Single-Nucleotide Polymorphisms of Artemisinin Resistance-Related *Pfubp1* and *Pfap2mu* Genes in *Plasmodium Falciparum*, Central China

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Abstract

Background

Molecular markers for monitoring resistance could help improve malaria treatment policies. Delayed clearance of *Plasmodium falciparum* by Artemisinin-based Combination Therapies (ACTs) has been reported in several countries. In addition to the *PfKelch13* (*pfk13*), new drug resistance genes, the ubiquitin-specific protease 1 (*pfubp1*) and the eadaptor protein complex 2 mu subunit (*pfap2mu*) have been identified as being linked to ACTs. This study investigated the prevalence of single-nucleotide polymorphisms (SNPs) in clinical *Plasmodium falciparum* isolates *pfubp1* and *pfap2mu* imported from Africa and Southeast Asia (SEA) to Wuhan, China, to provide baseline data for antimalarial resistance monitoring in this region.

Methods

Peripheral Blood samples were collected in Wuhan, China, from August 2011 to December 2019. The SNPs of *Pfubp1* and *pfap2mu* of *P. falciparum* were determined by nested PCR and Sanger sequencing.

Results

In total, 296 samples were collected. Subsequently, 92.23% (273/296) were successfully amplified and sequenced for the *Pfubp1*. There were 60.07% (164/273) wild strains and 39.93% (109/273) mutant strains. For the *pfap2mu* gene, it was divided into three fragments for amplification, 82.77% (245/296), 90.20% (267/296) and 94.59% (280/296) were sequenced successfully respectively. Genotypes reportedly associated with ACTs resistance detected in this study included *pfubp1* D1525E as well as E1528D and *pfap2mu* S160N. The mutation prevalence rates were 10.99% (30/273), 13.19% (36/273) and 11.24% (30/267), respectively.

Conclusions

The existence of mutation sites of known clearance genes detected in the isolates in this study, including D1525E and E1528D in the *pfubp1* gene, and S160N in the *pfap2mu* gene, further proved the risk of ACTs resistance. Constant vigilance is therefore needed to protect the effectiveness of ACTs, and to prevent the spread of drug-resistant *P. falciparum*. Further studies in malaria-endemic countries are needed to further validate potential genetic markers for monitoring parasite populations in Africa and SEA.

1. Background

As one of the three major global public health problems, malaria has seriously endangered human health and affected social and economic development. Of these, *Plasmodium falciparum* is the most common, and also the cause of utmost severe malaria and deaths worldwide. According to the World Health Organization (WHO), an estimated 229 million malaria cases occurred in 2019, with 94% of them occurring in Africa. Additionally, there were approximately 409,000 deaths from malaria globally [1]. In

2020, after four consecutive years of reporting zero indigenous malaria cases, in China. And it was recently certified malaria elimination by the WHO [2]. Although China has made substantial progress in the fight against malaria, significant challenges remain. The risk of cross-border transmission of imported malaria increases with the rise in global travel of people. In particular, imported cases from Africa and Southeast Asia (SEA) [3]. Enhancing malaria surveillance has been emphasized by WHO as a core intervention to accelerate process towards eradication [4].

Malaria control was temporarily relieved with the discovery of Artemisinin (ART) in the 1970s. In an attempt to prevent the emergence of resistance, Artemisinin-based Combination Therapies (ACTs) is recommended by the WHO as first-line treatment for uncomplicated falciparum malaria [5]. If the effectiveness of ACTs against *P. falciparum* is not sustained, the emergence of drug-resistant parasites is a major threat to global malaria control and elimination efforts. Evidence first appeared near Cambodia border, where delayed clearance of the parasite was detected [6–9]. Others believe that this is a sign of ART resistance [7], and by others as “treatment failure” [10]. Despite the recent reports, ART-resistant parasites have been confirmed in Africa [11, 12]. Still now, the emergence and development of resistance of *P. falciparum* to ACTs has become a grave concern. The *P. falciparum* has developed resistance to ACTs and urgent action is needed to prevent the spread of drug-resistant parasites throughout Africa and SEA.

Molecular markers of antimalarial drug resistance have long been used to monitor the emergence and spread of antimalarial drug resistance in malaria endemic areas. The level of parasite drug sensitivity has been linked to the single-nucleotide polymorphisms (SNPs) or haplotypes of the genes, and these markers are relevant to the study of the efficacy of antimalarial drugs [13]. Mutations in the *PfKelch13* (*pfk13*) still considered to be important determinants of ART resistance [8]. Recently, the *pfk13* mutations have been identified as molecular markers of partial ART resistance. However, it doesn't account for all ART resistance [14]. Other possible genetic markers of ART resistance have been identified by genome-wide sequencing of drug- pressured mutants of the rodent malaria parasite *P. chabaudi* [14]. The new candidates of ubiquitin-specific protease 1 (*pfubp1*) and eadaptor protein complex 2 mu subunit (*pfap2mu*), both proved to be related to ART resistance and had polymorphic homologs in *P. falciparum* [14, 15]. Study reported that in cases of ART treatment recrudescence, no mutations in *pfk13* were observed, but mutations in *pfubp1* and *pfap2mu* were detected [16]. The *pfubp1* gene mutations is at codon 1525 and 1528, which change from aspartate to glutamate (D1525E) and glutamate to aspartate (E1528D), respectively. The mutation of *pfap2mu* gene is at codon 160, which changes from serine to aspartic acid or threonine (S160N/T) [13]. Until now, the role of these two genes in ACTs is not clearly understood. Therefore, more study is needed to verify these polymorphisms and their role in antimalarial resistance.

In the present study, polymorphisms of *pfubp1* and *pfap2mu* for *P. falciparum* isolates imported from Africa and SEA in Wuhan, China were surveyed. This survey will offer valuable information for rational medication for malaria patients in clinical practice, preventing the spread of drug-resistant *P. falciparum* in Africa, SEA and China.

2. Materials And Methods

2.1 Collection of samples

A cross-sectional study was performed in Wuhan, Hubei Province, China. From August 2011 to December 2019, peripheral blood 3 ml of returned migrant workers from African countries was collected at major hospitals in the region. Follow the previous steps for the next step [17]. Before inclusion, these filter papers were numbered and stored at -20°C with in polyethylene bag. Written Informed consent was obtained from each participant and parent or legal guardian(s) legal guardian.

2.2 Determination of *P. falciparum* gene mutations

Parasite DNA was extracted from pre-treatment blood-spots samples on filter paper using the TIANamp Blood Spots DNA Kit as per the manufacturer's protocol. Approximately 50 µl supernatant containing genomic DNA (gDNA) was obtained and stored at -20°C. The target fragments of the *pfubp1* and *pfap2mu* genes were amplified from the gDNA sample via nested PCR, following a previously published protocol [18] with minor modifications. the *pfubp1* and *pfap2mu* genes were successfully amplified. A gDNA sample extracted from the 3D7 parasite strain was used as a positive control. The primer sequences of the two genes are shown in Table S1. The reaction system and procedure for PCR are listed in Table S2. After all reactions, 3.0 µl PCR products were analyzed by agarose gel (1.5%) electrophoresis. The remaining PCR were purified for Sanger sequencing (Genewiz, Soochow, China).

2.3 Data Analysis

DNA sequences were analyzed using DNASTar software (DNASTAR Inc., Madison, WI, USA), and SNPs in *pfubp1* and *pfap2mu* were detected using PF3D7_1218300 and PF3D7_0104300 (PlasmoDB: <http://plasmodb.org/plasmo/>) as a reference sequence. Data were recorded using Excel software (Microsoft Excel; version 2016) and the prevalence of individual SNPs at each site was determined. Graphing was done using GraphPad Prism 5 (GraphPad Software, Inc). And statistical analysis was SPSS 25 (SPSS Inc., Chicago, IL, USA). *P*-value < 0.05 was considered significant.

3. Results

3.1 General information

A total of 296 returnees from Africa and SEA who were diagnosed with *P. falciparum* and treated with ACTs were collected from 2011 to 2019 (Table S3). Including 122 cases in West Africa (WA), 78 cases in Central Africa (CA), 59 cases in South Africa (SA), 26 cases in East Africa (EA), 6 cases in North Africa (NA), and 5 cases in SEA. These cases were mainly from 29 countries in African and SEA, mainly concentrated in Congo (14.86%, 44/296), Nigeria (14.53%, 43/296), followed by Angola (11.15%, 33/296), Liberia (7.43%, 22/296), and Mozambique (4.72%, 14/296).

3.2 Mutation prevalence of *pfubp1* gene

For *pfubp1* investigation and evaluation, a 304-bp region was amplified and sequenced. A total of 296 samples were sequenced through quality assurance analysis, of which 23 were of low quality. Of the 273 samples successfully sequenced, these cases were mainly from Congo, Nigeria and Angola, accounting for 14.65% (40/273), 13.55% (37/273) and 12.09% (33/273), respectively (Table S3). The proportion of isolates with no mutations in the *pfubp1* gene, that is wild type sequence as the 3D7 strain, was 60.07% (164/273). A total of 109 isolates (39.93%, 109/273) were found to harbour 10 different SNPs. There were 8 non-synonymous mutations including D1510N, E1518Y, E1519D, E1519K, E1519V, D1525E, E1528D and E1528G. And 2 synonymous mutations were N1515N and N1518N. Among, the unreported loci were D1510N and N1515N. Most isolates had more than one mutation in the *pfubp1* gene (Fig. 1A).

This survey found that the main epidemic mutation sites were D1525E and E1528D. For the D1525E locus, the frequencies of wild type, mutant, and mixed types were 83.15% (227/273), 10.99% (30/273) and 5.86% (16/273), respectively. The prevalence of mutation in WA, CA, SA, EA, NA, and SEA were 16.07%, 22.86%, 12.28%, 8.33%, 40.00% and 20.00% respectively (Table 1). These mutations were also mainly focused in Congo (20%, 6/30), Nigeria (13.33%, 4/30), (10%, 3/30) each in Liberia and Equatorial Guinea. In addition, one case was found in SEA (Fig. 2A). For the D1525E, Among the WA, CA, and SA regions. After statistical analysis, but it was not statistically significant ($P = 0.068$). For the period from 2011 to 2019 (Table 2), the annual trend results showed that the mutation rate of D1525E was 28.57% in 2011 and 38.38% in 2012. By 2013 and 2014, it had decreased to 10.26% and 8.82% respectively. Then the mutation rate rose sharply to 24.24% in 2015 and 18.60% in 2016. By 2017 it had fallen to 0.00%. In 2018, it began to increase from 6.67–17.02% in 2019. Due to the extremely low number of samples in 2011 and 2017, they were excluded. After trend analysis was performed for the remaining samples, there was no statistical significance ($F = 0.895$, $P = 0.388$).

For the E1528D locus, the frequencies of wild type, mutant type, and mixed types were 79.12% (216/273), 13.19% (36/273) and 7.69% (21/273), respectively. The prevalence of mutation in WA, CA, SA, EA, NA, and SEA were 29.46%, 14.29%, 14.04%, 12.50%, 40.00%, and 20.00%, respectively (Table 1). For the E1528D, among the WA, CA, and SA regions. After statistical analysis, which was statistically significant ($P = 0.016$). And comparison between regions, WA and CA also showed differences ($P = 0.042$). These mutations were also mainly focused in Liberia (22.22%, 8/36), (13.89%, 5/36) each in Nigeria and Congo, and Sierra Leone (8.33%, 3/36). Similarly, one case has been found in SEA (Fig. 2B). From 2011 to 2019 (Table 2), the annual trend results showed that the mutation rate of E1528D was 28.57% in 2011 and 31.25% in 2012. By 2013 and 2014, it had decreased to 20.51% and 23.53% respectively. By 2015, the mutation rate was 27.27%. Between 2016 and 2017, it dropped again to 16.28% and 12.50%. It rose to 20.00% in 2018, and then fell to 12.77% in 2019. For trend analysis, which was statistically significant ($F = 7.708$, $P = 0.039$).

3.3 Mutation prevalence of *pfap2mu* gene

In order to investigate and evaluate *pfap2mu*, which divided into three regions for amplification and sequencing. A total of 296 samples were amplified and sequence. The sizes are 841 bp, 578 bp, and 753 bp, respectively. The quality assurance analysis determined that the three fragments of the *pfap2mu* gene failed to be sequenced in 29 cases, 51 cases and 16 cases respectively. A total of 32 different SNPs were found to harbour in the samples successfully sequenced. There were 20 non-synonymous mutations including V127L, R146K, Q149H, S160N, N162I, N184H, V196I, K199T, N200Y, A236T, N239K, G315S, A337S, L363H, Y407H, S476A, I478F, L579F, S594F and *595Q (*, the stop codon). And 12 synonymous mutations including Y6Y, I100I, E163E, K186K, R188R, K199K, T287T, L442L, T443T, S476S, N533N and D548D. Among the unreported loci, non-synonymous mutations including V127L, R146K, N184H, V196I, A236T, N239K, G315S, L363H, Y407H, L579F, S594F and *595Q. And synonymous mutations including Y6Y, I100I, K186K, T287T, L442L, T443T, N533N and D548D. Most isolates had more than one mutation in the *pfap2mu* gene (Fig. 1B).

Among them, Y6Y to I100I were detected in the first segment. According to sequencing quality assurance determined, 17.23% (51/296) were sequencing failures. The successful sequencing rate was 82.77% (245/296). Most cases were concentrated in Congo 13.47% (33/245), Nigeria 13.06% (32/245) and Angola 12.24% (30/245). The prevalence of I100I mutation was 4.49% (11/245) and 1.22% (3/245) of mixed type. Mutation cases were mainly focused on Congo 36.36 (4/11) and Mozambique 18.18% (2/11). Only one mutant of Y6V was detected in Ivory Coast. And one case mixed type of V127L occurred in South Sudan (Table S3).

The second amplified fragment detected sites from R146K to V196I. According to sequencing quality assurance determined, 9.79% (29/296) were sequencing failures. The successful sequencing rate was 90.20% (267/296) (Table S3). Most cases were concentrated in Congo 14.98% (40/267), Nigeria 14.61% (39/267) and Angola 11.99% (32/267). The most prevalent mutation was S160N, E163E, R188R, and K199T in these isolates. The prevalence of S160N mutation was 11.24% (30/267) and 1.87% (5/267) of mixed type. Mutations were mainly concentrated in Angola and Congo with 5 cases each, and Cameroon and Equatorial Guinea with 4 cases each (Fig. 3). The mutation prevalence of E163E, R188R, and K199T were 6.37% (17/267), 2.25% (6/267) and 5.24% (14/267), respectively. The R188R mutations were mainly concentrated in Angola and Congo, and the K199T mutations were mainly concentrated in Angola, Congo and Zambia (Table S3).

The third fragment examined sites from Y407H to *595Q. A total of 296 samples were sequenced and 16 were of low quality as determined by quality assurance analysis. Among the 280 samples successfully sequenced, these cases were mainly from Congo, Nigeria and Angola, accounting for 15.36% (43/280), 13.93% (39/280) and 11.79% (33/280), respectively (Table S3).

For known delayed clearance locus S160N. The prevalence of S160N mutation was 11.24% (30/267). The prevalence of mutation in WA, CA, SA, EA, NA, and SEA were 9.26 %, 19.72%, 25.00%, 10.53%, 13.64% and 20.00%, respectively (Table 1). For the S160N, among the WA, CA, and SA regions. After statistical analysis, but it was not statistically significant ($P = 0.102$). Mutations were mainly concentrated in Angola

and Congo with 5 cases each, and Cameroon and Equatorial Guinea with 4 cases each (Fig. 3). From 2011 to 2019, according to the annual trend results of S160N (Table 2). During 2011, the mutation rate was 50%. In 2012, 2013 and 2014, the values dropped sharply to 19.35%, 8.33% and 6.06% respectively. Then it rose to 20.00% and 18.00% during 2015 and 2016, and from 2017 to 2019 it began to decline gradually again, to 14.90%, 9.38% and 7.84% respectively. For trend analysis, which was no statistically significant ($F = 0.327$, $P = 0.592$).

3.4 Prevalence of the *pfubp1* and *pfap2mu* haplotypes

The results showed a number of SNPs that were being inherited together on the gene as haplotypes in some of the isolates. However, the prevalence of the haplotypes was low (Table 3). Eighteen different haplotypes were observed: two for *pfubp1* and sixteen for *pfap2mu*. The most prevalent haplotype for the *pfubp1* gene was D1525E-E1528D, which was observed in 0.73% (2/273) of the isolates with mutations. They were distributed in Congo and Liberia. The other haplotypes were all present singly.

4. Discussion

Over the past several decades, the emergence and rapid spread of resistance to *P. falciparum* has become a major contributor to the global malaria burden [19]. Up to now, there is no indigenous malaria cases reported in China [20]. It is worth noting that malaria endemic areas including Africa and SEA are the main source of imported malaria in China including Wuhan [21, 22]. With the widespread use of ACTs in malaria-endemic areas such as Africa and SEA, resistance to ART has begun to emerge [6, 7, 11, 12, 21]. A molecular marker is being sought for early detection of malaria parasite resistance to ART. The use of molecular markers to track and determine the early development of drug resistance in *P. falciparum* is a powerful tool that can be implemented in any region.

For *pfubp1* gene, most isolates 60.07% observed were wild type without mutation. Mainly concentrated in Nigeria, Congo and Angola, with the proportions of 15.85%, 14.63% and 14.02%, respectively. In a study of Ethiopian and Tanzanian, the wild type isolates of the *pfubp1* gene were 92.8% and 30.9%, respectively [23]. In a survey, the wild type prevalence was 41.5% in Kenya [24]. These results are generally consistent with the findings of this study, and all of them have a high prevalence rate of wild type. However, a study in Ghana detected a low wild type prevalence of 11.1% for the *pfubp1* gene [13]. For those isolates that were mutated, some had more than one gene mutation. And most of them are non-synonymous mutations. Among the known delayed clearance genotypes, the wild type of D1525E and E1528D were 83.15% and 79.12%, respectively. Their mutation prevalence were 10.99% and 13.19%. In another study of imported malaria in Africa, mutations in the *pfubp1* gene D1525E and E1528D were 2.6% and 6.5% [25]. In particular, the mutation of D1528E found in isolates from Ghana, Kenya and Tanzania were 7.4%, 17.1% and 10.5%, respectively [13, 23, 24]. It is basically consistent with the survey in this research. There are no reports of D1525E and E1528D being found in SEA. However, mutations of D1525E and E1528D have also been found in SEA. Based on the changes in mutation rates of D1525E and E1528D between 2011 and 2019, they were 17.02% and 12.77%, respectively. And when the mutation rate of *pfk13* was

monitored in previous studies from 2017 to 2019 [21], no mutations were been observed. The mutation rate suggests that ART should continue to consider this strategy for treating *P. falciparum* in Africa, SEA, and China. However, the presence of the mutation rate advises that new ART resistance genes may have been widely involved in multiple regions and need to be further studied. Therefore, it is urgent to continue and further investigate the *pfubp1* gene. In particular, sites associated with known delayed clearance in ART resistance.

For the *pfap2mu* gene, the proportion of wild type isolates were 93.47%, 61.81% and 91.07%, respectively. Most of them are concentrated in Nigeria, Congo and Angola. According to a report from Ghana, during 2015–2016, the wild type isolates of the *pfap2mu* gene was 11.1% [13]. For those isolates that were mutated, some had more than one gene mutation. And most of them are non-synonymous mutations. Among the known delayed clearance genotypes, the wild type of S160N was 86.89%. They mutation prevalence were 11.24%. According to the change in mutation rate of S160N from 2011 to 2019, it has now dropped to 7.84%. It displays that ART medication is still in a safe state. But it is not to be taken lightly. In a survey in Kenya [26], the prevalence of S160N/T of the *pfap2mu* was 70.21%, and the mutation was 29%. In addition, S160N was found in isolates from a UK patient from Angola who had failed artemether-lumifantrine (AL) treatment, so the use of AL could enhance the role of the mutation in recurrent parasitic disease [16]. There are no reports of S160N being found in SEA. However, mutations of S160N have also been found in SEA. Notably, insertions/deletions (indels) identified of the *pfap2mu* gene was detected in Ghana, Kenya, and UK patients from Liberia and Uganda [13, 16, 26]. But this was not found in the present study, which may be due to regional and sample differences. In most cases, ACTs are still effective against *P. falciparum* of African and SEA origin, but the cases presented here suggest that this favorable situation may worsen in the future. The presence of molecular markers associated with drug resistance is important for their role in regulating drug sensitivity and subsequent dynamic prediction of drug resistance [21, 22]. ART resistance to the global spread of malaria treatment will be a serious blow. Therefore, an in-depth understanding of drug action and drug resistance mechanism is crucial for early detection of drug resistance.

Several limitations of the current study are worth noting. First, the sample size in 2011 and 2017 was not large for statistical analysis due to irresistible reasons. Second, for samples that failed in amplification and sequencing, leading to failure in SNPs analysis. Advanced gene-editing tools, especially CRISPR/Cas9 technique or CRISPR/Cas12a technique [27, 28], should be considered for further research. CRISPR/Cas9 technology can quickly locate key ART-related sites in targeted genes. The artificial introduction of CRISPR/Cas9 mutations can effectively shorten the process of finding drug-resistant sites compared to natural mutations under long-term drug pressure. It will provide a useful tool for finding new mutations in drug-resistant genes. The CRISPR/Cas12a technology takes advantage of Cas12a enzyme to perform specific cleavage, which can accurately and efficiently distinguish genotypes for any SNP sites.

5. Conclusions

Overall, this study investigated the frequency and spatial distribution of mutations associated with ART resistance in the *pfubp1* and *pfap2mu* genes from imported *P. falciparum* isolates in Wuhan, China. The results of this study provide valuable information for potential molecular markers of ART resistance. This thus highlights the possibility of endemic parasites in use that are less sensitive to ACTs. Therefore, constant vigilance is therefore needed to protect the effectiveness of ACTs. Further studies in malaria-endemic countries are needed to further validate potential genetic markers for monitoring parasite populations in Africa and SEA.

Declarations

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WJC and JL conceived and designed the study. KW coordinated the field collections of patient isolates. KW carried out a microscopic examination. WJC, KW and XNS performed the experiments. WJC and WW analysed the data. WJC, WXD and JL wrote the paper. All the authors read and approved the final manuscript.

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Availability of data and material

The datasets analysed in this study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable

Ethics approval and consent to participate

The current study was approved by the ethics committees of the Hubei University of Medicine and Wuhan City Center for Disease Prevention and Control Ethics Committee. Informed consent was obtained from all participated individuals.

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Tables

Due to technical limitations, table 1 to 3 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Distribution of mutant isolates with known clearance delay of S160N pfap2mu gene in different countries. * NO. is the number of mutations

Supplementary Files

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