Evaluation of the Effect of Gene Duplication by Genome Editing on Drug Resistance in Plasmodium falciparum

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The emergence and spread of drug-resistant Plasmodium falciparum have compromised antimalarial efficacy and threatened the global malaria elimination campaign using artemisinin combination therapies. The impacts of amino acid substitutions in antimalarial drug resistance-associated genes on drug susceptibility have been investigated; however, the effects of amplification of those genes remain unexplored due to the lack of robust genetic approaches. Here, we generated transgenic P. falciparum parasites with an additional copy of a drug resistance-associated gene using the highly efficient CRISPR/Cas9 system and investigated their drug response. Insertion of a drug resistance-associated gene expression cassette in the genome resulted in a roughly twofold increase of mRNA levels of the target gene mdr1, which encodes multidrug resistance protein 1. The gene duplication event contributed to resistance to mefloquine, lumefantrine, and dihydroartemisinin, while the duplication of a genomic region encoding plasmepsin 2 and plasmepsin 3 did not affect resistance to antimalarial drugs, including piperaquine. We further demonstrated that mdr1 mRNA expression levels are strongly associated with mefloquine resistance in several field-derived P. falciparum lines with various genetic backgrounds. This study provides compelling evidence that mdr1 could serve as a molecular marker for the surveillance of mefloquine-resistant parasites. Long DNA integration into parasite genomes using the CRISPR/Cas9 system provides a useful tool for the evaluation of the effect of copy number variation on drug response.

Keywords: Plasmodium falciparum, CRISPR/Cas9, mdr1, plasmepsin, drug resistance

Abbreviations: mdr1, multidrug resistance protein 1; pm2, plasmepsin 2; pm3, plasmepsin 3.
INTRODUCTION

The WHO estimated that there were 241 million cases and 627,000 deaths due to malaria in 2020 (World Malaria Report, 2021). The emergence of drug-resistant parasites makes malaria control difficult, as evidenced by the fact that chloroquine-resistant *Plasmodium falciparum* is now spread globally (Ecker et al., 2012). Currently, the WHO recommends artemisinin-based combination therapies (ACTs), in which artemisinin is used as a first-line drug along with partner drugs such as mefloquine, lumefantrine, piperaquine, and amodiaquine. ACTs have greatly contributed to the decrease in malarial deaths (Bhatt et al., 2015; World Malaria Report, 2021). However, recent epidemiological studies indicate that ACT treatment failures are increasing worldwide, particularly in the Greater Mekong Subregion (GMS) (Ashley et al., 2014; Amato et al., 2017; Witkowski et al., 2017; Ikeda et al., 2018). One possible reason for ACT failures is the emergence of strains with mutations in *kelch13*, which are associated with artemisinin resistance (Ariey et al., 2014; van der Pluijm et al., 2019; Balikagala et al., 2021). In addition, ACT failure may result from resistance acquired from partner drugs (Leang et al., 2013; Ashley et al., 2014; Nsanzabana, 2019). Triple ACTs (TACTs), which add another partner drug with a conventional ACT, were introduced and have increased antimalarial efficacy (Dini et al., 2018; Okell et al., 2018; van der Pluijm et al., 2020). The selection of effective partner drugs is important for successful treatment by ACTs; the identification of suitable molecular markers associated with partner drug resistance is required for optimal ACT design and implementation.

Recent epidemiological studies suggested that partner drug resistance is due to amino acid substitutions or copy number variations of several genes. Mefloquine resistance is considered to be conferred by an increase in the copy number of the gene that encodes multidrug resistance protein 1 (*PF3D7_0523000, mdr1*), a protein homologous to the human drug efflux pump (Price et al., 2004). Piperaquine resistance is reported to be increased by the addition of a second partner drug with a conventional ACT (Leang et al., 2013; Ashley et al., 2014; Nsanzabana, 2019). Triple ACTs introduced and have increased antimalarial efficacy (Dini et al., 2018; Okell et al., 2018; van der Pluijm et al., 2020). The selection of effective partner drugs is important for successful treatment by ACTs; the identification of suitable molecular markers associated with partner drug resistance is required for optimal ACT design and implementation.

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sgRNA, an oligonucleotide pair was annealed and cloned into the pf-gRNA plasmid as described. The plasmid expressing sgRNA was modified by a csp-sgRNA_1F and csp-sgRNA_1R primer set to integrate within the circumsporozoite protein locus (csp, PF3D7_0304600) and named psgRNA-cspHR1. To integrate the mdr1 promoter region within the csp locus, the plasmid expressing sgRNA to recognize csp-HR1 was generated with the csp-gRNA_2F and csp-sgRNA_2R primer set, and the resulting plasmid was named psgRNA-cspHR2. The donor DNA plasmid pDonor_mdr1 and PCR-fragment were used for the generation of the pfcas9-mdr1 parasite. First, the mdr1 fragment including ORF and 3’ untranslated regions (UTRs) was inserted using an In-Fusion HD cloning kit into the EcoRI and HindIII sites of the donor template DNA plasmid, to replace the GFP coding sequence. Next, to add the 3-kbp promoter of mdr1 to the csp locus, the donor DNA fragment was used in which the homologous region of the csp locus and mdr1 region containing the promoter and 700-bp ORF was fused by overlap PCR. To prevent re-cleavage by the Cas9 enzyme, the donor DNA lacked 20 bp of the csp sequence in the homologous region. The donor DNA plasmids pDonor_pm2 and pDonor_pm3 were used for the generation of pfcas9-pm2 or pfcas9-pm3 parasites, respectively. The donor DNA plasmids were modified by integrating the pm2 fragment including promoter and 3’-UTR and amplified using the primer set pm2_HindIII-F and pm2_EcoRI-R, or the pm3 fragment amplified using the primer set pm3_HindIII-F and pm3_EcoRI-R as described above. The donor DNA to generate the pfcas9-pm2/3 parasite was a fragment that fused the 3’-UTR of pm2 with the pm3 expression cassette by overlap PCR. All donor DNA plasmids were linearized for transfection. The sequences of oligonucleotides used for sgRNA-expressing plasmid or donor DNA constructions are listed in Supplementary Table 1.

Generation of Transgenic Parasites

Transfection of P. falciparum by CRISPR/Cas9 was performed as described (Mohring et al., 2019; Morita et al., 2021). Tightly synchronized mature schizonts were transfected using the FP-158 program on a Nucleofector 4D device (LONZA, Basel, Switzerland). First, for transfections to integrate the target gene into the csp locus, the sgRNA-csp and linear donor DNA were used. Next, the sgRNA-cspHR1 and linear donor DNA were used to add the mdr1 promoter region. To generate the pfcs9-pm2/3 line, the pfcs9-pm2 line was transfected with linear donor DNA and the sgRNA-cspHR2. Each linear repair DNA template (25 µg) plus plasmid containing the sgRNA (25 µg) was dissolved in 100 µl of P3 Primary Cell 4D-Nucleofector X Kit (LONZA) and were mixed with purified schizonts (1 × 10⁸). Pyrimethamine-mediated (25 ng/ml) selection of the transgenic parasites was initiated 72 h after transfection and continued for 10 days. The emergence of transgenic parasites was monitored using a diagnostic PCR assay, followed by 1 µM of 5-fluorocytosine treatment for 5 days to eliminate plasmid-containing parasites. Parasite clones were further selected by limiting dilution, in which by diagnostics the target gene was integrated. The diagnostic PCR products were sequenced by standard Sanger sequencing to confirm the absence of undesired mutations in the target gene coding regions. To generate pfcas9-mdr1 and pfcas9-pm2-3, synchronized mature schizonts of the plasmid-free primary transgenic parasite clone were used for the secondary transfection. Removal of the sgRNA plasmid from the primary transgenic parasites was confirmed by the restoration of pyrimethamine susceptibility. The sequences of primers used for diagnostic PCR are listed in Supplementary Table 1.

Transcription of Target Genes

To quantify mdr1 transcript levels, total RNA was purified from parasites 6 to 10 h post-infection (hpi), when the expression of mdr1 reached the maximum level (https://plasmodb.org/plasmo). For assay of pm2 and pm3 transcript levels, total RNAs were purified from parasites 26 to 30 hpi. Total RNA was isolated using TRIzol™ (Thermo Fisher Scientific, Waltham, MA, USA) and purified from three biological independent samples for each parasite strain. The cDNAs were synthesized from 1 µg of each purified total RNA using a PrimeScript RT reagent kit with a gDNA Eraser according to the manufacturer’s instructions (TAKARA Bio, Kusatsu, Japan). Quantitative reverse transcription PCR assays were performed in triplicate using cDNA as a template with Power SYBR® Green Master Mix (Thermo Fisher Scientific) and a StepOne Plus device (Thermo Fisher Scientific). Target gene expression levels were calculated based on the CT values using the ddCt method. All real-time PCRs were performed using the seryl tRNA synthetase gene (PF3D7_0717700) as an internal control and at least two independent experiments. Primers are listed in Supplementary Table 1.

Southern Blotting

Southern hybridization analysis was performed as described with some modifications (Shinzawa et al., 2020; Nishi et al., 2021). Briefly, to detect the mdr1 locus, genomic DNA was extracted from blood-stage parasites (2 µg) and digested with the restriction enzyme ScaI. The primers mdr1-probe-F and mdr1-probe-R were used to generate the DNA probe. To detect the pm2 locus, parasite genomic DNA was digested with the restriction enzymes Scal and Sall. For detecting the pm3 locus, parasite genomic DNA was digested with the restriction enzymes Scal, Sall, and BamHI. The fragments were separated on a 0.8% agarose gel and transferred to nylon membranes. DNA probes to characterize the pm2 and pm3 loci were generated with the following respective primer pairs: pm2-probe-R and pm3-probe-F and pm3-probe-R. The positive control was the plasmid used to generate the transgenic parasites in this study. The PCR products were labeled with digoxigenin (DIG) and used as hybridization probes. Chemiluminescence signals were detected using ChemiDoc MP (Bio-Rad, Hercules, CA, USA).
Fitness assays were performed as described with some modifications (Nishi et al., 2021). Tightly sorbitol-synchronized ring-stage parasites were inoculated at 0.1% parasitemia and 2% hemocrit in a drug-free complete medium. The culture medium was changed, and the parasitemia was monitored by Giemsa stain daily for 4 days.

Preparation of Antimalarial Drug Stock Solutions
Melqoquine, piperaquine, lumefantrine, and dihydroartemisinin were purchased from Tokyo Chemical Industry Co., Ltd. Tokyo, Japan. Stock solutions of 50 mM of melqoquine, 2 mM of lumefantrine, and 100 mM of dihydroartemisinin were prepared in dimethyl sulfoxide (Wako, Tokyo, Japan) and 1 mM of piperaquine in 0.5% lactic acid (Wako), with ultrasonic treatment and warming at 60°C to dissolve. The stock solutions were aliquoted and stored at −20°C until use.

Measurement of the IC50 Values of Antimalarial Drugs
Parasites were synchronized by the sorbitol-based method, and 48 h later, the parasitemia was adjusted to 0.5% in blood at 2% hematocrit. The final drug concentrations tested ranged from 0 to 800 nM for melqoquine, 0 to 100 nM for piperaquine, 0 to 1,600 nM for lumefantrine, and 0 to 80 nM for dihydroartemisinin. Parasite culture (50 μl) and diluted drug medium (50 μl) were dispensed at a final parasitemia of 0.25% in quadruplicate to wells of a flat-bottom 96-well plate. Piperaquine and lumefantrine were incubated for 96 h, melqoquine for 144 h due to its longer half-life, and dihydroartemisinin for 48 h due to its rapid loss of drug activity. Parasite growth in each well was assessed using SYBR Green I fluorescence, as described above for the in vitro IC50 drug susceptibility assays. Good concordance was observed between cell count values by a SYBR Green assay versus microscopy-based counting of Giemsa-stained thin blood smears. Assays were repeated in duplicate at least twice.

Copy Number Variation Analysis of mdr1
To quantify mdr1 copy numbers, total genomic DNAs were purified from the transgenic parasites as described above. Real-time genomic PCR assays were performed in triplicate with Power SYBR® Green Master Mix (Thermo Fisher Scientific) and a StepOne Plus device (Thermo Fisher Scientific) using genomic DNA as a template. To calculate the mdr1 copy number, PF3D7_0523800 was used as an internal control. The primer sets for mdr1 and PF3D7_0523800 were validated using pfCas9 and pfCas-mdr1 genomic DNA. For laboratory strains, the reactions of real-time genomic PCR were performed using 0.1 ng of genomic DNA. Quantification of mdr1 copy numbers was determined by ddCt methods. All real-time PCRs were performed in two independent experiments. Primers are listed in Supplementary Table 1.

RESULTS
Generation of Transgenic Plasmodium falciparum Parasites With an Additional Copy of Drug Resistance-Associated Genes
To investigate a possible direct causal association of drug resistance with copy number amplification of mdr1, pm2, and pm3, we generated transgenic P. falciparum parasites with an additional copy of each gene by CRISPR/Cas9-mediated knocking-in of long genomic regions. The introduced expression cassettes contained the target gene coding regions together with the promoter and 3′-UTRs, predicted by amplification-free RNA-seq data to mimic its original expression pattern (Chappell et al., 2020). The above resistance-associated genes were introduced into the circumsporozoite protein (csp) locus of a Streptococcus pyogenes Cas9-expressing 3D7 parental strain (pfCas9, parental). The csp locus is considered to be dispensable for asexual parasite proliferation, with no known knock-out phenotype (Nishi et al., 2021). To knock-in mdr1, the fragment corresponding to the coding region and 3′-UTR was introduced into the csp locus, followed by the insertion of an upstream 3-kb promoter region (pfCas9-mdr1; Figure 1A). For pm2 and pm3, transgenic parasites were generated with an additional copy of pm2 or pm3 (pfCas9-pm2 or pfCas9-pm3; Figure 1A). To investigate a possible synergistic effect of pm2 and pm3, an expression cassette of pm3 was introduced into the genome of pfCas9-pm2 downstream of the inserted pm2, to generate transgenic parasites containing...
additional copies of both pm2 and pm3 (pfcas9-pm2/3; Figure 1A). Genotyping PCR and Southern hybridization analyses showed the correct integration of the additional gene copy at the csp locus in the respective transgenic parasites (Figures 1B–E). There were no undesired mutations in the coding regions of the inserted genes (data not shown), supporting that the additional copies were correctly introduced.

**Increase in mRNA Expression of Target Genes Following Copy Number Amplification**

To evaluate the relationship between copy number amplification and mRNA expression, the transcript levels of each gene were compared between parental and transgenic parasites. The mdr1 transcript levels were examined by quantitative reverse transcription PCR at the ring stage (6–10 hpi), and those of pm2 and pm3 were examined at the trophozoite stage (26–30 hpi), at their highest transcription timing according to the transcriptomics data (Toenhake et al., 2018; Wichers et al., 2019; Chappell et al., 2020). The results demonstrated that the mdr1 transcript level in ring-stage parasites of the pfcas9-mdr1 line was about 2-fold higher than that of parental parasites (Figure 2A and Supplementary Table 2). The insertion of each pm2 or pm3 expression cassette, or both pm2 and pm3 increased their target gene mRNA levels by 1.5- to 2-fold compared to parental parasites (Figures 2B, C and Supplementary Table 2). All transgenic strains appeared to be normal with respect to intra-erythrocytic development (Figure 2D). The results were consistent for two independent clones of each transgenic parasite. In summary, these results demonstrated that an additional copy of a drug resistance-associated gene within the csp locus increased the respective mRNA expression about twofold without any transcriptional suppression, and the increased expression did not affect the asexual stage development.

**Drug Response of the Transgenic Parasites With Amplification of Drug Resistance-Associated Genes**

To examine the effects of an additional copy of mdr1 on drug susceptibility, we measured the 50% inhibitory concentration (IC50) values of pfcas9-mdr1 for the common ACT partner drugs mefloquine, lumefantrine, and piperaquine, and an artemisinin derivative, dihydroartemisinin (Table 1 and Figure 3). The IC50 values of mefloquine for two clones of pfcas9-mdr1 were 38.7 and 37.8 nM, which were ~1.8-fold higher than those of parental parasites (IC50 value; 21.0 nM; Figure 3A). The pfcas9-mdr1 line
survived with no morphological changes, even at a mefloquine concentration that killed the parental line (Supplementary Figure 1). For lumefantrine, the IC_{50} values of the two clones were 30.3 and 30.1 nM. These values were ~1.4-fold increase compared to the parental line (IC_{50}; 21.2 nM; Figure 3B). Although the increased expression of mdr1 mediated by an additional copy insertion confers resistance to mefloquine and lumefantrine, parental and transgenic parasites were not significantly different for piperaquine sensitivity (Figure 3C). In addition, the amplification of mdr1 slightly increased the IC_{50} values for dihydroartemisinin (Figure 3D).

We then analyzed the effects of increased copy numbers of pm2 and pm3 on ACT-component drugs by measuring IC_{50} (Table 2). Insertion of pm2 or pm3 had no effect on the resistance of ACT partner drugs, namely, mefloquine, lumefantrine, and piperaquine, nor on an artemisinin derivative, dihydroartemisinin.

**TABLE 1** IC_{50} values of mefloquine, piperaquine, lumefantrine, and dihydroartemisinin with pfcas9-mdr1.

| Parasite line       | Mean ± SEM | Statistical outputs | Mean ± SEM | Statistical outputs |
|---------------------|------------|---------------------|------------|---------------------|
|                     | MFQ IC_{50} (nM) | N | Notation | p-Value | LMF IC_{50} (nM) | N | Notation | p-Value |
| Parental            | 21.0 ± 1.8  | 5  | N.A.     | N.A.    | 21.2 ± 1.6  | 4  | N.A.     | N.A.    |
| pfcas9-mdr1 clone#1 | 38.7 ± 3.2  | 5  | **       | 0.001   | 30.3 ± 2.1  | 5  | *        | 0.012   |
| pfcas9-mdr1 clone#2 | 37.8 ± 4.4  | 3  | **       | 0.006   | 30.2 ± 1.4  | 4  | **       | 0.006   |

| Parasite line       | Mean ± SEM | Statistical outputs | Mean ± SEM | Statistical outputs |
|---------------------|------------|---------------------|------------|---------------------|
|                     | PPQ IC_{50} (nM) | N | Notation | p-Value | DHA IC_{50} (nM) | N | Notation | p-Value |
| Parental            | 25.3 ± 3.0  | 3  | N.A.     | N.A.    | 3.17 ± 0.14  | 9  | N.A.     | N.A.    |
| pfcas9-mdr1 clone#1 | 21.9 ± 0.2  | 3  | n.s.     | 0.325   | 3.73 ± 0.14  | 8  | *        | 0.017   |
| pfcas9-mdr1 clone#2 | 22.4 ± 1.3  | 3  | n.s.     | 0.421   | 3.89 ± 0.30  | 7  | *        | 0.035   |

*Statistical comparisons were made against the parental line. N, number of independent repeats (assays conducted with technical duplicates). Statistics employed unpaired Student’s t-tests.

*p < 0.05; **p < 0.01.
These results were consistent with a report demonstrating that episomal overexpression of pm2 or pm3 did not confer drug resistance (Loesbanluechai et al., 2019). Furthermore, no significant changes were observed in drug resistance by insertion of both pm2 and pm3 (Figures 4A–D). The amplification of the pm2 and pm3 region has been reported to be associated with reduced susceptibility of piperaquine using a PSA (Witkowski et al., 2017). Therefore, we performed a PSA using pfcas9-pm2/3 transgenic parasites and found that an additional copy of both pm2 and pm3 had no impact on drug response compared to parental parasites (Figures 4E, F and Table 2). These results indicate that, at least in the genetic background of the P. falciparum reference strain 3D7, a 1.5- to 2-fold increase in pm2 and pm3 expression levels is not sufficient to confer detectable drug resistance to piperaquine and other drugs including artemisinin.

### TABLE 2

| Parasite line               | MFQ IC50 (nM) | N  | Notation | p-Value | LMF IC50 (nM) | N  | Notation | p-Value |
|-----------------------------|---------------|----|----------|---------|---------------|----|----------|---------|
| parental                    | 26.7 ± 2.2    | 3  | N.A.     |         | 18.9 ± 2.0    | 3  | N.A.     |         |
| pfcas9-pm2 clone#1          | 18.6 ± 2.5    | 3  | n.s.     | 0.071   | N.A.          |    |          |         |
| pfcas9-pm2 clone#2          | 20.5 ± 3.2    | 3  | n.s.     | 0.193   | N.A.          |    |          |         |
| pfcas9-pm3 clone#1          | 24.4 ± 1.8    | 3  | n.s.     | 0.472   | N.A.          |    |          |         |
| pfcas9-pm3 clone#2          | 22.8 ± 0.6    | 3  | n.s.     | 0.169   | N.A.          |    |          |         |
| pfcas9-pm2/3 clone#1        | 24.8 ± 0.5    | 3  | n.s.     | 0.454   | 18.7 ± 1.5    | 3  | n.s.     | 0.960   |
| pfcas9-pm2/3 clone#2        | 27.1 ± 2.1    | 3  | n.s.     | 0.895   | 18.9 ± 0.8    | 3  | n.s.     | 1.000   |

| Parasite line               | PPQ IC50 (nM) | N  | Notation | p-Value | DHA IC50 (nM) | N  | Notation | p-Value |
|-----------------------------|---------------|----|----------|---------|---------------|----|----------|---------|
| parental                    | 26.3 ± 0.6    | 4  | N.A.     |         | 4.04 ± 0.49   | 4  | N.A.     |         |
| pfcas9-pm2 clone#1          | 29.2 ± 1.5    | 3  | n.s.     | 0.105   | N.A.          |    |          |         |
| pfcas9-pm2 clone#2          | 26.9 ± 5.1    | 3  | n.s.     | 0.902   | N.A.          |    |          |         |
| pfcas9-pm3 clone#1          | 33.1 ± 3.7    | 3  | n.s.     | 0.085   | N.A.          |    |          |         |
| pfcas9-pm3 clone#2          | 31.8 ± 3.3    | 3  | n.s.     | 0.109   | N.A.          |    |          |         |
| pfcas9-pm2/3 clone#1        | 29.3 ± 1.1    | 3  | n.s.     | 0.052   | 3.96 ± 0.59   | 5  | n.s.     | 0.924   |
| pfcas9-pm2/3 clone#2        | 31.3 ± 2.7    | 4  | n.s.     | 0.121   | 3.83 ± 0.65   | 5  | n.s.     | 0.816   |

| Parasite line               | PSA survival rate (%) | N  | Statistical outputs |
|-----------------------------|-----------------------|----|---------------------|
| parental                    | 2.23                  | 2  | N.A.                |
| pfcas9-pm2/3 clone#1        | 2.32                  | 2  | N.A.                |
| pfcas9-pm2/3 clone#2        | 2.25                  | 2  | N.A.                |

Statistical comparisons were made against the parental line. N, number of independent repeats (assays conducted with technical duplicates). Statistics employed unpaired Student’s t-tests. MFQ, mefloquine; LMF, lumefantrine; PPQ, piperaquine; DHA, dihydroartemisinin; n.s., not significant; N.A., not applicable; PSA, piperaquine survival assay.

(Figures 4A–D).
**mdr1 Transcript Levels Correlate With Mefloquine Resistance, But Not Lumefantrine and Dihydroartemisinin**

Copy number variation analysis of patient-derived *P. falciparum* DNA has shown that *mdr1* is associated with antimalarial drug susceptibility (Price et al., 2004; Price et al., 2006; Phompradit et al., 2014; Phyo et al., 2016; Montenegro et al., 2021). We demonstrated that an increase in *mdr1* mRNA expression reduced the susceptibility for mefloquine, lumefantrine, and dihydroartemisinin (Figure 3). We next examined whether *mdr1* transcript levels directly correlate with drug resistance using multiple laboratory strains. We used 3D7 and 7G8 as *mdr1* single-copy reference strains and Dd2 and FCR3 lines as *mdr1* multiple copy reference strains (Price et al., 2004; Veiga et al., 2012). The Cambodian IPC_5188 and IPC_5202 isolates, which respectively show susceptible and resistance phenotypes in a ring-survival assay with artemisinin, were used as strains in which the *mdr1* copy number was unknown (Ariey et al., 2014). First, a real-time genomic PCR assay was developed to determine *mdr1* genome copy number by the ddCt method, with PF3D7_0523800 as an internal control gene, which locates on the same chromosome as *mdr1* but outside of the genomic multiplication region of known drug-resistant strains. The results showed that when 3D7, which has one copy of *mdr1*, was used as a control, 7G8 had a single copy of *mdr1*, and Dd2 and FCR3 had multiple copies as reported (Supplementary Table 3). The Cambodian isolates IPC_5188 and IPC_5202 were found to contain single copies and multiple copies, respectively (Figure 5A and Supplementary Table 3). The subsequent quantitative reverse transcription PCR assays revealed that the strains with multi-copy *mdr1* showed higher expression levels than lines with a single copy of *mdr1* (Figures 5B–D and Supplementary Table 3). We then examined whether there were significant correlations between the *mdr1* expression levels of these laboratory strains and their susceptibility to mefloquine, lumefantrine, or dihydroartemisinin (Figures 5C, D).

**DISCUSSION**

Recent studies show that the evolution of *P. falciparum* drug resistance has rapidly compromised the clinical efficacy of ACTs (Ashley et al., 2014; Amato et al., 2017; Witkowski et al., 2017).
The design of effective ACT strategies requires an understanding of the local spread of drug-resistant parasites, especially regional resistance to partner drugs. In this report, we determined if duplications of \textit{mdr1}, \textit{pm2}, or \textit{pm3}, which were observed in field-derived drug-resistant parasites, could reduce the susceptibility against ACT partner drugs. By applying the CRISPR/Cas9 genome editing system, we successfully generated transgenic parasites containing additional copies of \textit{mdr1}, \textit{pm2}, \textit{pm3}, or a combination of \textit{pm2} and \textit{pm3}; these gene duplications resulted in approximately twofold increases in target gene transcript levels.

We proved by using transgenic parasites that an increased mRNA expression level of \textit{mdr1} directly decreased sensitivity to mefloquine, lumefantrine, and dihydroartemisinin. In various genetic backgrounds, the high correlation between the expression level of \textit{mdr1} and the IC\textsubscript{50} values of mefloquine indicated that the \textit{mdr1} copy number was the most important genetic factor contributing to mefloquine resistance. However, \textit{mdr1} mRNA levels do not correlate with copy number, and this indicates the role of additional aspects that determine \textit{mdr1} expression levels, such as transcription-related factors. Expression levels of \textit{mdr1} were not tightly correlated with sensitivity to lumefantrine and dihydroartemisinin, although insertion of an additional copy of \textit{mdr1} resulted in decreased sensitivities. These results suggest that the sensitivity to those drugs is affected by other factors in the genetic background rather than \textit{mdr1} expression. Our findings are consistent with the epidemiology-based copy number variation analyses of \textit{mdr1}, which reported that mefloquine resistance was strongly associated with \textit{mdr1} copy number. The mRNA expression level of \textit{mdr1} should be determined to judge mefloquine-resistant parasites; however, measuring transcript levels is difficult using patient blood due to RNA instability. Therefore, the \textit{mdr1} copy number remains an important marker for surveillance of drug-resistant parasites, and epidemiological investigations should continue.
We also clearly demonstrated that insertion of an additional pm2 or pm3 copy into the *P. falciparum* 3D7 strain had no impact on its antimalarial drug sensitivity. These observations agree with the determination that increased expression of pm2 or pm3 via episomal plasmids had no effect on piperquine susceptibility (Loesbanluechhai et al., 2019). The correlation between piperquine resistance and pm2/3 amplification has been reported only in Southeast Asia (Huang et al., 2020). Therefore, increasing pm2/3 expression in the genetic background of strains of Southeast Asian origin may confer piperquine resistance. In addition, mutations in the chloroquine resistance transporter that differ from mutations conferring chloroquine resistance have been shown to reduce piperquine sensitivity (Duru et al., 2015; Agrawal et al., 2017; Dhingra et al., 2017; Ross et al., 2018). Also, many parasites with a single copy of pm2 and pm3 have been isolated, which have failed dihydroartemisinin/piperquine combination treatment in epidemiological studies (Huang et al., 2020; Si et al., 2021). Currently, the pm2/3 copy number variation is being used as a molecular surveillance resistance marker, but further studies are needed to discover the molecular basis of piperquine resistance and epistatic interactions.

Epidemiology studies have predicted relationships between antimalarial drug resistance and various genetic factors. Genome editing in combination with Cas9-constitutive-expressing parasites and linearized-donor DNA templates enables the knock-in of large-sized DNAs, such as gene coding sequences including regulatory regions (Nishi et al., 2021). This system is useful to evaluate the causality between copy numbers of drug resistance-associated genes and drug resistance. Additional asexually dispensable gene loci are available for the generation of transgenic parasites with higher copy numbers of target genes and subsequent analysis of correlation with drug resistance, of importance since field isolates with three or more copies of specific genomic regions have been found (Ahoudi et al., 2021). Improvement of this system could allow us to find novel associations between gene copy number and antimalarial drug resistance by further copy number variation analysis in field studies. Identifying the relevance of the association through a genome editing-based gene amplification such as used in this study would provide important information for determining drug strategies for malaria control.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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**ETHICS STATEMENT**

Ethical approvals for the use of human red blood cells (RBCs) and plasma from the Japanese Red Cross Tokyo Blood Center were obtained from the Medical Research Ethical Committee of the Tokyo Medical and Dental University. Written informed consent from the patients/ participants or patients/participants legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

**AUTHOR CONTRIBUTIONS**

RK, SI, and NS conceived and designed experiments. RK and NS conducted experiments and analyzed the data. All authors wrote the manuscript and contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.915656/full#supplementary-material
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