	1.82		118.36	85.55		17.61	13.82		34.48	14.48		580.54	59.41		3	4	
	II-g	absent	40	7.99	5.29	0.604	1.4	1.2	0.952	109.29	73.67	0.563	13.98	10.1	0.628	37.38	18	0.028*	630.76	117.34	0.196	23	17	0.516
		present	6	6.74	6.59		1.43	0.84		90.09	86.07		16.23	13.44		57.41	31.91		701.15	157.6		4	2	
	II-h	absent	33	7.63	5.61	0.696	1.43	1.19	0.767	103.72	72.77	0.662	15.19	10.93	0.347	40.22	22.37	0.911	620.23	117.25	0.085	19	14	0.538
		present	13	8.33	5.04		1.32	1.1		114.57	81.69		11.94	9.05		39.44	17.79		689.96	129.66		8	5	
	IV-e	absent	41	8.08	5.18	0.374	1.36	1.04	0.504	109.42	72.04	0.499	14.46	9.88	0.721	40.78	21.38	0.475	643.61	128.66	0.570	26	15	0.085
		present	5	5.77	7.4		1.73	2.02		85.18	100.5		12.67	15.75		33.58	18.04		609.82	70.35		1	4	
	IV-h	absent	28	7.04	5.21	0.223	1.5	1.36	0.490	101.76	79.59	0.574	13.36	11.02	0.466	38.72	22.19	0.612	644.26	132.55	0.771	16	12	0.518
		present	18	9.05	5.64		1.25	0.73		114.61	67.64		15.69	9.62		41.98	19.42		633.22	111.48		11	7	
	IV-k	absent	34	7.74	5.74	0.862	1.34	1	0.564	103.27	74.97	0.596	14.3	10.82	0.977	41.52	21.98	0.412	626.27	115.5	0.210	18	16	0.161
		present	12	8.06	4.57		1.57	1.55		116.76	76.01		14.19	9.75		35.67	18		678.68	142.18		9	3	
	V-a	absent	26	7.28	4.84	0.442	1.52	1.18	0.439	95.1	72.62	0.230	13.39	10.19	0.523	43.05	22.25	0.265	644.35	116.45	0.786	14	12	0.324
		present	20	8.53	6.13		1.25	1.14		121.98	76.3		15.41	10.92		36.02	19.04		634.21	135.11		13	7	
	V-b	absent	42	8.12	5.52	0.237	1.45	1.19	0.373	113.64	74.46	0.042*	15.02	10.59	0.115	39.94	21.29	0.958	644.14	125.38	0.462	25	17	0.552
		present	4	4.74	3.03		0.9	0.52		34.86	14.84		6.38	3.67		40.54	20.29		595.87	106.37		2	2	
	V-c	absent	40	7.78	5.69	0.886	1.33	1.12	0.309	105.22	76.87	0.717	14.67	10.96	0.505	40.28	21.34	0.818	642.68	130.71	0.702	24	16	0.484
		present	6	8.12	3.28		1.85	1.41		117.25	62.3		11.58	5.92		38.13	20.19		621.65	60.59		3	3	
	V-d	absent	32	7.63	5.37	0.716	1.32	1.1	0.470	104.73	73.49	0.781	12.84	9.75	0.163	37.42	18.25	0.213	623.23	115.21	0.168	18	14	0.430
		present	14	8.27	5.69		1.59	1.29		111.48	79.78		17.53	11.59		45.87	26.02		678.14	137.61		9	5	

FIG 3 SNPs and microindels and their associations with IC_{50} values and parasite clearance rates. We used one-way ANOVA to compare associations between IC_{50}s and the presence (+) or absence (−) of the SNP or microindel type. Chi-square analysis was used to compare associations between high (<36-h) or low (>90-h) parasite clearance rates and the presence (+) or absence (−) of the SNP or microindel type. *, $P < 0.05$ (significant findings are also highlighted in gray, for additional emphasis. #, the number of samples bearing each genotype. Results represent only the analysis for genotypes with allele frequencies of >8%.

for MI-II-g type and PPQ IC_{50}s ($P = 0.028$), with mean IC_{50} of 37.4 nM in the absence of the polymorphism and of 57.4 nM in isolates containing this type of polymorphism. The MI-V-b type showed a trend of association with MQ susceptibility ($P = 0.042$) that encompassed an increased number of asparagines (N) from 10 (mean IC_{50} of 113.6 nM) to 14 (mean IC_{50} of 34.9 nM) (Fig. 3).

Interestingly, we observed the PCT phenotype to be influenced by MI polymorphisms. For MI-II, the presence of the type II-a allele was significantly more frequent in those parasites with high clearance rates ($P = 0.025$) (Fig. 3).

DISCUSSION

The PfMRP2 ABC transporter has been found to be located in the plasma membrane (13). Conceptually, its localization and predicted function suggest this ABC transporter is of potential relevance in the efflux of xenobiotics from the parasite cytoplasm, as reported for the structurally related PfMRP1 (14).

The present work shows that *Pfmrp2* harbors frequent and complex natural variations, including SNPs and MIs based on tandem repeats. The accumulation of these polymorphisms gives rise to a significant level of diversity in this protein, more extensive than that documented for any of the two previously well-investigated ABC transporters in *P. falciparum*, Pgh-1 and PfMRP1. This high diversity implies the existence of PfMRP2 natural variants with different sizes and possible conformations and consequent varied transporting capacities.

Compared with the first characterization of this gene by Mu et al., in which 97 isolates from geographically distinct regions were analyzed, our investigations revealed a notably higher frequency of SNP/kb (2.8 versus 1.3 SNP/kb) (33). Of note, since the main goal of our work was to understand the influence of the *Pfmrp2* gene in drug susceptibility and parasite clearance, the set of parasites used in our study was expected to represent a relatively homogeneous sample. As such, and as in most other similar types of studies, the observed polymorphism frequencies should not be taken as representative of Southeast Asian *P. falciparum* populations as a whole. On the other hand, we expect most of the observed polymorphisms to be relatively common (i.e., >1% frequency) in the targeted region. Also such a low prevalence can rapidly increase in time upon drug pressure. Such events can be relevant in the context of this work, due to the notorious tendency for Southeast Asia parasite populations to develop resistance.

Our data are suggestive of an involvement of this gene in modulating parasite drug sensitivity to at least some of the drugs tested. In the particular case of CQ, and acknowledging that all the analyzed Thai isolates carry the *pfcrt* 76T allele (21), we observed an association between the parasite sensitivity and a synonymous SNP coding for an asparagine at aa 1138 (GA**T** → GA**C**). Explaining the effect of a synonymous mutation in a phenotype has been a challenge in other areas of drug transport proteins. The presently most favored hypothesis is based on preferential codon usage. In *P. falciparum*, the two possible triplets that code for the asparagine amino acid have very distinct frequencies. Codon usage analysis describes the triplet GAU appearing at a greater frequency (55%) than the triplet GAC (only 9%) (34). The use of alternative codons with different levels of intracellular availability in terms of loaded tRNA molecules is thought to influence translation rates. This hypothesis has been experimentally put forward through investigations of the synonymous t3434c SNP in the human P-glycoprotein, which is known to influence the exposure of some orally administered drugs (35). Indeed, it has been demonstrated that the nucleotide change disturbs the pace of the mRNA translation

process, leading to changes in the conformational dynamics of the nascent protein toward its final three-dimensional shape (36, 37). Such a mechanistic hypothesis is worthy of investigation in the context of *P. falciparum* biology.

As documented for other ABC transporters in *P. falciparum* (32), PfMRP2 has divergent insertions throughout its protein sequence, which normally occur within protein domain linkers. Although already observed in other *P. falciparum* ABC transporters, namely, Pgh-1, the number and extension of polymorphisms of these structures in PfMRP2 are unprecedented (Fig. 1 and 2). All MI regions found are localized outside the TM domains. Their exclusion from such protein regions is probably related to the inability of the transmembrane domains to accommodate this type of polymorphism, as the polymorphisms most likely alter the intramembrane α-helix structure, leading to functionally deleterious distortions in the three-dimensional structure of the protein. Considering their location, the extension of these polymorphisms is expected to have a nonnegligible effect in the protein structure and potentially in its function. Unfortunately, in contrast with our previous work on the ABC transporter Pgh-1 (32), the large number of variable insertions/deletions impaired the development of a meaningful homology structure model to validate its impact on the protein's function.

Suggestively, associative trends were revealed between variations in the MI-II region and the IC_{50}s of PPQ, a 4-aminoquinoline structurally related to CQ. These data are consistent with the fact that CQ and PPQ show a degree of cross-resistance (22). An increase of asparagines from 10 to 14 at the MI-V region was also found associated with increased MQ susceptibility. It is conceivable that these variations alter the steady-state conformations of the cytoplasmic regions of the protein and with that, the interaction with the antimalarial drugs, affecting their efflux out of the parasite. The proposed potential role of PfMRP2 in the drug response to CQ and MQ is further supported by the recent DNA microarray-based observations that associated *Pfmrp2* promoter polymorphisms with the parasite CQ and MQ responses *in vitro* (20).

Recently, Okombo et al. (38) found associations between variation in the size of the PfMRP2 MI-III and the *in vitro* parasite responses to lumefantrine. Since this MI region was not polymorphic in our analyzed set of isolates (all have the DNNNTS sequence deleted), our study does not confirm or deny the previously reported observation. It is possible that this might be related to the fact that, although the present study includes a substantial number of laboratory-adapted strains, the herein-revealed biodiversity of this gene will demand future studies with larger sample sizes for definitive determination of the role of PfMRP2 in the parasite response to antimalarial drugs.

We are reporting for the first time a likely influence of the variable MI regions on the parasite clearance time upon ACT treatment (artesunate-mefloquine). Although these data are preliminary, they suggest a potential role of PfMRP2 in the capacity for the parasite to withstand clinical levels of artemisinin (and possibly mefloquine). It is possible that some of these variants involve fitness costs that become evident upon exposure to a stressor, like the aforementioned drugs. Though a change in fitness characteristics by itself may not be considered a resistance response (39), our data point to the potential contribution of PfMRP2 in the capacity of the parasite in evading the action of these powerful but short-term drugs.

The likely influence of PfMRP2 on the parasite response to multiple drugs mirrors that reported for PfMRP1 and its proposed role in CQ, artemisinin, quinine, mefloquine, and lumefantrine (14, 16, 40, 41). The present report adds to several independent lines of evidence by our investigators and others, suggesting the importance of MRP-like proteins in parasite drug responses, highlighting the genetic variances found herein with their potential use as molecular sentinel tools.

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