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EPIDEMIOLOGY AND SURVEILLANCE

Prevalence of *Plasmodium falciparum* Kelch 13 (*PfK13*) and Ubiquitin-Specific Protease 1 (*pfubp1*) Gene Polymorphisms in Returning Travelers from Africa Reported in Eastern China

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ABSTRACT Delayed clearance of *Plasmodium falciparum* by artemisinin-based combination therapies (ACTs) has already been observed for African isolates. Here, we aimed to investigate the prevalence, among travelers returning from African countries, of polymorphisms in two genes correlated with delayed parasite clearance (encoding *P. falciparum* Kelch 13 [*PfK13*] and ubiquitin-specific protease 1 [*pfubp1*]) reported in eastern China and to provide baseline data for antimalarial drug resistance (ART) surveillance and evaluation. A total of 153 filter paper blood spots collected in 2017–2019 from patients with uncomplicated *P. falciparum* cases in Anhui and Shandong Provinces were included in this study. Among them, 3.3% (5/153) of the isolates carried *PfK13* mutations, and 3 of them harbored the same synonymous mutation, C469C. A total of 13.1% (20/153) of the isolates were found to contain *pfubp1* mutations, and all were nonsynonymous. The *pfubp1* genotypes associated with ART that occurred in this study included E1528D (6.5% [10/153]) and D1525E (2.6% [4/153]). However, a high prevalence of the previously unreported mutation E1531D (5.9% [9/153]) was also detected. In addition, two types of deletions (encoding KID and KIE, respectively) and two types of insertions (encoding KYE and KYDKYD, respectively) were found in 16 isolates and 6 isolates, respectively. This study showed limited variation in *PfK13* among travelers returning from African countries and suggested other potential molecular markers, such as *pfubp1*, for use in the surveillance of African isolates in ACT susceptibility studies. Further clinical trial research is under way to investigate these *PfK13* and *pfubp1* mutations, as well as other candidate molecular markers, and their roles in delaying parasite clearance.

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

Imported malaria, especially imported *Plasmodium falciparum* malaria from Africa, has become a great threat to malaria elimination in China (1). In 2019, 1,013 cases of imported *P. falciparum* malaria from five African countries, including Nigeria, the Democratic Republic of the Congo, Côte d'Ivoire, Guinea, and Ghana, have been reported, accounting for 51.9% of the total *P. falciparum* cases reported in China (2). Malaria still remains a major challenge in sub-Saharan Africa, where 228 million cases and 405,000 malaria-related deaths were reported in 2018 (3). Artemisinin-based combination therapies (ACTs) were the first-line drugs recommended by the World Health Organization (WHO), and an estimated 214 million ACT treatments, most of which were used in African countries, were delivered in 2018. However, the emergence

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and development of *Plasmodium falciparum* resistance to ACTs, which initially occurred near the Thailand-Cambodia border, have become a grave concern (4, 5). To solve this problem, the WHO has launched efforts to contain antimalarial drug resistance (ART), including therapeutic efficacy studies (TESs) and integrated drug efficacy surveillance (iDES), particularly in the Greater Mekong Subregion (GMS) (6). In addition, molecular markers revealing genetic changes in these parasites were found to be highly associated with ACT resistance. Therefore, knowledge of these molecular markers at the country or regional level and monitoring of the genomes of the parasite population are crucial for the early detection of emerging resistance.

The gene encoding *Plasmodium falciparum* Kelch 13 (*PfK13*) was identified as a molecular marker for ACT-resistant isolates in 2014 (4). To date, the WHO has reported a total of nine single nucleotide polymorphism (SNP) sites (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, and C580Y) that were validated *in vivo* and *in vitro* as associated with ACT resistance (7). These SNPs were found mainly in the GMS and were closely associated with delayed clearance following ACT treatment (8, 9).

Among *P. falciparum* isolates from Africa, A578S was the most common mutation site and showed no relationship to clinical or *in vitro* ACT resistance (10, 11). In eastern China, the ART-related *PfK13* mutation R561H was identified as the main mutation site in Zhejiang Province among migrant workers from Rwanda (12). In 2017, Jiangsu Province reported a patient who had returned from Equatorial Guinea and was harboring the M579I site, which was confirmed to be linked to ACT resistance, with a 2.29% *in vitro* survival rate by ring-stage survival assay (13), suggesting that careful surveillance of African parasite populations is still warranted.

Another molecular marker, encoding *P. falciparum* ubiquitin-specific protease (*pfubp1*), was adopted as a major molecular marker for monitoring ACT resistance in Africa by recent surveillance studies (14, 15). It is known that *ubp1* encodes a deubiquitinating (DUB) enzyme that is responsible for the cleavage of ubiquitin from any protein or peptide to which it is joined (16). A homologue of *pfubp1* was first discovered in the rodent malaria parasite *Plasmodium chabaudi* (*pcubp1* encodes ubiquitin carboxy-terminal hydrolase 1) and found to be associated with ACT resistance (17). Two studies in Kenya and Ghana showed that the codon changes D1525E (from aspartic acid to glutamic acid) and E1528D (from glutamic acid to aspartic acid) are closely associated with delayed parasite clearance (18, 19). More studies are therefore required to validate *pfubp1* and its role in ACT resistance.

The increase in imported *P. falciparum* cases from Africa, which has become the main infection source in China (20), is a major risk factor for the spread of ACT resistance. Here, we aimed to investigate the genetic diversity of the *PfK13* propeller and *pfubp1* alleles in Anhui and Shandong Provinces in eastern China and to examine the *PfK13* and *pfubp1* mutation status of the pathogen population so as to monitor and evaluate the potential emergence of ACT resistance.

RESULTS

Epidemiological and clinical study. A total of 153 *P. falciparum* malaria cases were involved in this study, and all were imported from African countries (Fig. 1). The average patient age was 42 years, and 147 patients (147/153 [96.1%]) were male. The numbers of cases reported in 2017, 2018, and 2019 were 41, 44, and 68, respectively. The *P. falciparum* cases were mainly imported from the Democratic Republic of the Congo ($n = 28$), Nigeria ($n = 27$), and Angola ($n = 16$). None of the patients who stayed in hospital ($n = 86$) were positive on day 3 after ACT administration as detected by microscopy.

Polymorphisms in the *PfK13* propeller. To investigate *PfK13* propeller polymorphisms, all 153 *P. falciparum* samples were successfully sequenced and analyzed (Table 1). A total of five isolates with *PfK13* propeller mutation sites were observed. Among them, three isolates harbored the same synonymous mutation (C469C) and had come from Nigeria, Ghana, and Liberia. Another isolate, from the Democratic Republic of the

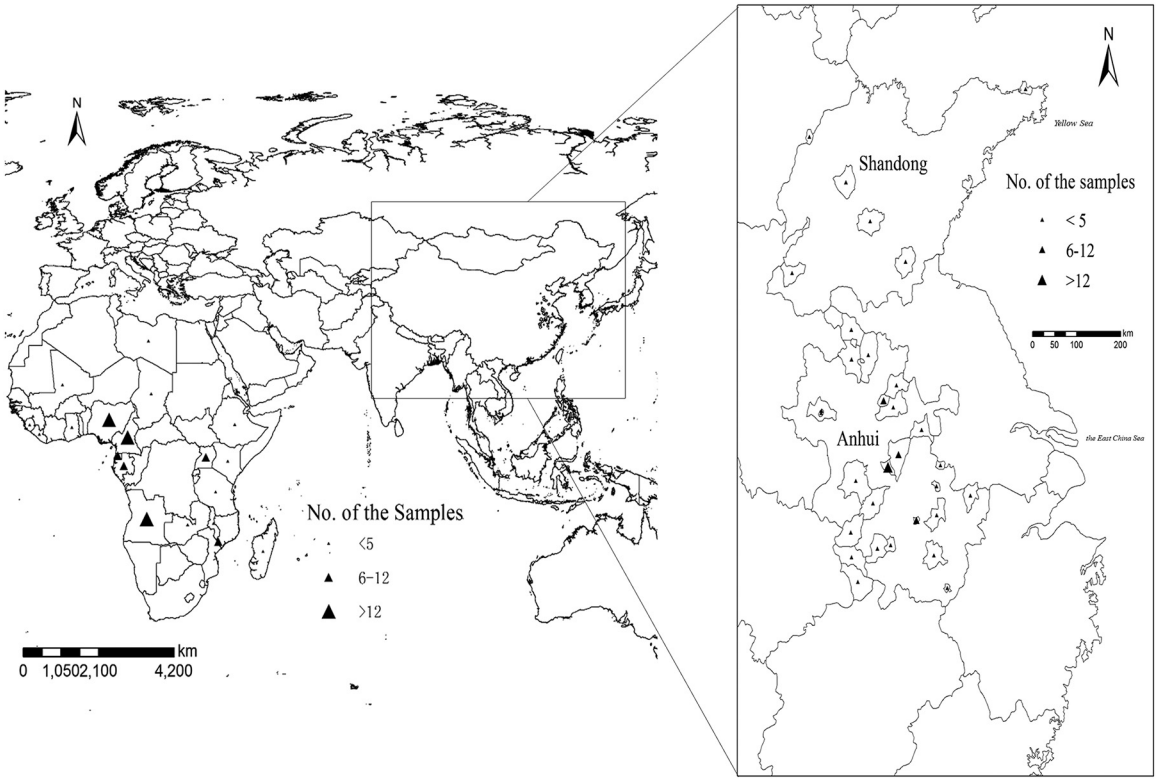

FIG 1 Study sample collection sites in source countries in Africa and in counties in Shandong and Anhui. All countries and counties are labeled according to the number of samples obtained using ArcGIS 10.1.

Congo, reported in 2019, harbored the synonymous mutation site T537T. The remaining isolate, from Uganda, was found to carry V589A (Table 1).

Polymorphisms in *pfubp1*. For *pfubp1* investigation and evaluation, a 300-bp region was amplified and sequenced. All 153 samples were successfully sequenced. A total of 20 isolates (13.1% [20/153]) were found to harbor seven different SNPs. Among them, E1528D was the major polymorphism ($n = 10$) (6.5% [10/153]), followed by E1531D ($n = 9$) (5.9% [9/153]) and D1525E ($n = 4$) (2.6% [4/153]) (Table 2). The E1528D and E1531D sites were found mainly in patients returning from Nigeria ($n = 4$) and the Democratic Republic of the Congo ($n = 2$), respectively (Fig. 2). Fourteen of the isolates had single mutation sites, while the remaining six were found to harbor mixed mutant alleles: three isolates were found to harbor both D1525E and E1531D, two isolates were found to harbor both E1528D and E1531D, and one isolate was found with both V1497M and E1531D.

Insertions and deletions in *pfubp1* isolates. The multiple nucleotide sequence alignment results showed that nine isolates were genotyped with deletions of AAATA CGAC (encoding KID) at amino acid residues 1520 to 1522, and seven isolates were identified with deletions of AAATACGAA (encoding KIE) at amino acid residues 1526 to

TABLE 1 Prevalence of *P. falciparum PfK13* polymorphisms in travelers returning from Africa in Shandong and Anhui Provinces, 2017–2019

Study site	No. of wild-type genes	*PfK13* mutation			Yr
		Site	No. of isolates	Prevalence (%)	
Anhui	41	0	0	0	2017
	42	C469C	2	4.8	2018
	41	C469C	1	2.4	2019
Shandong	24	T573T V589A	2	8.3	2019

TABLE 2 Prevalence of *pfubp1* polymorphisms in travelers returning from Africa in Shandong and Anhui Provinces, 2017–2019

Study site	No. of wild-type genes	*pfubp1* mutation			Yr
		Site	No. of isolates	Prevalence (%)	
Anhui	40	D1525E	1	2.4	2017
		E1528D	1	2.4	
	38	D1525E	2	4.5	2018
		E1528D	3	6.7	
		E1531D	5	11.1	
	32	V1497M	1	2.4	2019
		D1525E	1	2.4	
		E1528D	6	14.3	
		E1531D	4	9.5	
Shandong	24	K1523P	1	3.8	2019
		E1531G	1	3.8	

1528 (Fig. S1). In addition, we found that four isolates had insertions of AAATATGAA (encoding KYE) at amino acid residues 1526 to 1528, and two isolates had insertions of AAATATGACAAATATGAC (encoding KYDKYD) at amino acid residues 1519 to 1524 (Fig. S1).

DISCUSSION

The resistance of *P. falciparum* to ACTs is one of the major challenges facing malaria elimination worldwide (21). The previous work of our group focused on the molecular markers found in migrant populations along the China-Myanmar and China-Vietnam borders, because these regions were at high risk due to the ART that emerged and spread from the Thailand-Myanmar border in the GMS (11, 22, 23). In this study, we aimed to determine genetically whether new candidate SNPs in K13 or *pfubp1* existed among patients returning from African countries, in order to understand the population

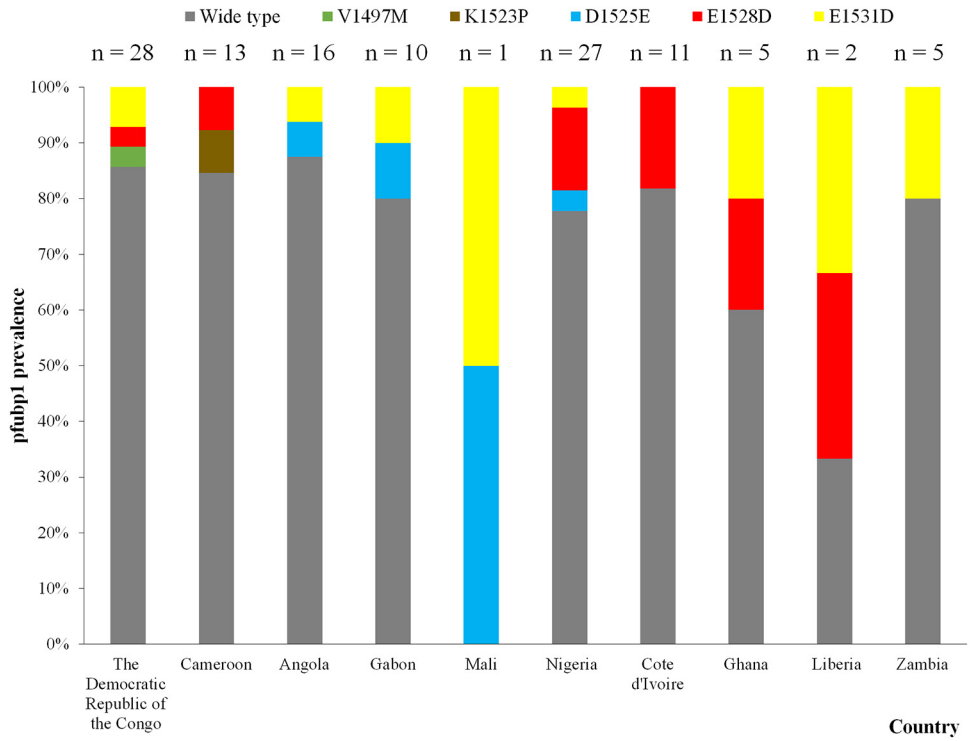

FIG 2 Prevalences of *pfubp1* in travelers returning from source countries. The numbers of samples for the source countries, including wild-type and mutated sequences, are indicated.

genetics of K13 and *pfubp1* polymorphisms and to explore the relationship between molecular markers and clinical drug effectiveness.

Our findings provide evidence that a limited number of *PfK13* polymorphisms were present among isolates from Africa, in agreement with many other reports (24–26). Interestingly, synonymous mutations accounted for the majority of the K13 mutant alleles observed. We found four isolates harboring two synonymous mutations, C469C and T537T, which have no correlation with ART. However, the widely reported mutation A578S was not observed in this study. The treatment outcomes of these patients showed that all the isolates were sensitive to ACT, and no positive parasite results were observed on day 3 after ACT administration.

Since a limited set of *PfK13* molecular markers occurred in the African isolates, we introduced another marker, *pfubp1*, to explore its role in delayed parasite clearance among African isolates. This assay detected the known delayed parasite clearance genotypes D1525E and E1528D, which were observed in 2.6% ($n = 4$) and 6.5% ($n = 10$) of the isolates, respectively. D1528E was also found in isolates from Ghana, Kenya, and Tanzania (14, 15, 27), and the prevalence of E1528D was lower than those among the Ghanaian (7.4% [6/81]), Kenyan (17.1% [6/35]), and Tanzanian (10.5% [2/19]) isolates. Notably, among the African isolates, especially those from Nigeria (which reported four isolates with *pfubp1* D1528E), the ART of the parasite populations increases in the presence of long-term drug use. In addition, we observed a novel mutation, E1531D, at rates as high as 5.9% in the African isolates. Therefore, new mutations such as E1531D may be selected, and their prevalence may increase with the continuing use of ACTs to treat African isolates.

Combining the SNP results of *PfK13* and *pfubp1*, we found that one isolate from Ghana simultaneously harbored the C469C K13 allele and the E1528D and E1531D alleles of *pfbup1*, while another two isolates harbored the T537T and V589A K13 alleles and the KID and KIE *pfubp1* deletions. This surprising result may provide a basis for further investigation into possible correlations among these mutations using additional K13 and *pfubp1* genotypes from different geographical isolates.

Interestingly, we found two types of deletions and two types of insertions in the multiple sequence alignment between the mutated isolates and wild-type isolates. The KYE insertion at bp 4576 to 4584 was also observed in isolates from Ghana, Kenya, and Burkina Faso and in a patient returning from Liberia to the United Kingdom (14, 15, 28). The KYDKYD insertion at bp 4555 to 4572 had not been observed previously, nor had either of the deletions (KID and KIE). The most common deletions and insertions in *pfubp1* in this study were lysine, asparagine, and glutamic acid residues. These resulted from deletions/insertions of the amino acids KID, KIE, KYE, and KYDKYD at positions ranging from 1519 to 1528. These deletions/insertions led to frameshifts that were caused by a deletion or insertion of either a thymidine or a guanosine nucleotide. Although the treatment outcomes of the patients harboring these isolates indicated that they were still sensitive to ACTs, more studies of the roles of these deletions and insertions in the relationship between phenotype and clinical outcome are needed to validate these results.

Conclusion. The present results showing molecular markers including *PfK13* and *pfubp1* in African isolates have implications for the development of ART with continuing use of ACTs. The findings of this study indicated the existence of limited *PfK13* polymorphisms among African isolates and suggested the adoption of other potential molecular markers of ART, such as *pfubp1*, to carry out molecular surveillance for clinical analysis of *P. falciparum* ART. Further research is under way to investigate and elucidate both *PfK13* and *pfubp1*, as well as other candidate molecular markers, and their roles in delayed parasite clearance in clinical trials.

MATERIALS AND METHODS

Study sites and samples. This study was conducted in Anhui and Shandong Provinces in eastern China, which were predominant centers of imported *P. falciparum* malaria. Anhui Province covers 105 counties with 70.6 million people and experienced a malaria resurgence in 2005–2008 that was due mainly to the accumulation of residual foci of *P. vivax* (29). Shandong Province has a long coastline

measuring 3,024.4 km. It contains 137 counties and has a population of 97.9 million. Economic trade overseas is frequent. The number of imported *P. falciparum* cases, especially those from Africa, has increased significantly in these two provinces, and 240 *P. falciparum* cases were reported in 2018, accounting for 13.6% of all *P. falciparum* cases nationwide (2).

A total of 153 *P. falciparum*-infected blood samples of travelers returning from Africa from 2017 to 2019 were collected and examined at enrollment, including 127 cases from Anhui and 26 cases from Shandong (Fig. 1). Approximately 100 μl of finger prick blood was spotted onto a piece of 3MM Whatman filter paper (GE Healthcare, Boston, MA, USA) and air dried. Each of the samples was labeled with a study number and stored at −4°C until extraction.

Individual epidemiological information was also collected from a Web-based reporting system (China Information System for Disease Control and Prevention) and analyzed in this study.

Treatment and follow-up. Patients were treated with dihydroartemisinin and piperaquine according to national antimalarial regulations, with a total adult dose of 2.5 mg dihydroartemisinin/kg of body weight and 20 mg/kg piperaquine for 3 days (30). The thick and thin blood smears of the patients who stayed in the hospital for treatment were collected on day 3. Giemsa-stained blood slides were prepared for the identification of *Plasmodium* to species level. Slides were examined and read by an expert microscopist certified as level 1 by the WHO. Slides were considered negative when no asexual parasites were found after 1,000 white blood cells (WBCs) had been counted.

Genetic polymorphisms. The *Plasmodium falciparum* genomic DNA from the approximately 10-μl blood samples collected was extracted with a QIAamp DNA blood kit (Qiagen, Valencia, CA) as described previously (4). Polymorphisms in the *PfK13* gene (PF3D7_1343700) were determined by nested PCR amplification of an 849-bp fragment (from amino acids 427 to 709) as described previously (14). Polymorphisms in *pfubp1* (PF3D7_0104300) were identified in the 300-bp region (from amino acids 1463 to 1563) by using a slightly revised PCR method (14). A DNA sample extracted from the 3D7 parasite strain was used as a positive control. The primers used for the *pfubp1* nested PCR were as follows: nest 1 forward primer, CGCCCGTACTATGAAGAAGATC; nest 1 reverse primer, GGCTTTTACCTGAACTGTTCAGG; nest 2 forward primer, CGTAAACAGAATATTCAGGATTGG; nest 2 reverse primer, CTAGCCCTTTATTATCA TTATCGT.

Amplification of the *pfubp1* polymorphisms was performed with 40 cycles of a 3-step PCR procedure with 30 s annealing at 52°C (nest 1) or 55°C (nest 2) and elongation at 72°C for 90 s. The PCR products were collected and sent for Sanger sequencing (Shanghai Bunan Biological 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 carried out using the MAFFT Web-based tool with Clustal Omega Sequence Alignment Editor (https://www.ebi.ac.uk/Tools/msa/clustalo/). Sequences with poor quality after three sequencing attempts were not included in the analysis. A map showing the study sites and the numbers of isolates was created by ArcGIS 10.1 (Environmental Systems Research Institute, Inc.). Version 4.0.2 of the R 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 constituent ratios of *pfubp1* and *PfK13* polymorphisms between Anhui and Shandong in 2017–2019. GraphPad Prism 8.3.0 (GraphPad Software Inc., San Diego, CA, USA) was used to plot the distribution of *pfubp1* and *PfK13* mutations.

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; no. 2019008).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We declare that we have no competing interests.

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