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Evaluation of Antimalarial Resistance Marker Polymorphism in Returned Migrant Workers in China

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Imported malaria has been a great challenge for public health in China due to decreased locally transmitted cases and frequent exchange worldwide. *Plasmodium falciparum* has been mainly responsible for the increasing impact. Currently, artesunate plus amodiaquine, one of the artemisinin combination therapies recommended by the World Health Organization, has been mainly used against uncomplicated *P. falciparum* malaria in China. However, drug resistance marker polymorphism in returning migrant workers has not been demonstrated. Here, we have evaluated the prevalence of *pfmdr1* and *pfcrt* polymorphisms, as well as the K13 propeller gene, a molecular marker of artemisinin resistance, in migrant workers returned from Ghana to Shanglin County, Guangxi Province, China, in 2013. A total of 118 blood samples were randomly selected and used for the assay. Mutations of the *pfmdr1* gene that covered codons 86, 184, 1034, and 1246 were found in 11 isolates. Mutations at codon N86Y (9.7%) were more frequent than at others, and $Y_{86}Y_{184}S_{1034}D_{1246}$ was the most prevalent (63.6%) of the four haplotypes. Mutations of the *pfcrt* gene that covered codons 74, 75, and 76 were observed in 17 isolates, and $M_{74}N_{75}T_{76}$ was common (70.6%) in three haplotypes. Eight different genotypes of the K13 propeller were first observed in 10 samples in China, 2 synonymous mutations (V487V and A627A) and 6 nonsynonymous mutations. C580Y was the most prevalent (2.7%) in all the samples. The data presented might be helpful for enrichment of molecular surveillance of antimalarial resistance and will be useful for developing and updating antimalarial guidance in China.

Malaria is the most important parasitic protozoan infection that poses serious threats to human health globally (1). China has had success in reducing the morbidity and mortality of malaria to low levels through continuous and large-scale interventions (2). Despite the large decreases in local cases, imported malaria has been a great challenge to public health due to frequent exchanges worldwide. *Plasmodium falciparum*, the deadly species, has been mainly responsible for the increasing impact (3). Imported malaria may pose high risks to malaria-free localities in which *Anopheles* mosquitoes are prevalent during the transmission season, and severe malaria is mainly caused by *P. falciparum* without timely diagnosis and effective treatment. In China, malaria cases significantly increased in 2013 ($n = 4{,}128$), mainly due to the large number of migrant workers who had returned from Ghana with *P. falciparum* infection ($n = 1{,}046$; 25.3%) reported in Shanglin County, Guangxi Province, China. In response to such an emergency, artesunate (AS) plus amodiaquine (AQ), one of the artemisinin combination therapies (ACTs) recommended by the World Health Organization (WHO) and China, was mainly used against uncomplicated *P. falciparum* infection (4).

AS plus AQ is one of the WHO-recommended ACTs to treat uncomplicated *P. falciparum* malaria worldwide (5). It was adopted as the first-line treatment in 2006 by WHO (6), and it was found that the combination of AS plus AQ is therapeutically superior to a combination of chloroquine (CQ) plus pyrimethamine-sulfadoxine (SP) and significantly reduced gametocyte carriage following treatment (7). The efficacy and tolerability of AS plus AQ has been tested formally in several clinical trials in different epidemiological settings in Africa (8–12). In China, AQ has been widely used as a monotherapy for nearly 30 years, and drug tolerance/resistance was first observed in Yunnan Province, China; the evidence showed that it also elicits cross-resistance against CQ and piperaquine (PQ) (13, 14). On the other hand, AQ was shown to have synergism in combination with AS in some regions of China (15). Because of this, AS plus AQ was adopted as one of the four ACTs against uncomplicated *P. falciparum* malaria in China. At present, no artemisinin resistance has been observed in China, but emergent resistance in the Greater Mekong Subregion (GMS) (Cambodia, Laos, Myanmar, Thailand, Vietnam, and Yunnan Province, China) poses a great challenge for the control and elimination of malaria in China (16, 17). In Africa, the emergence and spread of CQ, AQ, and antifolate antimalarial resistance has long been observed (18, 19), and a decreased response to AS plus AQ was also observed (12). Although it has yet to be established whether artemisinin resistance has spread westward, the spread of resistant parasites to sub-Saharan Africa would be disastrous (20). Due to the increasing importation of *P. falciparum* malaria from Africa recently and the fact that little was known about the current drug resistance, particularly among Chinese migrant workers, a study was urgently needed to provide some useful suggestions for rational administration. The aim of this study was to evaluate the drug resistance polymorphism of migrant workers

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in Shanglin County, Guangxi Province, which will be used as evidence for further molecular surveillance of drug resistance and also will be useful for developing and updating antimalarial guidance.

MATERIALS AND METHODS

Study design. Imported *P. falciparum* malaria has sharply increased in Shanglin County, mainly due to migrant workers who had returned from Ghana. To estimate the incidence of malaria and the prevalence of polymorphisms of drug resistance-related molecular markers in migrant workers due to be administered AS plus AQ (Guilin Pharmaceutical Co. Ltd., Guilin, Guangxi Autonomous Region, China), and in order to provide useful suggestions for future treatment of such clustered imported *P. falciparum* malaria, all individual case information was carefully reviewed and analyzed, and bioassays were performed by a random-sampling method.

Study sites. We conducted this study in Shanglin County, Guangxi Province, located in south China. Reported malaria cases significantly increased in 2013 in the county due to the clustered migrant workers who had returned from Ghana, with a sharp peak observed in June.

Samples for study. Blood samples from migrant workers with fever were screened for malaria, and 1,251 samples with malaria confirmed at enrollment in Shanglin County were labeled with study numbers, names, and dates and stored in sealed frozen tubes at −80°C until use. Since migrant workers who had returned from Ghana accounted for most (n = 1,046) of the total *P. falciparum* cases (n = 1,104), we randomly selected for PCR 118 samples of *P. falciparum* from the migrant workers who had returned from Ghana to evaluate the polymorphism.

Laboratory methods. DNA was isolated from selected blood samples with a QIAamp DNA minikit (Qiagen, Valencia, CA). Known *P. falciparum* polymorphisms were assessed at the following alleles: *pfmdr1* N86Y, Y184F, S1034C, N1042D, and D1246Y and *pfcrt* M74I, N75E, and K76T. Also, we investigated the mutation of the *PF3D7_1343700* kelch propeller domain (PF13_0238, also called the K13 propeller), a molecular marker of artemisinin resistance. Polymorphisms were evaluated using nested PCR, followed by restriction fragment length polymorphism (RFLP) analysis, as described previously (21, 22). Sequencing was carried out by Shanghai DNA BioTechnologies Co., Ltd. (Shanghai, China). Sequences were analyzed with the BLAST program (http://blast.ncbi.nlm.nih.gov/). Multiple nucleotide sequence alignments and analysis were performed using the BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Data analysis. Data were analyzed with Microsoft Excel and SAS version 9.2. The chi-square (χ^2) test or Fisher's exact test was used to assess differences. P values were calculated and were considered statistically significant at <0.05.

Ethical considerations. The study was reviewed and approved by the ethical committee of the Chinese Center for Disease Control and Prevention (China CDC).

RESULTS

Enrollment. A total of 1,251 migrant workers were diagnosed with malaria in 2013: 1,104 with *P. falciparum* infections, 107 with *Plasmodium vivax* infections, 21 with *Plasmodium ovale* infections, 9 with *Plasmodium malariae* infections, and 10 with mixed infections. Among the 1,046 migrant workers with *P. falciparum* infections who had returned from Ghana, 118 subjects were enrolled in the study, and blood samples were collected from them and analyzed (Fig. 1).

Epidemiologic profile of malaria in Guangxi, 2013. Reported cases increased sharply in 2013, particularly in June and July, due to the clustered migrant workers who had returned from Ghana. In total, 1,251 malaria cases were recorded in the province during the whole year in a Web-based reporting system, all of which were considered imported malaria, and this corresponded to a 468.6% increase from the 220 cases reported in 2012 ($P < 0.05$) (Fig. 2). A proportion (84.1%) of the total malaria cases and 87.0% of the total *P. falciparum* malaria cases were reported in Shanglin County, and most of them (98.2%; n = 1,033) were in workers who had returned from Ghana.

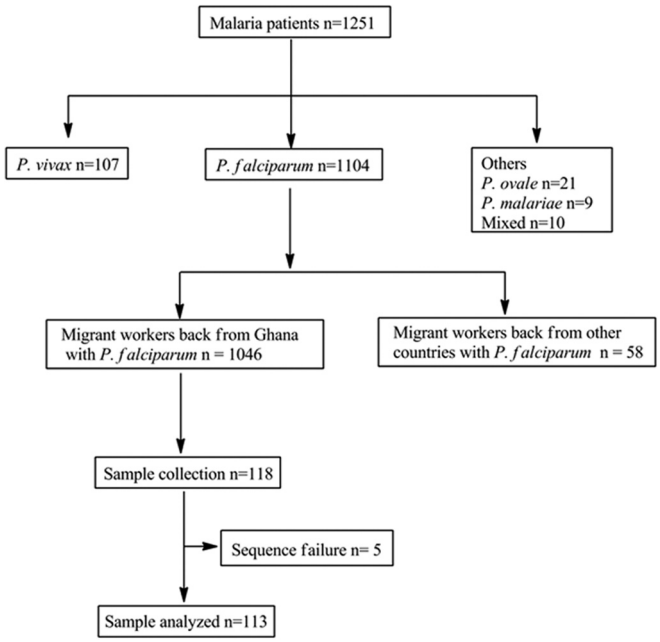

FIG 1 Screening, enrollment, and follow-up of subject patients.

Mutational analysis of *pfcrt* and *pfmdr1*. Of the 8 mutation types, no N1042D *pfmdr1* mutation was found, and it is not discussed further here. Mutations of the *pfmdr1* gene that covered codons 86, 184, 1034, and 1246 were sequenced successfully in 11 isolates. Mutations at codon N86Y (9.7%) was more frequent than the others (Table 1), and $Y_{86}Y_{184}S_{1034}D_{1246}$ was the most prevalent (63.6%) of the four reported haplotypes (Table 2). Mutations of the *pfcrt* gene that covered codons 74, 75, and 76 were found in 17 isolates. Three different *pfcrt* genotypes were found, among which $M_{74}N_{75}T_{76}$ was common (70.6%) (Table 2).

Gene polymorphism of the K13 propeller. Eight different genotypes were observed in 10 samples, including 2 synonymous mutations and 6 nonsynonymous mutations (Table 3). The synonymous mutations were V487V and A627A, with prevalences of 0.9% (n = 1) and 0.9% (n = 1), respectively. Of the 6 nonsynonymous mutations, C580Y was the most prevalent (2.7%; n = 3) and was found in all the samples. Furthermore, one patient was found to have a mixed $V_{487}L_{692}$ genotype.

DISCUSSION

ACT has been recommended as the first-line therapy to address the resistance of *P. falciparum* to monotherapies and to improve treatment outcomes. AS plus AQ, dihydroartemisinin (DHA) plus PQ, and the other two ACTs were the preferred antimalarials used against uncomplicated *P. falciparum* in China. However, widespread artemisinin resistance was observed in the GMS and resistance to CQ, AQ, and other antimalarials was observed in Africa (16, 23–25), which may represent a great challenge from resistant parasites imported into China with the migrant popula-

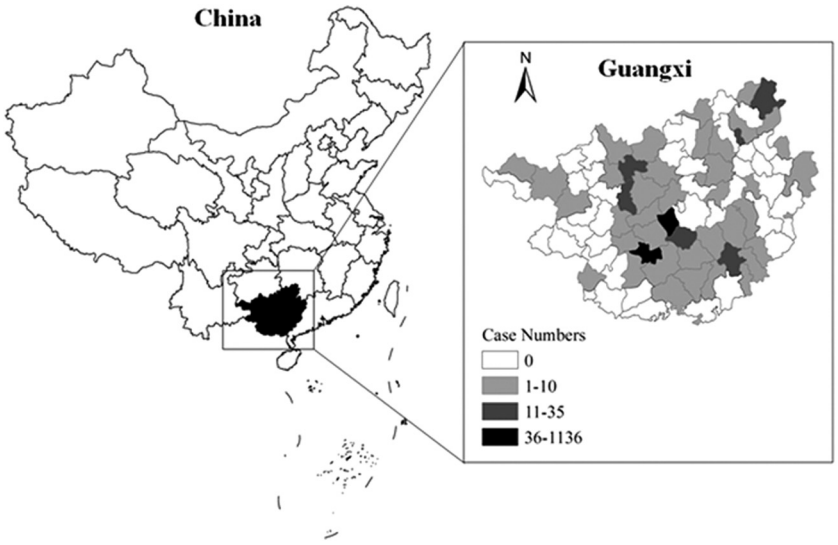

FIG 2 Malaria in Guangxi Province, China, in 2013. The map was created using ArcGIS 10.1 (Environmental Systems Research Institute, Inc.).

tion, immediately affecting therapy efficacy. Therefore, we designed this study to evaluate the drug resistance marker polymorphism and the prevalence of an artemisinin resistance marker (K13 propeller) in migrant workers in order to provide useful suggestions for rational administration in China.

Guangxi Province is located in south China and is classified as a "malaria unstable" region (26). Malaria incidence decreased to 0.47/100,000 in 2012, and no local *P. falciparum* malaria has been observed since 2003 (27, 28). However, due to the large number of migrant workers returning from Ghana, malaria cases increased sharply in 2013. This was because most Chinese migrant workers in Ghana were deported by the Ghana government, and most of them were from Shanglin County.

Both *pfmdr1* and *pfcrt* were selected for chloroquine resistance, and they also have been reported to be associated with *in vitro* responses to AS and AQ (12, 29). The results showed a high prevalence of *pfmdr1* N86Y, which was not frequently found along the China-Myanmar border (30). This could be explained by the fact that a high prevalence of the *pfmdr1* N86Y allele was seen in Ghana due to excess use of CQ (31), and it was also observed in other regions of Africa after extensive use of AS plus AQ (32, 33); also, most of the *pfmdr1* gene copy number amplifications in field samples harbor an asparagine at amino acid position 86 of the *pfmdr1* gene (34). Codons 184, 1034, and 1246 were also found in the studied samples, and this was similar to the results of Danquah et al. in Burkina Faso, which may also be due to differences in transmission intensity and drug usage (29). The $Y_{86}Y_{184}S_{1034}Y_{1246}$ haplotype was detected more frequently than others, also suggesting the widespread occurrence of the N86Y allele in migrant workers.

pfcrt K76T has been widely used as a reliable marker for CQ resistance, and it was found at a high prevalence in China (35). This was consistent with our study results showing that *pfcrt* K76T played the predominant role in the *pfcrt* genotypes. Molecular studies have shown that parasites transfected with *pfcrt* of the $S_{72}V_{73}M_{74}N_{75}T_{76}$ haplotype have decreased sensitivity to AQ and its active metabolite (36, 37), and this haplotype was detected in 19% of infected individuals in Tanzania after the increasing use of AQ in the region (38). However, two other haplotypes, $I_{74}N_{75}T_{76}$ and $M_{74}E_{75}K_{76}$, were also found in our study, suggesting that selection of other *pfcrt* haplotypes is still needed. Another factor that may contribute to the high prevalence of *pfcrt* K76T is the continued use of chloroquine as a first-line drug for *P. vivax* infection over several decades in Ghana, which indicates that the chloroquine pressure for the maintenance of the *pfcrt* mutation in *P. falciparum* is still present in the country (31).

TABLE 1 Selection of *P. falciparum* polymorphisms treated with artesunate plus amodiaquine

Gene	SNP[a]	Occurrence of mutation		
		n	%	95% CI[b]
pfmdr1 ($n = 11$)	N86Y	11	9.7	4.31–15.12
	Y184F	2	1.8	0.55–3.01
	S1034C	1	0.9	0.34–1.54
	N1042D	0	0	0
	D1246Y	2	1.8	0.49–3.10
pfcrt ($n = 17$)	M74I	1	0.9	0.27–1.51
	N75E	4	4.4	2.31–6.52
	K76T	13	11.5	6.05–16.87

[a] SNP, single-nucleotide polymorphism.
[b] CI, confidence interval.

TABLE 2 Prevalences of genotypes of candidate genes *pfmdr1* and *pfcrt*

Candidate gene	Genotype[a]	Prevalence (%) of mutation in selected samples
pfmdr1 ($n = 11$)	$\mathbf{Y}_{86}\mathbf{F}_{184}S_{1034}D_{1246}$ ($n = 2$)	18.2
	$\mathbf{Y}_{86}Y_{184}S_{1034}D_{1246}$ ($n = 7$)	63.6
	$\mathbf{Y}_{86}Y_{184}\mathbf{C}_{1034}\mathbf{Y}_{1246}$ ($n = 1$)	9.1
	$\mathbf{Y}_{86}Y_{184}S_{1034}\mathbf{Y}_{1246}$ ($n = 1$)	9.1
pfcrt ($n = 17$)	$\mathbf{I}_{74}N_{75}\mathbf{T}_{76}$ ($n = 1$)	5.9
	$M_{74}N_{75}\mathbf{T}_{76}$ ($n = 12$)	70.6
	$M_{74}\mathbf{E}_{75}K_{76}$ ($n = 4$)	23.5

[a] The mutated amino acids are in boldface.

TABLE 3 Polymorphisms observed in the K13 propeller in *P. falciparum* isolates[a]

Codon position	Amino acid reference	Nucleotide reference	Amino acid mutation	Nucleotide mutation[d]	Prevalence of mutation (%)
487[b,c]	V	GTA	V	gt**G**	0.9 ($n = 1$)
539	R	AGA	T	a**C**a	0.9 ($n = 1$)
575[c]	R	AGA	T	a**C**a	1.8 ($n = 2$)
580	C	TGT	Y	t**T**t	2.7 ($n = 3$)
580[c]	C	TGT	F	t**A**t	0.9 ($n = 1$)
584	D	GAT	V	g**T**t	0.9 ($n = 1$)
627[b,c]	A	GCT	A	gc**A**	0.9 ($n = 1$)
692[c]	V	GTT	L	**C**tt	0.9 ($n = 1$)

[a] Cases were all imported from Ghana.
[b] Synonymous mutation.
[c] Mutated site not previously reported.
[d] Mutations are in boldface.

Artemisinin and its derivatives have been used for falciparum malaria treatment in China since the late 1970s (39). *In vitro* assays have shown that the susceptibility of *P. falciparum* to artemisinins is declining in China, but no evidence has been detected for artemisinin resistance (40). Here, we have also investigated the prevalence of K13 propeller gene polymorphism in migrant workers to understand the status of artemisinin resistance. Two synonymous mutations, V487V and A627A, were observed, with prevalences of 0.9% ($n = 1$), and neither had been reported before. Six nonsynonymous mutations were also found, and three of them were unreported. Also, the results showed that C580Y was the predominant allele (2.7%; $n = 3$); it was identified in slow-clearing parasites in malaria patients treated with artemisinin, which was supported by the work of Ariey et al., who frequently detected it from 2001 and 2002 to 2011 and 2012 in Pailin and Battambang in Cambodia (41). The M476I allele, which can significantly increase resistance to artemisinin, was not found in this study. This indicated that the mutated K13 propeller gene alleles exist in migrant workers returning from Ghana, which may raise concerns about the emergence of artemisinin resistance in Africa, and the findings support further clinical trials associated with K13 propeller mutations, which will be useful to identify additional genetic loci involved in monitoring the emergence of artemisinin resistance.

According to the antimalarial strategy of China, piperaquine phosphate is the recommended antimalarial chemoprophylaxis to be used in the area of *P. falciparum* endemicity. However, Chinese travelers often take ACTs to treat malaria in Africa. Despite the fact that AS plus AQ was introduced in Ghana as the first-line drug for treatment of uncomplicated malaria in 2004 (42), no evidence has shown that antimalarial resistance has occurred due to drug abuse. Therefore, more related research should be carried out to determine whether mutation of the K13 propeller has possibly occurred in the infecting *Plasmodium* species or if it has appeared in the general population in the GMS.

The prevalence of the K13 propeller gene polymorphism detected in migrant workers in China revealed that the use of antimalarials should be based on the resistance status in the importing country and that rational use of antimalarials against the *Plasmodium* species imported from Africa and Southeast Asia should be adopted. In addition, routine monitoring and surveillance, as recommended by the WHO global plan for artemisinin resistance containment, should be continuously strengthened. It is also necessary to carry out additional clinical investigations to complement sentinel surveillance, including analysis either of drug markers or risk factors or of new approaches to monitor resistance.

In conclusion, high prevalences of *pfmdr1*N86Y and D1246Y and *pfcrt* K76T alleles were observed in migrant workers who had returned from Ghana to Shanglin County, Guangxi Province. In addition, we have reported for the first time 8 K13 propeller gene polymorphisms, 2 synonymous mutations and 6 nonsynonymous mutations, among which the C580Y allele has the predominant role. The present data might be helpful for enrichment of molecular surveillance of antimalarial resistance and will be useful for developing and updating the antimalarial guidance in China.

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We declare that we have no competing interests.

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