Increased in the Prevalence of *Plasmodium Falciparum* With *Kelch13* C580Y Mutations and the Decline in *pfprt* and *pfmdr1* Mutant Alleles in Papua New Guinea

Naoko Yoshida

Juntendo University: Juntendo Daigaku

Masato Yamauchi

Juntendo University

Ryosuke Morikawa

Juntendo University

Francis Hombhanje

Divine Word University

Toshihiro Mita (✉ tmita@juntendo.ac.jp)

Juntendo university <https://orcid.org/0000-0001-8180-2344>

Research

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Abstract

Background

The C580Y mutation in *Plasmodium falciparum kelch13* (*pfk13*) is the most commonly observed variant in artemisinin-resistant isolates in the Greater Mekong Subregion (GMS). Until 2017, it had not been identified outside GMS, except for Guyana. In 2017, we identified three parasites carrying the C580Y mutation in Papua New Guinea (PNG). As the C580Y allele rapidly spread in the GMS, there is concern that this mutant is now spreading in PNG.

Methods

In 2020, we conducted a cross-sectional survey at two clinics in Wewak, PNG. Symptomatic patients infected with *P. falciparum* were treated with artemether plus lumefantrine following a national treatment policy. Blood samples were obtained before treatment, and polymorphisms in *pfk13*, *pfcr1*, and *pfmdr1* were determined. Parasite positivity was examined on day 3.

Results

A total of 94 patients were included in this analysis. The prevalence of C580Y was significantly increased (2.2% in 2017, 5.7% in 2018, and 6.4% in 2020; $p = 4.2 \times 10^{-3}$). A significant upward trend of wild-type prevalence was found for *pfcr1* (1.9% in 2016 to 46.7% in 2020; $p = 8.9 \times 10^{-16}$) and *pfmdr1* (59.5% in 2016 to 91.4% in 2020; $p = 2.3 \times 10^{-6}$). Among 26 patients, including three with C580Y infections successfully followed on day 3, none showed positive parasitemia.

Conclusions

Under the conditions of significant increases in *pfcr1* K76 and *pfmdr1* N86 alleles in PNG, the increase in *pfk13* C580Y mutants may be a warning indicator of the emergence of parasites resistant to the currently used first-line treatment regimen of artemether plus lumefantrine. Therefore, nationwide surveillance of molecular markers for drug resistance and assessment of its therapeutic effect, are important.

Background

Artemisinin (ART)-based combination therapy (ACT) is a widely used first-line treatment for uncomplicated malaria. Malaria deaths have markedly decreased since the introduction of the treatment in the early 2000s [1]. However, the emergence of ART-resistant *P. falciparum* was first reported in the Greater Mekong Subregion (GMS) in 2006 [2–4]. Since then, ART-resistant parasites have rapidly spread in the region, partly because of the emergence of resistance to partner drug(s) of ACT [5, 6]. Therefore, the emergence and spread of RT-resistant parasites outside the GMS has become a global concern.

Propeller polymorphisms of the *pfk13* gene are useful molecular markers to monitor the emergence and spread of ART resistance [4, 7]. To date, ten or more non-synonymous mutations in *pfk13* have been

validated as polymorphisms for ART resistance. These include N458Y, Y493H, R539T, I543T, R561H, and C580Y [8]. In particular, C580Y has gradually outcompeted the other mutations and become dominant in some parts of the GMR region (GMSR) [5, 9, 10]. C580Y is considered the most useful molecular marker to trace the spread of ART resistance in GMS. However, outside the GMSR, this mutation has been detected only in Guyana [11, 12] and, as we reported more recently, in Papua New Guinea (PNG) [13].

In PNG, chloroquine plus sulfadoxine/pyrimethamine had been adopted as the official first-line treatment regimen for uncomplicated malaria by 2010. This therapy was subsequently replaced with artemether plus lumefantrine (AL). In 2017, we identified three *P. falciparum* parasites harboring C580Y in Wewak, East Sepik. Population-genetic analysis using whole-genome and haplotypes of *pfk13* flanking microsatellite markers suggested that the C580Y in PNG did not migrate from Southeast Asia. Rather, it had independently emerged from another region in New Guinea [13]. Considering the aggressive increase in the C580Y harboring parasites in GMSR, there is a growing concern that a similar phenomenon may occur in PNG. Therefore, it is essential to assess whether parasites harboring C580Y have increased in the parasite population in PNG.

In addition to the issue of ART resistance, our previous ex vivo drug study also found that most *P. falciparum* parasites were resistant to chloroquine despite the discontinuation of chloroquine use in the early 2010s [14]. This is contrary to the observations in many African countries where chloroquine susceptibility recovered years after discontinuation [15–28].

To evaluate whether levels of ART-resistant molecular markers have increased since the first emergence and whether chloroquine resistance persists, a molecular epidemiological study was performed in 2020 in Wewak, East Sepik. Ex vivo drug susceptibility studies had previously been conducted there in 2002, 2003, and 2016–2018 [13, 14, 29–31]. The results we describe show a significant increase in C580Y prevalence and the potential recovery of chloroquine susceptibility.

Methods

Study design and site

This study was conducted at two clinics (Wirui Urban and Town) in January and February 2020 in Wewak District, East Sepik Province, PNG [14]. The study area comprises a lowland swamp along the coast. High transmission rates of malaria occur throughout the year, with seasonal fluctuations [32]. All four *Plasmodium* species for human malaria were observed in this region, with *P. falciparum* predominant.

Ethical approval for the study was obtained from the Medical Research Ethical Committee of Juntendo University (No. 2017070) and the Medical Research Advisory Committee of the PNG National Department of Health (MRAC No.16.41).

Patients and blood collection

In both clinics, patients with suspected malarial symptoms were screened using the Rapid Diagnosis Test (RDT) (Carestart™ Malaria HRP2/pLDH COMBO, Access Bio Inc., NJ, USA). Patients > 1 year of age with *Plasmodium*-positive results were recruited for the study and were enrolled after obtaining informed consent from the patients or their guardians. Blood samples (100 µl) were obtained by finger prick and transferred onto ET31CHR chromatography filter paper (Whatman Limited, Kent, UK). After drying at room temperature, the samples were separated in a plastic bag and stored at –20°C. Thick and thin blood smears were prepared and stained with 2% Giemsa for 45 min for parasite counting. All *P. falciparum* positive patients were treated with the AL regimen according to national guidelines and were asked to visit the clinics to evaluate parasite positivity on day 3 of treatment.

Malaria PCR, genotyping of *pfk13*, *pfcr1*, and *pfmdr1*

Parasite DNA was extracted from one-quarter of each blood spot using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). *P. falciparum* positivity was confirmed by species-specific PCR as previously described [33]. Polymorphisms were determined by direct sequencing, as previously described [14] and included the *pfk13* gene (propeller domain amino acid positions 427–726), *P. falciparum* chloroquine resistance transporter gene (*pfcr1*; amino acid positions 72–76), and *P. falciparum* multidrug resistance-1 gene (*pfmdr1*; amino acid positions 86, 184, 1034, 1042, and 1246). Allele prevalence of *pfk13*, *pfcr1*, and *pfmdr1* in 2020 were compared to those in 2002, 2003, and 2016–2018 [13, 14, 29]. The genotypes of *pfk13* in 2018 were analyzed using the same samples previously reported [14].

Statistical analysis

Statistical analysis was performed using R software (version 4.1.0), with the Chi-square test for trend (Cochran-Armitage trend test). Statistical significance was set at $p < 0.05$.

Results

Patients and *Plasmodium* sp. specific PCR

Among the 335 patients screened with RDT, 118 had positive results for *Plasmodium* (Fig. 1). Of these, species-specific PCR revealed that 13 were parasite negative and nine were other species, resulting in 96 patients with *P. falciparum* (Additional file 1). Two patients who received an intramuscular injection of artemether within two weeks prior to enrollment were excluded from further analysis. Finally, 94 samples were used for molecular analysis. There were no significant differences in the background characteristics of the enrolled patients between the two clinics (Table 1). The median age was 17 years and the median parasitemia was 0.74%. No significant difference was observed between patient number, age, sex, and average parasitemia in each year (Additional file 2).

Table 1
Characteristics of studied patients

Characteristic	Day 1 (n = 94)	Day 3 (n = 27)
Sampling clinics; n		
Wirui	46	7
Town	48	20
Age; n		
0–9	10	4
10–19	41	7
20–	43	16
Median (IQR)	17 (13,26.5)	25 (14.5,33)
Sex; n		
Male	47	12
Female	47	15
Symptoms; n (%)		
Muscle or joint aches	24 (26)	4 (15)
Chill/Shivering	36 (38)	4 (15)
Headache	60 (64)	9 (33)
Nausea/Vomiting	18 (19)	1 (4)
Abdominal pain	14 (15)	0
Diarrhea	7 (7)	2 (7)
Cough	24 (26)	3 (11)
Convulsions	8 (9)	1 (4)
Temperature (> 37.5°C)	47 (50)	2 (7)
Parasitemia*, Median (%) (IQR)	0.74 (0.35, 1.5)	0
IQR = Inter Quartile Range		

Frequency of polymorphisms in *pfk13*, *pfcr1*, and *pfmdr1*

C580Y was the only mutation observed in *pfk13* and was identified in six patients (5.2%) in 2018 and six (6.4%) in 2020 (Fig. 2). Since the first detection of C580Y in three patients (2.2%) in 2017 [13], there has been a statistically significant increase in the prevalence of C580Y ($p = 4.2 \times 10^{-3}$, Cochran-Armitage trend test).

In both *pfcr1* and *pfmdr1*, the majority of parasites harbored chloroquine resistant types in 2002 and 2003 [29]. However, a marked shift of allele prevalence to chloroquine-sensitive types was observed after the mid-2010s (Fig. 2). In *pfcr1*, there were two haplotypes: wild-type (CVMNK) and mutant (SVMNT; mutation underlined). Wild-type prevalence significantly increased from 1.9% in 2016 to 46.7% in 2020 ($p = 8.9 \times 10^{-16}$, Cochran-Armitage trend test). In *pfmdr1*, the N86 allele also significantly increased from 59.5% in 2016 to 91.4% in 2020 ($p = 2.3 \times 10^{-6}$, Cochran-Armitage trend test). The transition to the chloroquine-sensitive form of *pfmdr1* occurred at least three years earlier than that of *pfcr1*. In fact, the prevalence of the *pfmdr1* N86 allele in 2016 was higher than that of K76 in *pfcr1* in 2020. Although polymorphisms were observed at positions 184 and 1042, the prevalence fluctuated annually without any upward or downward trends. Only the wild-type allele was found throughout the study period at positions 1034 and 1246.

Follow-up of patients on day 3

Among the 94 patients treated with AL, 27 were successfully followed-up with clinical assessment on day 3. The patients included three infected with the C580Y mutant. Only one patient was afebrile on day 3. Prevalent symptoms remaining on day 3 were headache (33%) and muscle/joint pain (15%). All follow-up patients showed an absence of parasites on day 3 smears. There were no cases of early treatment failure.

Discussion

We commenced an epidemiological study related to malaria drug resistance in 2002 in East Sepik, PNG [29, 30]. After a long interval from 2004 to 2015, the study was restarted in 2016 [14]. In 2017, we identified three *pfk13* C580Y mutants [13]. This raised concern that ART-resistant *P. falciparum* parasites may have already emerged and spread in the study area. In this study, the prevalence of *pfk13* C580Y was relatively low, but it had increased significantly since the first detection. In general, drug-resistant malaria increases slowly at the beginning of an emergence, but increases rapidly as the frequency of resistant parasites increases. Indeed, C580Y frequencies gradually increased approximately five years from the initial detection, but then rapidly expanded and even overtook the other *pfk13* alleles in Cambodia and Western Thailand [5, 9, 10]. Therefore, even though the current prevalence of *pfk13* C580Y is low in PNG, rapid expansion in the near future could be anticipated.

On the other hand, regional malaria epidemiological factors in PNG may suppress the rapid increase in ART-resistant parasites. First, residents in our study area developed higher levels of herd immunity to malaria than those in the GMSR because of higher malaria transmission intensity in PNG [34, 35]. This

can considerably influence the clearance of ART-resistant parasites from human hosts [36] and may slow the rate of increase in the C580Y allele in the region. Second, because ART is primarily used in ACT, the presence of resistant parasites to ACT partner drugs significantly affects the diffusion rate of C580Y. In the GMSR, parasites resistant to partner drug(s), mefloquine, and piperaquine have already emerged and spread [37, 38]. In particular, parasites harboring both *pfk13* C580Y and plasmepsin 2/3 copy number variants, which are molecular markers for piperaquine resistance, have rapidly increased in West Cambodia [5, 39]. In contrast, in PNG there is no evidence that parasites are resistant to lumefantrine, the currently used partner drug of ACT. Our previous ex vivo drug susceptibility study from 2016 to 2018 also demonstrated that the average IC_{50} to lumefantrine was 4.6 nM and no parasite fulfilled the criteria of ex vivo lumefantrine resistance [14]. Furthermore, we found that no patient exhibited parasite positivity on day 3, although the follow-up number was small.

The effects of the C580Y mutation on ART resistance are important determinants of the survival of drug-resistant parasites. However, the introduction of C580Y into *P. falciparum* clones did not substantially increase the level of in vitro ART resistance compared to other mutations, such as R539T [40]. In addition, drug-resistant mutations generally confer a decrease in parasite fitness, which often leads to a survival disadvantage [41–44]. Several laboratory studies have demonstrated that the growth rates of C580Y harboring transgenic parasites were equal to or less than those of transgenic parasites with other *pfk13* mutations, such as R561H, E252Q, and G538V, suggesting that C580Y incurs at least a similar level of fitness impairment to the other *pfk13* mutations [40, 45, 46]. These laboratory findings are inconsistent with the field observations in the GMSR where the C580Y mutant has outcompeted other mutants [5, 9, 10]. This implies that some unique background genetic changes in the South-East Asia (SEA) parasites play a beneficial role in the survival of the SEA parasites harboring the C580Y allele. This might include compensation for the harmful effects of the C580Y mutation. Several single nucleotide polymorphisms (SNPs) have been identified in SEA *pfk13* isolates [47]. However, among these SNPs, only one (ferredoxin D193Y) was found in our PNG C580Y mutants [13], suggesting that PNG C580Y mutants do not possess the same background genetic changes as SEA C580Y mutants.

As for chloroquine susceptibility, we previously reported that the average IC_{50s} values were still high (80.5–106.6 nM) with a low prevalence of *pfcr* K76 wild-type (2.3–11.7%) during 2016–2018 [14]. In 2020, however, the prevalence of *pfcr* K76 wild-type rapidly increased to 46.7%. Since a significant association between the *pfcr* K76 wild-type allele and ex vivo chloroquine susceptibility was confirmed in our study area [14], the observed rapid increase in the *pfcr* K76 allele suggests that chloroquine sensitivity has been recovering. The significant increase in *pfmdr1* N86 wild-type from 59.5–91.4% in the three years to 2020 also suggests the potential resurgence of chloroquine sensitivity. Although this phenomenon has been widely observed in African countries [15–26, 28], it is very rare in SEA [48].

We previously found that decreased ex vivo susceptibility to lumefantrine was significantly associated with *pfmdr1* N86 [14], consistent with a previous transfection study showing that an allelic change from N86Y to N86 increased IC_{50} for lumefantrine three to four times [49]. There are considerable reports that *pfmdr1* N86 wild-type is selected by AL treatment [50, 51]. Furthermore, a recent meta-analysis of 60 AL

clinical trials revealed that only 38% of patients treated with AL were symptomatic when the infection recurred [52]. If patients are asymptomatic at the time of recurrence, they are unlikely to seek treatment, resulting in the persistence of parasitemia. This would increase the chance that the parasites could be transmitted to other human hosts, which could subsequently spread the drug-resistant mutations in the parasite population. In addition, a specific *pfmdr1* haplotype (multicopy *pfmdr1* in addition to N86 and Y184F) has increased and become predominant in Cambodia and Vietnam [53]. Gene editing experiments showed that this haplotype significantly reduces parasite susceptibility to lumefantrine [53]. In our study site, although this haplotype was not detected [13], all these genetic changes (multicopy *pfmdr1*, N86, and Y184F) were individually observed. Therefore, in addition to the assessment of the relapse rate following AL, it is necessary to monitor the appearance of this haplotype.

Conclusions

Since its first identification in 2017, *pfk13* C580Y harboring *P. falciparum* parasites have been increasing in Wewak, East Sepik, PNG. A significant increase in *pfcr1* K76 and *pfmdr1* N86 was also observed. This suggests a possible recovery of chloroquine sensitivity and, on the other hand, a decrease in sensitivity to lumefantrine, the ACT partner drug. The increasing frequency of *pfk13* C580Y mutants under these circumstances is a warning sign that parasites resistant to AL will emerge in the near future. Thus, it is important to enhance continuous monitoring to detect early signs of the emergence of ACT-resistant parasites.

Abbreviations

ACT: artemisinin combination therapy

ART: artemisinin

AL: artemether plus lumefantrine

GMS: Greater Mekong Subregion

IC₅₀: 50% growth inhibitory concentration

IQR: Inter Quartile Range

kelch13: *pfk13*

PCR: polymerase chain reaction

PNG: Papua New Guinea

RDT: rapid diagnostic test

SEA: Southeast Asia

SNP: Single nucleotide polymorphism

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Medical Research Ethical Committee of Juntendo University (No. 2017070) and the Medical Research Advisory Committee of the Papua New Guinea National Department of Health (MRAC No. 16.41).

Consent for publication

Prior to participation, all study subjects consented to the publication of study results in the medical literature in an anonymized manner.

Availability of data and materials

The primary datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

All authors declare no competing interests.

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Authors' contributions

NY and TM designed and coordinated the study; MY, ST, and FH performed the field study; NY and RM performed the laboratory work; NY and TM analyzed and interpreted the data; NY and TM wrote the manuscript. All the authors contributed significantly to this work. All authors read and approved the final manuscript.

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Figures

Figure 1

Flow chart of the study

Figure 2

Temporal changes in *pfk13*, *pfcr1* and *pfmdr1* allele prevalence in 2002, 2003, and 2016–2020. The allele type corresponding to each color is blue for the wild-type, yellow for the mix, and red for the mutant.

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