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Characterization of *pfmdr1*, *pfcrt*, *pfK13*, *pfubp1*, and *pfap2mu* in Travelers Returning from Africa with *Plasmodium falciparum* Infections Reported in China from 2014 to 2018

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ABSTRACT The artemisinin-based combination therapies (ACTs) used to treat *Plasmodium falciparum* in Africa are threatened by the emergence of parasites in Asia that carry variants of the Kelch 13 (K13) locus with delayed clearance in response to ACTs. Single nucleotide polymorphisms (SNPs) in other molecular markers, such as *ap2mu* and *ubp1*, were associated with artemisinin resistance in rodent malaria and clinical failure in African malaria patients. Here, we characterized the polymorphisms in *pfmdr1*, *pfcrt*, *pfK13*, *pfubp1*, and *pfap2mu* among African isolates reported in Shandong and Guangxi provinces in China. Among 144 patients with *P. falciparum* returning from Africa from 2014 to 2018, *pfmdr1* N86Y (8.3%) and *pfcrt* K76T (2.1%) were the major mutant alleles. The most common genotype for *pfcrt* was $I_{74}E_{75}T_{76}$ (8.3%), followed by $E_{75}T_{76}$ (2.1%). For K13 polymorphisms, a limited number of mutated alleles were observed, and A578S was the most frequently detected allele in 3 isolates (2.1%). A total of 27.1% (20/144) of the isolates were found to contain *pfubp1* mutations, including 6 nonsynonymous and 2 synonymous mutations. The *pfubp1* genotypes associated with artemisinin resistance were D1525E (10.4%) and E1528D (8.3%). Furthermore, 11 SNPs were identified in *pfap2mu*, and S160N was the major polymorphism (4.2%). Additionally, 4 different types of insertions were found in *pfap2mu*, and the codon AAT, encoding aspartic acid, was more frequently observed at codons 226 (18.8%) and 326 (10.7%). Moreover, 4 different types of insertions were observed in *pfubp1* at codon 1520, which was the most common (6.3%). These findings indicate a certain degree of variation in other potential molecular markers, such as *pfubp1* and *pfap2mu*, and their roles in either the parasite's mechanism of resistance or the mode of action should be evaluated or elucidated further.

KEYWORDS *Plasmodium falciparum*, Kelch 13, *pfubp1*, *pfap2mu*, antimalarial drug resistance, Africa

Malaria treatment currently relies on artemisinin-based combination therapies (ACTs), which are the first-line drugs used to treat uncomplicated *Plasmodium falciparum* as recommended by the World Health Organization (WHO) (1). The emergence and spread of *Plasmodium falciparum* resistant to ACTs were first reported in western Cambodia and are a grave concern facing the Greater Mekong Subregion (GMS) (2, 3). This has also prompted global concern, given that resistance to

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chloroquine, mefloquine, and other antimalarials arose in the same area and then spread to sub-Saharan Africa (4, 5). Molecular markers revealing the genetic changes have been fundamental in monitoring existing or predicting future drug resistance reported somewhere (6, 7). Information about the molecular markers of antimalarial drug (e.g., artemisinin-based combination therapies [ACTs]) resistance is vital for containment and elimination.

Single nucleotide polymorphisms (SNPs) at the country or region level have been fundamental in monitoring the genomes of the parasite population. SNPs in the *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*) gene, especially the N86Y allele, have shown to be associated with resistance to chloroquine (CQ), amodiaquine, mefloquine, and quinine (8, 9). The *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene K76T mutation has been confirmed to be closely associated with CQ resistance (10, 11). The *P. falciparum* Kelch 13 (*pfK13*) gene, identified as a molecular marker for ACT-resistant isolates first observed at the Thai-Cambodia border in 2014, has spread to 5 countries in the GMS (2, 12, 13). So far, 10 of the *pfK13* mutations (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, and C580Y) have been validated *in vitro* and *in vivo* as associated with delayed clearance following ACT treatment (14). Two other molecular markers, the *P. falciparum* ubiquitin-specific protease (*pfubp1*) and clathrin vesicle-associated adaptor 2 (*pfap2mu*), which are the polymorphic homologs of *pcubp1* (*pcubp1* encodes ubiquitin carboxy-terminal hydrolase 1) and *pcap2mu* (*pcap2mu* encodes clathrin vesicle-associated adaptor 2, μ subunit), were both shown to be markers of parasites with reduced sensitivity to ACTs *in vivo* (15, 16). Two studies in Kenya and Ghana showed that the codon changes D1525E and E1528D are closely associated with reduced susceptibility to ACTs *in vitro* (17, 18).

In this study, we characterized *pfmdr1*, *pfcrt*, *pfK13*, *pfubp1*, and *pfap2mu* mutations in African isolates reported in Shandong and Guangxi from 2014 to 2018. Our aim was to investigate the prevalence of these molecular markers of the mutated alleles conferring ACT resistance.

RESULTS

Demographics of *P. falciparum* infection. A total of 144 *P. falciparum* blood samples from Shandong and Guangxi by GPS location were used in this study (Fig. 1). Among all the participants, 141 (97.9%) were male, 80 (55.6%) were inpatients, and 119 (82.6%) patients had the typical symptoms of sweating, cold, and fever. For Shandong and Guangxi, the median age was 42 and 38 years old; the interval from onset to first diagnosis was 3.65 and 2.47 days, and the interval from first diagnosis to confirmed diagnosis was 1.68 and 0.64 days, respectively (Table 1). No obvious difference was found among all the demographic characteristics. The *P. falciparum* cases were mainly imported from Ghana ($n = 43$, 29.9%), Cameroon ($n = 32$, 22.2%), and the Democratic Republic of the Congo ($n = 10$, 6.9%).

***pfmdr1* polymorphisms.** We have successfully amplified *pfmdr1* for all 144 isolates and sequenced the obtained fragment product. The results showed that 5 polymorphisms were found in the *pfmdr1* gene in 22 *P. falciparum* isolates (15.3%), including 3 nonsynonymous polymorphisms found in 13 isolates and 3 synonymous polymorphisms in 9 isolates. Among the nonsynonymous polymorphisms, N86Y was the major mutant allele found in 12 isolates (8.3%), and G102G, which was observed in 6 isolates (4.2%), was the most common of the synonymous polymorphisms. N86Y was found mainly in the workers returning from Cameroon ($n = 3$) and the Democratic Republic of the Congo ($n = 3$), which accounted for 9.4% (3/32) and 33.3% (3/9) of the isolates collected from those two countries, respectively. The other mutant alleles included I91T and S100S, which were only present in one isolate, except for L108L, which was found in 2 isolates (1.4%) (Table 2). In addition, we have found only 1 $Y_{86}G_{102}$ genotype in the isolates imported from Cameroon (Table 3). The distribution of *pfmdr1* by imported country and by year is listed in Table S2.

***pfcrt* polymorphisms.** All 144 isolates were successfully amplified and sequenced in this study. A total of 3 nonsynonymous polymorphisms were identified in 5 isolates (3.5%). Among all 144 isolates, the mutant allele codon K76T was found as the major

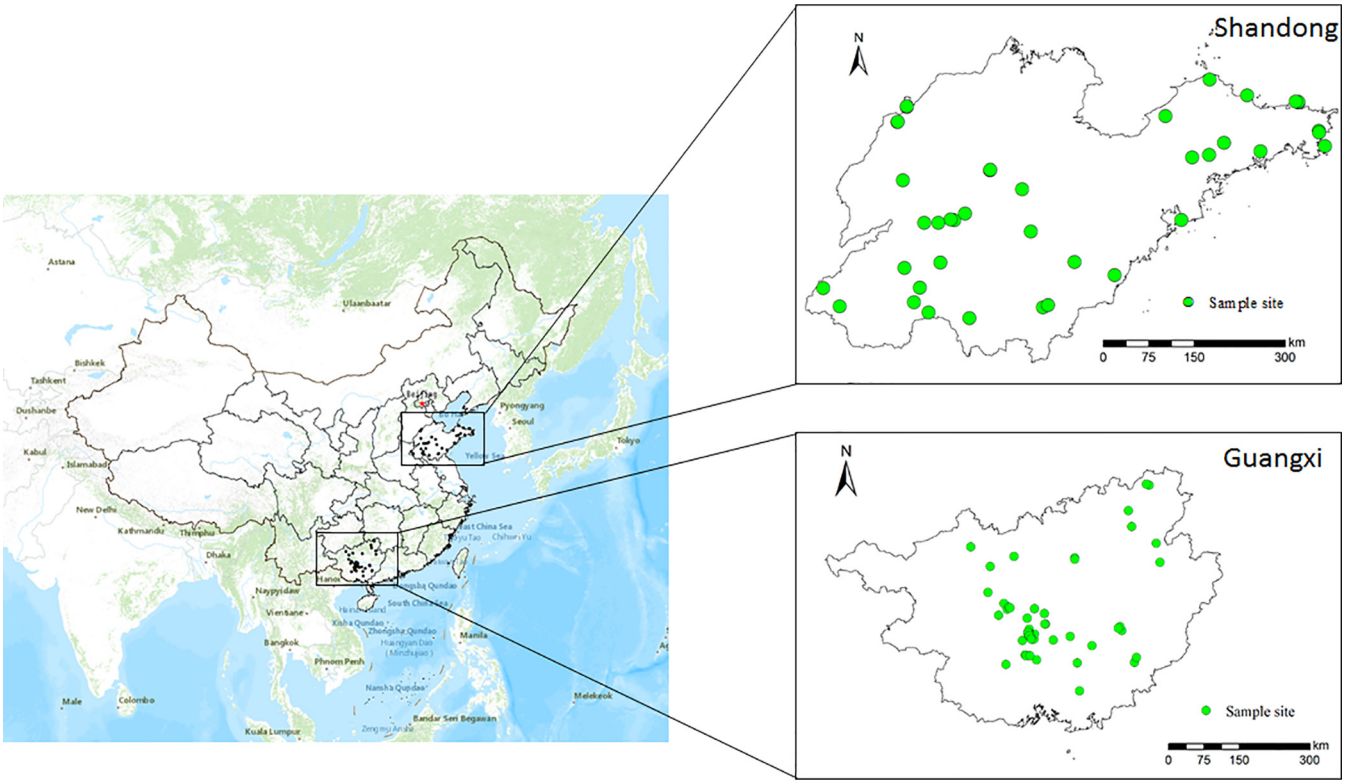

FIG 1 Study sample collection sites in Shandong and Guangxi. All counties are labeled by GPS location using ArcGIS 10.1.

polymorphism in 3 isolates (2.1%) (Table 2). In addition, 2 different genotypes involving codons 74, 75, and 76 were found with 12 isolates harboring $I_{74}E_{75}T_{76}$ (8.3%) and 3 isolates harboring $E_{75}T_{76}$ (2.1%) (Table 3). $I_{74}E_{75}T_{76}$ was mainly found in the workers returning from the Democratic Republic of the Congo ($n = 4$, 44.4%, 4/9) and Ghana ($n = 3$, 18.8%, 3/16). The distribution of *pfcrt* by year and imported country is listed in Table S2.

***pfK13* polymorphisms.** The *pfK13* gene was successfully amplified and sequenced in all 144 *P. falciparum* isolates. None of the isolates harbored the polymorphisms that were associated with delayed clearance after ACT treatment. We identified 4 polymorphisms in the *pfK13* gene, and 2 of them were nonsynonymous polymorphisms, whereas the other 2 were synonymous polymorphisms. The mutant allele A578S was the more frequently detected allele in 3 isolates (2.1%), while the mutant allele D584E was only found in 1 isolate from Ghana (0.7%) (Table 2). Among the synonymous polymorphisms, C469C and Y500Y were found in one isolate each from travelers returning from Nigeria and the Democratic Republic of the Congo, respectively. The distribution of *pfK13* by year and imported country is listed in Table S2.

***pfubp1* polymorphisms.** We successfully amplified and sequenced the *pfubp1* gene in all the 144 *P. falciparum* isolates. Referring to the *pfubp1* polymorphisms, 6 nonsynonymous polymorphisms and 2 synonymous polymorphisms were identified in 39 isolates (27.1%). Among the nonsynonymous polymorphisms, D1525E and E1528D were the most common polymorphisms observed in 15 (10.4%) and 12 (8.3%) isolates, respectively. The mutated alleles D1486D and N1518N were the two synonymous polymorphisms found in 5 isolates (3.5%) (Table 2). Only 1 genotype, $D_{1528}D_{1531}$, was found in 1 isolate from Cameroon (Table 3). The distribution of *pfubp1* by year and imported country is listed in Table S2.

***pfap2mu* polymorphisms.** We successfully amplified and sequenced the *pfap2mu* gene in 144 (100%) isolates. The results showed that 11 polymorphisms were identified, including 6 nonsynonymous polymorphisms and 5 synonymous polymorphisms. Among the 6 nonsynonymous polymorphisms, S160N was the major polymorphism found in

TABLE 1 Characteristics of study participants with *P. falciparum* by study sites in Shandong and Guangxi Provinces, 2014 to 2018

	Characteristics					
Study site	Male, no. (%)	Age, y, median	Inpatients, no. (%)	Experienced symptoms, no. (sweat, cold, fever) (%)	Interval from onset to first diagnosis, mean	Interval from first diagnosis to confirmed diagnosis, mean
Shandong ($n = 40$)	40 (100.0)	42	25 (62.5)	31 (77.5)	3.65	1.68
Guangxi ($n = 104$)	101 (97.1)	38	55 (53.9)	88 (84.6)	2.47	0.64
P value	0.56	1.358	0.452	0.332	1.236	1.97

6 isolates (4.2%), followed by the K199T allele, which was found in 3 isolates (2.1%). Among the 5 synonymous mutations, G163G was the most observed mutation in 7 isolates (4.9%) (Table 2). A total of 5 different genotypes were found, including $N_{160}P_{281}$, $N_{160}T_{362}$, $G_{163}R_{188}$, $G_{163}E_{236}$, and $R_{334}R_{336}R_{346}$, and each was identified only once (Table 3). The distribution of *pfap2my* by year and imported country is listed in Table S2.

Insertions and deletions in *pfubp1* and *pfap2mu*. A variety of insertions and deletions were identified by sequencing 6 regions of *pfubp1* and *pfap2mu*, as shown

TABLE 2 Mutations in *pfmdr1*, *pfcrt*, *pfK13*, *pfubp1*, and *pfap2mu* in the study sites, 2014 to 2018

Gene	Genotype	Subtotal	Amino acid reference	Nucleotide reference	Amino acid mutation	Nucleotide mutation[a]	Prevalence (%)
pfmdr1	Y_{86}	12	N	AAT	Y	**T**AT	8.3
	T_{91}	1	I	ATT	T	**A**CT	0.7
	S_{100}	1	S	TCT	S	**T**CC	0.7
	G_{102}	6	G	GGT	G	GG**C**	4.2
	L_{108}	2	L	TTA	L	TT**G**	1.4
pfcrt	I_{74}	1	M	ATG	I	AT**T**	0.7
	K_{75}	1	N	AAT	K	AA**A**	0.7
	T_{76}	3	K	AAA	T	A**C**A	2.1
pfK13	C_{469}	1	C	TGC	C	TG**T**	0.7
	Y_{500}	1	Y	TAT	Y	TA**C**	0.7
	S_{578}	3	A	GCT	S	**T**CT	2.1
	D_{584}	1	E	GAA	D	GA**T**	0.7
pfap2mu	K_{146}	2	R	AGA	K	A**A**A	1.4
	Q_{146}	2	Q	CAG	Q	CA**A**	1.4
	N_{160}	6	S	AGT	N	A**A**T	4.2
	I_{162}	1	I	ATT	I	AT**C**	0.7
	G_{163}	7	G	GAA	G	GA**G**	4.9
	R_{188}	5	R	AGA	R	AG**G**	3.5
	T_{199}	3	K	AAA	T	A**C**A	2.1
	I_{235}	1	T	ACA	I	A**T**A	0.7
	K_{266}	1	E	GAA	K	**A**AA	0.7
	N_{269}	1	N	AAT	N	AA**C**	0.7
	S_{288}	1	L	TTA	S	T**C**A	0.7
pfubp1	D_{1486}	1	D	GAT	D	GA**C**	0.7
	N_{1518}	4	N	AAC	N	AA**T**	2.8
	E_{1525}	15	D	GAC	E	GA**A**	10.4
	D_{1528}	12	E	GAA	D	GA**C**	8.3
	D_{1531}	1	E	GAA	D	GA**C**	0.7
	E_{1537}	4	K	AAA	E	**G**AA	2.8
	G_{1539}	1	D	GAT	G	G**G**T	0.7
	P_{1544}	1	H	CAT	P	C**T**T	0.7

[a]Mutations are in boldface.

TABLE 3 Genotype of *pfmdr1*, *pfcrt*, *pfubp1*, and *pfap2mu* with *P. falciparum* in Shandong and Guangxi Provinces, 2014 to 2018

	Guangxi			Shandong		
Gene	Genotype[a]	Subtotal	Proportion[b] (%)	Genotype	Subtotal	Proportion (%)
pfmdr1	**Y**$_{86}$G$_{102}$	1	1.0	ND[c]	ND	ND
pfcrt	**E**$_{75}$**T**$_{76}$	1	1.0	**E**$_{75}$**T**$_{76}$	2	5.0
	I$_{74}$**E**$_{75}$**T**$_{76}$	9	8.7	**I**$_{74}$**E**$_{75}$**T**$_{76}$	3	7.5
pfap2mu	**N**$_{160}$**P**$_{281}$	1	1.0	ND	ND	ND
	N$_{160}$T$_{362}$	1	1.0	ND	ND	ND
	G$_{163}$R$_{188}$	1	1.0	G$_{163}$**E**$_{236}$	1	2.5
	R$_{334}$**R**$_{336}$**R**$_{346}$	1	1.0	ND	ND	ND
pfubp1	**D**$_{1528}$**D**$_{1531}$	3	2.9	**D**$_{1528}$**D**$_{1531}$	1	2.5

[a]Mutations are in boldface.
[b]The proportion refers to the mutated genotype samples accounting for the whole samples collected at the study site.
[c]ND, no mutated genotype detected at the study site.

in Table 4. A total of 4 different types of insertions were found in *pfap2mu*, among which the codon AAT, encoding aspartic acid, at codon 226 was the most prevalent (18.8%, 28/149), followed by codon 326, which also showed the AAT insertion (10.7%, 16/149). Only 2 isolates with AAT deletion at codon 226 were found (1.3%, 2/149). For insertions in *pfubp1*, we found 4 different types of insertions, among which the AAATATGAC sequence, which encodes Lys-Tyr-Asp at codon 1520, was the most common (6.0%, 9/149). In addition, 2 types of deletions in *pfubp1* were observed in 30 isolates; 26 of them (17.4%, 26/149) contained the AAATATGAA sequence encoding Lys-Tyr-Glu at codon 1526, and 4 isolates harbored the deletion of Lys-Tyr-Asp at 1520.

DISCUSSION

The development and spread of artemisinin-resistant *P. falciparum* outside the GMS pose a great challenge particularly to sub-Saharan Africa, where it was reported in 2020 that it accounted for 90% of global malaria cases and 95% of malaria deaths (19). The previous population genetic analysis of whole-genome sequences showed that the isolates were classified to a cluster from Africa, suggesting that they may have originated in Africa rather than via migration from the GMS (5, 20). Since China has eliminated indigenous malaria but the imported malaria from Africa has increased significantly in most of China recently (21, 22), knowledge of the molecular markers associated with ACT resistance is crucial for its elimination and postelimination surveillance. Therefore, in this study, we have ge-

TABLE 4 Insertions/deletions in the *pfubp1* and *pfap2mu* genes and the consequent amino acid replacements

Gene	Codon	Reference sequence	Insertion(s)/deletion(s)	Prevalence (%)
pfap2mu	226	Asn ×7	Asn ×5	1.3
			Asn ×8	18.8
	233	Lys ×1	Lys ×2	1.3
	319	Asn ×5	Asn ×6	10.1
	326	Asn ×4	Asn ×5	10.7
pfubp1	1520	Lys-Tyr-Asp ×2	Lys-Tyr-Asp ×1	2.7
			Lys-Tyr-Asp ×3	6.0
			Lys-Tyr-Asp ×5	1.3
	1526	Lys-Tyr-Glu ×2	Lys-Tyr-Glu ×1	17.4
			Lys-Tyr-Glu ×3	4.0
			Lys-Tyr-Glu ×4	0.7

netically characterized *P. falciparum* infection in workers returning from Africa to Guangxi and Shandong Provinces and have explored the genetic signature at the *pfmdr1*, *pfcrt*, *pfK13*, *pfupb1*, and *pfap2mu* candidate drug-resistance marker loci.

We found that *pfmdr1* N86Y (8.3%) was the major mutant allele among all isolates from African countries, which is consistent with many other reports (23–25). The prevalence of *pfmdr1* N86Y from Cameroon in our study was 9.4% (3/32), and it was lower than that in Yaoundé (76%, 153/201) and Mfou (84%, 175/209) reported in 2012 (26) but similar to that in Cameroon reported in 2012 (13.0%, 25/190) (27). The changes in antimalarial treatment strategies and the simultaneous use of several formulations of ACT that are not officially recommended result in complex selective pressure, supporting the prediction of the evolution of *P. falciparum* resistance complexes. The prevalence of *pfmdr1* N86Y found in the Democratic Republic of the Congo (DRC) was similar to that recorded in other documents, which were recorded at intermediate frequencies of 37.9% (41/108) and 46.1% (47/102) in Kinshasa, and limited the impact of (re)treatment with artemether-lumefantrine (AL) or artesunate-amodiaquine (ASAQ) after selection for *pfmdr1* alleles and haplotypes in this country (28, 29). The low prevalence of *pfmdr1* 86Y from Cameroon (9.4%) and the DRC (33.3%) in this study compared with that in other studies may reveal a gradual return of the 4-aminoquinoline antimalarial sensitive genotype in the two countries recently (30).

Similar to the *pfmdr1* N86Y, the mutant *pfcrt* genotype $I_{74}E_{75}T_{76}$ was also mainly found in workers returning from the Democratic Republic of the Congo ($n = 4$, 44.4%), which is the country found to have the highest variability for chloroquine resistance (31). Interestingly, 3 of the isolates from the DRC simultaneously harbored the mutant genotypes of *pfmdr1* N86Y and *pfcrt* $I_{74}E_{75}T_{76}$, but $S_{72}V_{73}M_{74}N_{75}T_{76}$, which is related to amodiaquine resistance, was not detected in this study. The simultaneous presence of an intermediate prevalence of *pfmdr1* N86Y and *pfcrt* $I_{74}E_{75}T_{76}$ could be related to drug pressure such as chloroquine usage in the country. Referring to the use of chloroquine in the DRC, it was still in use despite its withdrawal from the national policy of malaria management in 2001 (32). In this study, most of the studied samples were *pfmdr1* and *pfcrt* wild types, and it is known that when the chloroquine pressure stops, the drug tends to recover its effectiveness against the parasite, while the use of AL seems to favor the return to the predominance of wild-type *pfcrt* K76 and *pfmdr1* N86 genotypes by an active selection (33). This could explain the high proportion of wild-type *pfmdr1* and *pfcrt* genotypes.

A limited number of K13 polymorphisms were found among the isolates from Africa, of which A578S was the most prevalent, which is consistent with many other reports (34, 35). The above K13 mutant alleles were found only in the African isolates and were absent from the southeast Asian isolates, suggesting that they may have originated in Africa (34).

Since a limited set of K13 molecular markers occurred in the African isolates, we introduced two other molecular markers, *pfubp1* and *pfap2mu*, to carry out the prevalence of those 2 molecular markers among African isolates. This assay detected the known delayed parasite clearance genotypes E1525D and D1528E in *pfubp1*, which were observed in 10.4% and 8.3% of the isolates, respectively. D1528E was also found in isolates from Ghana and Kenya, and the prevalence of E1528D was lower than that among the Ghanaian (7.4%) and Kenyan isolates (17.1%) (17, 36). Notably, among the African isolates, especially those from Nigeria (which had 4 isolates with *pfubp1* D1528E), the artemisinin resistance of the parasite populations will increase in the presence of long-term drug use.

The genotype of N160S in *pfap2mu* was shown in 4.2% of the isolates, lower than the percentage of isolates reported in Ghana (7.4%) (36). Previous studies showed that the differences in the amino acid encoded by N160S are associated with enhanced parasite survival *in vivo* following ACT treatment (37). Transgenic parasites carrying the mutated *pfap2mu* 160N were less sensitive to 2,8-dihydroxyadenine using a 48-h *in vitro* test. Another study from a UK patient who returned from Angola had S160N, thereby strengthening the role of that mutation in recurrent parasitemia during AL treatment (38). In addition to S160N, a high prevalence of the *pfap2mu* mutation, G163G (4.9%), was observed in this study following R188R (3.5%). Recently,

the *in vitro* drug susceptibility assays showed that not those expressing S160N, but parasites expressing the I592T variant, exhibited an 11% survival rate when exposed to 700 nM dihydroartemisinin (DHA) (39). Therefore, the recent implication of SNPs in these genes, confirming the existence of multiple pathways in either artemisinin resistance or the mode of action of ACTs, may raise a great concern in elucidating their molecular roles in *P. falciparum*.

Insertions and deletions identified in this study were similar to those observed in Ghana and Kenya (17, 36). The most common deletions and insertions observed in the isolates of *pfap2mu* resulted from an insertion of AAT, which led to an asparagine at codons 226, 326, and 319, respectively. The prevalence was slightly lower than that seen in Kenya. For the deletions and insertions in *pfubp1*, codon 1526 with a deletion of lysine-tyrosine-glutamic acid was observed. This resulted from deletions/insertions of the amino acids KYE and KYD, and most of them resulted in frameshifts, which were caused by a deletion or insertion of either a guanosine or a thymidine nucleotide.

Conclusion. In summary, this study identified the molecular markers *pfmdr1*, *pfcrt*, K13, *pfubp1*, and *pfap2mu* in African isolates, which has implications for the development of artemisinin with continuing use of ACTs. The findings of this study suggest that other novel molecular markers, such as *pfap2mu* and *pfubp1*, should be investigated and analyzed to explore their roles in conferring resistance. Further research is under way to elucidate the molecular roles of *pfap2mu* and *pfubp1* as well as other potential molecular markers in delayed parasite clearance in clinical trials.

MATERIALS AND METHODS

Study sites and samples. We have performed a study of isolates collected before ACT treatment in 34 counties in Guangxi and Shandong Provinces in eastern China from 2014 to 2018. Guangxi and Shandong provinces are affected by imported *P. falciparum*; the number of imported *P. falciparum* was 1,916 cases reported in those 2 provinces, accounting for nearly 16.5% of the total imported *P. falciparum* cases ($n = 11{,}614$) nationwide.

Molecular marker polymorphisms. A total of 144 *P. falciparum*-infected blood samples from 2014 to 2018 were collected and examined at enrollment (104 cases from Guangxi and 40 cases from Shandong). Approximately 100 μl of blood was obtained from a finger prick and spotted on a piece of 3MM Whatman filter paper (GE Healthcare, Boston, MA, USA), which was allowed to air dry. Each of the samples was labeled with a study number and stored at −20°C until extraction. The *Plasmodium falciparum* genomic DNA from approximately 20 μl of each dried blood sample was then extracted with a QIAamp DNA blood kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

To investigate polymorphisms in the *pfmdr1* (PF3D7_0523000), *pfcrt* (PF3D7_0709000), *pfK13* (PF3D7_1343700), *pfubp1* (PF3D7_0104300), and *pfap2mu* (PF3D7_1218300) genes, we amplified the purified DNA templates by following protocols for these genes as previously described (2, 17, 40). A DNA sample extracted from the 3D7 parasite strain was used as a positive control. To genotype the polymorphisms in *pfmdr1*, *pfcrt*, *pfK13*, *pfubp1*, and *pfap2mu*, the primes and the target fragment were obtained by nested PCR followed by restriction fragment length polymorphism assays (Table S1). The PCR products were collected and subjected to Sanger sequencing (Shanghai BioTechnologies Co., Ltd., Shanghai, China).

Data analysis. Sequences were analyzed with the BLAST program (http://blast.ncbi.nlm.nih.gov/). Multiple nucleotide sequence alignments and analysis were performed using the DNAMAN software editor. Sequences with poor quality after 3 sequencing attempts were not included in the analysis. R (version 4.0.2) statistical software (R Foundation for Statistical Computing, Vienna, Austria) was used to conduct statistical analyses, and the chi-square test was employed to test the different ratios of demographical characteristics including male, age, symptom, interval from onset to first diagnosis, and interval from first diagnosis to confirmed diagnosis between Guangxi and Shandong from 2014 to 2018. A map showing the study sites by Global Position System (GPS) location was created by ArcGIS 10.1 (Environmental Systems Research Institute, Inc.).

Ethical considerations. This study was reviewed and approved by the ethical committee of the National Institute of Parasitic Diseases, Chinese Centre for Disease Control and Prevention (NIPD, China CDC, number 2019008).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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Jun Feng provided the draft and made revisions. Dongmei Xu contributed on the method establishment and made revisions.

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