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REPORTING THE IMPACT OF ARTEMISININ RESISTANCE: MOLECULAR SURVEILLANCE OF *PFK13* AND *PFEXO* MUTATIONS IN *PLASMODIUM FALCIPARUM* IN SOUTHERN PROVINCES OF VIETNAM

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SUMMARY

Malaria, mainly caused by *Plasmodium falciparum (P. falciparum),* is a major global health concern. In Vietnam, resistance to artemisinin-based combination therapies (ACTs) is rising, jeopardizing malaria control efforts. This study focuses on mutations in the *pfK13* and *pfEXO* genes, particularly the E415G mutation in the *pfEXO* gene, in southern Vietnam. The study encompassed 421 patients distributed across two cohorts. The first cohort, comprising 63 patients from Binh Phuoc and Dak Nong, had uncomplicated *P. falciparum* malaria and constituted a segment of the Therapeutic Efficacy Studies (TES). These individuals received treatment in accordance with the 2009 World Health Organization (WHO) guidelines. The second cohort, comprising 358 patients from the Central Highlands, was established to evaluate the frequency of mutations in genes associated with artemisinin resistance. Molecular marker analysis, including Sanger sequencing for *pfK13* and ARMS-PCR for E415G in *pfEXO*, was conducted. The study also examined the association of these mutations with Day 3 parasitemia and treatment outcomes using Dihydroartemisinin-Piperaquine (DHA-PPQ). Most cases showed mutations in *pfK13,* linked to delayed parasite clearance and higher treatment failure, indicating *pfK13* as a key marker for artemisinin resistance. The E415G mutation in *pfEXO* was common but not significantly associated with resistance or treatment outcomes, though there was a tendency towards increased treatment failure among these patients. Our study implied the critical role of the C580Y mutation in the *pfK13* gene in artemisinin resistance and its impact on the efficacy of ACTs in Vietnam. The findings highlight the necessity of monitoring these mutations as molecular markers for drug resistance and call for the exploration of alternative treatment strategies in the face of evolving antimalarial drug resistance. This study contributes valuable insights to the molecular epidemiology of malaria in the southern provinces of Vietnam and

emphasizes the urgency of addressing artemisinin resistance in the global fight against malaria.

Keywords: *pfK13, pfEXO, E415G,* artemisinin resistance, *P. falciparum,* malaria.

INTRODUCTION

Malaria, a potentially fatal disease caused by parasites transmitted via *Anopheles mosquitoes*, poses a significant global health risk (Cowman *et al.,* 2016). Almost half the world's population is at risk, according to the World Malaria Report 2021 (WHO 2021). While malaria cases in the Greater Mekong Subregion (GMS) have decreased, the spread of antimalarial drug resistance, particularly in Vietnam, remains a concern (WHO 2021).

Since 2005, artemisinin-based combination therapies (ACTs) have been the standard treatment for uncomplicated falciparum malaria. These therapies effectively reduce parasite loads but are increasingly facing resistance challenges, jeopardizing global malaria control efforts. Resistance to dihydroartemisinin-piperaquine (DHA-PPQ), the primary treatment in Vietnam, is particularly alarming in the GMS (Ashley *et al.,* 2014). In Vietnam, the efficacy of DHA-PPQ has notably declined, with resistance emerging in various provinces (Thanh *et al.,* 2017).

Molecular markers, such as the *pfK13* kelch propeller and the E415G mutation, are key in assessing drug resistance (Ariey *et al.,* 2014;Boonyalai *et al.,* 2020). The *pfK13* gene, part of the kelch superfamily, plays a role in protein degradation and stress responses (Ariey *et al.,* 2014). Its mutations, associated with artemisinin resistance, have predominantly been found in Southeast Asia (Ashley *et al.,* 2014). The emergence of these mutations indicates independent development of resistance in various GMS areas, necessitating a shift from reduction to elimination strategies for multi-drug-resistant falciparum malaria (Ashley *et al.,* 2014). Another important marker, the E415G mutation in the *Plasmodium falciparum Exonuclease* (*pfEXO*) gene, is gaining attention as a potential indicator of antimalarial drug resistance (Amato *et al.,* 2017). This mutation, linked to treatment failures in Cambodia, has been identified in significant proportions in Vietnam's Central Highlands (Boonyalai *et al.,* 2020;Quang and Chavchich 2021).

This study aims to assess the prevalence of *pfK13* and E415G in *pfEXO* gene mutations in Southern Vietnam and evaluate their role as indicators of artemisinin resistance, vital for future malaria treatment strategies.

MATERIALS AND METHODS

Study sites and patients

A total of 421 uncomplicated falciparum malaria samples were collected from four provinces (Binh Phuoc, Gia Lai, Dak Nong, and Dak Lak) in southern Vietnam. These samples were categorized into two groups. The first cohort comprised 63 patients from Dak Nong (n = 19, August 2018 to May 2019) and Binh Phuoc (n = 44, August 2018 to May 2019). These patients participated in therapeutic efficacy studies (TES) following the WHO guidelines (WHO 2009). The inclusion criteria specified patients with an axillary temperature of ≥ 37.5 °C or a history of fever in the 24 hours before consultation.

Eligibility was also determined by a lack of prior antimalarial drug intake and a positive test for *P. falciparum* with a parasite density between 1,000 and 200,000 parasites/μL. Eligible patients received a three-day treatment of DHA (40 mg) combined with PPQ (320 mg) under the brand name Artenakin. Following administration, patients were monitored continuously for 60 minutes. The criteria for Adequate Clinical and Parasitological Response (ACPR) and treatment failure (Early Treatment Failure [ETF], Late Clinical Failure [LCF], and Late Parasitological Failure [LPF]) were based on the 2009 WHO guidelines (WHO 2009). These guidelines define outcomes for therapeutic efficacy studies with a 42-day follow-up. Informed consent was given by the patient or by a parent or guardian for children.

The second cohort was sampled from three *P. falciparum* endemic regions in the Central Highlands: Gia Lai (n = 215, August 2018 to May 2019), Dak Nong (n = 41, August 2018 to May 2019), and Dak Lak (n = 102, August 2018 to May 2019). The study included patients who were older than 16 years and younger than 70 years and who did not have any chronic diseases.

Genomic DNA extraction

Dried blood spots were collected, and then genomic DNA was extracted using Gene JET Whole Blood Genomic DNA Purification (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Extracted DNA was stored at –20 °C until amplified by PCR.

Detection of *pfK13* mutations

The *pfK13* gene was amplified using a nested PCR approach, with the sequences of the primer pairs listed in Table 1**.**

Table 1. Primer sequences used for amplification of the *pfK13* gene.

Primer name	Primer sequence (5'-3')
pfK13- 1F	GGGAATCTGGTGGTAACAGC
pfK13- 1R	CGGAGTGACCAAATCTGGGA
pfK13- 2F	GCCTTGTTGAAAGAAGCAGA
pfK13- 2R	GCCAAGCTGCCATTCATTTG

Two μL of genomic DNA were used as a template to amplify target genes. The amplification process involved 10 μL of DreamTaq Master Mix (2X) and 0.5 μL of each primer at a concentration of 10 pmol/μL for both the primary and secondary reactions. For the primary reaction, the following cycling parameters were used: 5 min at 94 °C, 25 cycles at 94 °C for 30 sec, 51 °C for 60 sec, 72 °C for 1 min 25 sec, and final extension for 5 min at 72 °C. For the nested PCR, 1 μL of 1/5 diluted primary PCR product was used as a template. Thermal cycling for the secondary PCR reaction included 5 min at 95 °C, 30 cycles at 94 °C for 45 sec, 50 °C for 45 sec, 72 °C for 45 sec, and a final extension for 5 min at 72 °C. The PCR products were visualized after electrophoresis on a 2% agarose gel stained with ethidium bromide. Secondary PCR products were purified by the GeneJET PCR Purification Kit (Thermo Scientific, USA)

and sequenced (Macrogen Inc., Seoul, Korea). The sequences were aligned with those of 3D7 retrieved from the *P. falciparum* database and deposited in GenBank (Accession No. NC_004331.3).

Detection of an E415G mutation in the *pfEXO* gene

A DNA fragment (749 bp) containing the *E415G* mutation region was amplified by the tetra-primer ARMS PCR technique. The oligonucleotide ARMS primers for the detection of the E415G mutation were designed based on the sequences of the *exonuclease* gene (PF3D7_1362500) via primer blast software (www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequences of four primers are listed in Table 2. The total reaction mixture (25 µL) contained 12.5 µL Gotaq Green Mastermix (2X), 0.3 µL of E415G-Fc and E415G-Ras-W primers, 1 µL of E415G-Fas-V and E415G-Rc primers, and 2 µL of DNA template. Thermal cycling steps were: 5 min at 95 °C, 40 cycles at 95 °C for 30 sec, 54 °C for 45 sec, 72 °C for 35 sec, and final extension for 5 min at 72 °C. The PCR products were visualized after electrophoresis on a 2% agarose gel stained with ethidium bromide. Samples from homozygous mutant alleles now produce two bands (749 bp and 474 bp), while those from wild-type alleles yield two bands (749 bp and 316 bp).

Table 2. Primers sequence to identify E415G.

Primer name	Sequence (5' → 3')
E415G-Fc	GGA ATG TGC TTT AAC GAA TGG
E415G-Fas-V	TAT GGT TAT AAC GAT AAA AC*G
E415G-Rc	GGT GTT CCT TCC TCT TTT CTT G
E415G-Ras-W	CCC AAT GAT TGT TTA CTT CG*T

Statistical analysis

Data management was conducted using Excel 2016. The frequency of mutations and the occurrence of samples with gene mutants were determined through simple counting methods. Mutation frequencies were then calculated and presented as frequencies and percentages. For clinical follow-up data, we considered the proportions of treatment failure, adequate clinical and parasitological response (ACPR), and day 3 parasite positivity following DHA-PPQ regimen. Categorical data were analyzed using the Chi-square test or Fisher's exact test. We compared the proportion of patients exhibiting day 3 parasite positivity with mutations in each target gene. The association between molecular markers and treatment failure of DHA-PPQ was examined using an unconditional logistic regression model, with a significance threshold set at $\alpha \leq 0.05$.

RESULTS

Demographic of study participants

The general characteristics of the study population are presented in Table 2. The first cohort, with patients from Binh Phuoc (44) and Dak Nong (19), had an average age of 30.75 ± 9.21 years, with a male to female ratio of 60:3. All had fever (axillary

temperature ≥ 37.5 °C), and parasitemia averaged 18,231 parasites/µL (range 560 to 154 666 parasites/µL). The second cohort, from Gia Lai, Dak Nong, and Dak Lak, was also predominantly male (91%, 326/358). Their average age was 32.89 ± 9.9 years, with a mean axillary temperature of 38.6 ± 0.6°C. Parasitemia here averaged 12 076 parasites/µL, with a range of 168 to 104 976 parasites/µL (Table 3).

Table 3. General characteristics of the study population.

Parameters	First cohort (n=63)	Second Cohort (n=358)
Mean Age	30.75 ±9.21	32.89 ± 9.9
Gender ratio (male/female)	60/3	326/358
Body temperature	38, 6 ± 0,58	38.6 ±0.6
Mean of parasitemia (range) (parasites/µL blood)	18 231 (560 – 154 666)	12 076 (168- 104 976)

Molecular markers analysis

Prevalance of mutations in P. falciparum parasites in Vietnam

The initial cohort revealed the C580Y mutation in the *pfK13* gene in 80.3% of investigated samples. This mutation was more prevalent in Binh Phuoc (86%) compared to Dak Nong (67%), and this difference wasn't statistically significant (P>0.05). The subsequent cohort confirmed the C580Y mutation as the predominant variant in the Central Highlands, found in 93.3% of isolates. Within this group, mutation frequencies in Dak Nong, Gia Lai, and Dak Lak were 91.9%, 92.5%, and 95.70%, respectively, with no significant variance across these areas (P>0.05). For the E415G mutation, the first cohort showed an 81.3% prevalence, with 84.4% in Binh Phuoc and 75% in Dak Nong, a nonsignificant variation (P>0.05). In the second cohort, 75.4% of isolates had the E415G mutation. Here, its prevalence was highest in Dak Lak (85.9%) and Dak Nong (80%), but significantly lower in Gia Lai (70%) (P<0.05). The combined C580Y/E415G genotype was common in both cohorts, ranging from 67.5 % to 77.3%.

Association between gene mutant and artemisinin resistance

The therapeutic efficacy of DHA-PPQ for treating uncomplicated *P. falciparum* malaria is currently evaluated over 42-day periods according to WHO guidelines. Monitoring parasite positivity after 72 hours (day 3) of treatment is crucial for determining resistance to ART.

In the first cohort study, 53 out of 63 patients treated with DHA-PPQ regimens completed the 42-day follow-up. Seven patients were lost to follow-up, and three patients were withdrawn due to the reinfection. By day 3 of treatment, 23.8% (15/63) of patients still had residual parasites. Twenty-one cases exhibited recurrent infection on days 14, 22, 28, 32, and 42 and were classified as either LCF or LPF. Overall, the ACPR efficacy of DHA-PPQ for treating *P. falciparum* was 50.8%, while the treatment failure rate was 49.2%.

Table 4. Prevalence of mutation from two cohort study.

Mutation/genotype	Provinces n (%)				Total
	Binh Phuoc	Dak Nong	Dak Lak	Gia Lai	
The first cohort study					
C580Y	37/43 (86)	12/18 (67)	NA	NA	**49/61 (80.3)**
E415G	27/32 (84.4)	12/16 (75)	NA	NA	**39/48 (81.3)**
C580Y/E415G	25/31 (81)	09/15 (60)	NA	NA	**34/44 (77.3)**
The second cohort study					
C580Y	NA	34//37 (91.9)	89/93 (95.7)	184/199 (92.5)	**307/329 (93.3)**
E415G	NA	32/40 (80)	79/92 (85.9)	149/213 (70)	**260/345 (75.4)**
C580Y/E415G	NA	26/37 (70.3)	69/84 (82)	121/199 (60.8)	**216/320 (67.5)**

Note: NA: not applicable; Bolded numbers in total coloumn indicate high prevalence rates of mutations.

Table 5. Mutations and the occurrence of *P. falciparum* parasites on day 3 of treatment.

Mutation		Occurrence of *P. falciparum* parasite on day 3		P
		Negative, n(%)	Positive, n(%)	
C580Y	Non mutation (n = 11)	11(100)	0(0)	**0.05**
	Mutation (n = 46)	33(71.7)	13(28.3)	
E415G	Non mutation (n = 8)	8(100)	0(0)	0.083
	Mutation (n = 37)	24(64.9)	13(35.1)	
C580/E415G	Mutation (n = 32)	19(59.4)	13(40.6)	**0.019**
	Non mution (n = 11)	11(100)	0(0)	

Note: Bolder number in P column indicate significant mutation effects on *P. falciparum* presence by day 3.

Between the C580Y mutation in the *pfK13* gene and slower parasite clearance, gene mutation analysis revealed a significant correlation with a 100% positivity rate on day 3 in patients with this mutation, compared to none in those with the wild-type gene. The E415G mutation was not statistically significant (P > 0.05), but all patients with this mutation had parasites on day 3. The combined C580Y/E415G genotype showed a higher parasite presence (40.6%) than those without the mutations (0%, p = 0.019) (Table 5).

Moving on to the role of mutations in candidate genes and their impact on the effectiveness of DHA-PPQ, we analyzed the association of mutations with the ACPR on day 42. The C580Ymutation was found to be significantly associated with a 50% treatment failure rate by day 42. In contrast, no significant correlation was found between the E415G mutation and treatment failure in patients treated with DHA-PPQ, despite patients with this mutation having a significantly higher rate of treatment failure (41.9%) compared to those with the wild-type gene (25%). Additionally, combined genotypes C580Y/E415G showed a noticeable but non-significant trend, with a 50% treatment failure rate (P = 0.141) compared to 18.2% in the non-mutated group. Finally, our result highlights the critical role of the C580Y mutation in both parasite persistence post-treatment and in treatment failure rates (Table 6).

Table 6. Association between mutation and treatment outcome of DHA-PPQ.

Mutation		**Treatment effectiveness**		**P**
		Treatment failure, n(%)	ACPR, n(%)	
C580Y	Non mutation (n = 10)	0(0)	10(100)	**0.003**
	Mutation (n = 40)	20(50)	20(50)	
E415G	Non mutation (n = 8)	2(25)	6(75)	0.369
	Mutation (n = 31)	13(41.9)	18(58.1)	
C580/E415G	Non mutaion (n = 11)	2(18.2)	9(81.8)	0.141
	Mutation (n = 26)	13(50)	13(50)	

Note: Bolded number in P column indicate statistically significant associations between mutations and treatment outcomes.

DISCUSSION

Artemisinin, a compound derived from the *Artemisia annua* plant, has revolutionized the treatment of malaria, particularly *P. falciparum* malaria, the most dangerous form. It's a core component of artemisinin-based combination therapies (ACTs), which are the first-line treatment for uncomplicated malaria. However, the emergence of ART resistance threatens global efforts to control and eventually eliminate malaria.

Our study offers crucial information regarding the molecular epidemiology *of P. falciparum* in the southern provinces of Vietnam, with a specific focus on the

prevalence of the C580Y mutation in the *pfK13* gene and the E415G mutation in the *pfEXO* gene. We observed a high prevalence of the C580Y mutation across various regions, notably in the Central Highlands. Despite the absence of statistically significant differences in mutation frequencies among the sampled localities, the consistently high prevalence noted in two cohort studies indicates the widespread nature of this mutation. Although the E415G mutation was prevalent, it did not show a statistically significant association with ART resistance or treatment outcomes. However, there was a trend toward increased treatment failure in patients with this mutation. The continued presence of parasites post-treatment, particularly in patients with the C580Y mutation, underscores the urgent need for alternative treatment strategies. Furthermore, the observed treatment failures associated with these mutations, especially the C580Y mutation, highlight the critical need to address this resistance.

The combined genotype of C580Y/E415G shows higher treatment failure rates, further complicating the treatment landscape. The *pfk13* gene, which encodes the Kelch protein in *P. falciparum*, has been at the center of research on ART resistance. Mutations in this gene are key indicators of resistance, as they can lead to delayed clearance of the parasite after treatment with ART. The World Health Organization (WHO) has reported over 200 mutations in the *pfk13* gene, with each potentially contributing to varying degrees of resistance (WHO 2014;WHO 2019). Each mutation may be associated with varying degrees of delayed parasite clearance following ART treatment, a key characteristic of ART resistance (WHO 2014;WHO 2019). Regionally, the prevalence of specific *pfk13* mutations varies. In Cambodia, Vietnam, and Lao PDR, mutations such as Y493H, R539T, I543T, P553L, and C580Y are commonly observed (Zaw *et al.,* 2020). In contrast, in western Thailand, Myanmar, and China, mutations like F446L, P553L, N458Y, R561H, P574L, and C580Y are more frequent (Ye *et al.,* 2016;Zaw *et al.,* 2020). Across Southeast Asia (SEA), P553L and C580Y mutations are prevalent, with the latter being more widespread in eastern Thailand (Zaw *et al.,* 2020). Clinical reports of ART resistance first surfaced in Binh Phuoc, Vietnam, in 2008 (WHO 2014). A molecular analysis of *pfK13* mutations in this province from 2009 to 2016 shows a significant increase in the proportion of the C580Y mutation, from 1.7% in 2009 –2010 to 79.1% in 2015 –2016 (Thuy-Nhien *et al.,* 2017). Conversely, the incidence of other mutations, such as I543T, Y493H, and R539T, decreased and eventually disappeared. This trend is mirrored in the Central Highlands of Vietnam, where there has been a steady increase in the prevalence of the C580Y mutation over the years (Thuy-Nhien *et al.,* 2017). Since 2017, the C580Y mutation has been the only mutation detected in malaria-endemic areas of Vietnam, including Binh Phuoc, Ninh Thuan, and Gia Lai (Thuy-Nhien *et al.,* 2017). More importantly, the study showed that the rapid increase of the *pfK13* mutation in these endemic areas has been accompanied by a significant change in the ACPR rates to DHA-PPQ treatment. Tthe ACPR rate was 93% in 2010 (Hien *et al.,* 2012), but it further dropped to 78% in 2015 (Thanh *et al.,* 2017). These findings indicate that the genetic changes have greatly impacted clinical outcomes, specifically affecting the efficacy of DHA-PPQ treatment for malaria in Vietnam. The C580Y mutation in the *pfk13*

gene has also emerged as a dominant marker in Cambodia, Myanmar, Thailand, and Laos. For instance, in Pailin, a regiob in western Cambodia where ART resistance was first described, the prevalence of parasites with the C580Y mutation increased significantly from 2008 to 2015, replacing other *pfK13* mutations (Hamilton *et al.,* 2019). Similarly, in Laos and Thailand, the prevalence of the C580Y mutation has been notably high in certain provinces (Hamilton *et al.,* 2019). The prevalence of *pfK13* mutations is dynamic, reflecting the changing nature of malaria and the parasite's adaptation to antimalarial drugs. The lack of diversity in detected mutations indicates a selective pressure favoring the C580Y variant, possibly contributing to its dominance in the parasite population (Hamilton *et al.,* 2019;Nair *et al.,* 2018).

The origin of the C580Y mutation has been a subject of extensive study in GMS (Amato *et al.,* 2018). This mutation, while not necessarily conferring a higher level of ART resistance compared to other *pfK13* mutations, seems to display enhanced fitness and transmissibility, as evidenced by its predominance over other mutations (Nair *et al.,* 2018). In contrast to Southeast Asia, Africa has reported a high occurrence of nonsynonymous mutations linked to delayed parasite clearance, albeit at a low frequency (Chenet *et al.,* 2016;Mita *et al.,* 2016). Many of these mutations have not proliferated within the local parasite populations. It's suggested that in Africa, ART resistance may be associated with other genetic factors, such as *pfcrt, pfmdr1, pfap2mu, pfubp1,* and *dhfr,* rather than *pfK13* mutations (Mita *et al.,* 2011). In South America, the *pfk13* C580Y mutant parasites have been identified in Guyana, with their genetic profiles differing from those in Southeast Asia, indicating an independent origin.

In our study, we observed a significant prevalence of parasites having the E415G mutation in the *pfEXO* gene across various locations. Similar to the C580Y mutation in the *pfK13* gene, the E415G mutation is predominantly found in eastern mainland Southeast Asia, a region that employs DHA-PPQ as an antimalarial combination therapy (Amato *et al.,* 2017). Intriguingly, the E415G mutation is completely absent in regions where this specific antimalarial therapy is not in use (Amato *et al.,* 2017). In Cambodia, the *pfK13* C580Y and E415G mutations, along with plasmepsin 2/3 gene amplification, have been strongly correlated with DHA-PPQ treatment ineffectiveness (Amato et al., 2017; Boonyalai et al., 2020). These genetic factors are associated with high survival rates of the malaria parasite in piperaquine survival assays. The colineage, known as KEL1/PLA1, containing the *pfK13* C580Y allele and amplified plasmepsin 2/3 genes, initially emerged in western Cambodia in 2008 and subsequently spread to neighboring regions (Amato *et al.,* 2018;Hamilton *et al.,* 2019;Imwong *et al.,* 2020;Imwong *et al.,* 2017). As a consequence, the DHA-PPQ cure rate in these areas has also experienced a rapid decline (Hamilton *et al.,* 2019). Moreover, the E415G mutation, along with specific *pfcrt* mutations, namely T93S, H97Y, F145I, I218F, M343L, C350R, and G353V, can confer resistance to piperaquine, an antimalarial drug used in combination with DHA in Cambodia (Boonyalai *et al.,* 2020).

The C580Y mutation in *pfK13* and the E415G mutation in the *pfEXO* gene of *P. falciparum* are both significant in the context of ART resistance, a critical issue in the treatment of malaria (Boonyalai *et al.,*

2022;Zaw *et al.,* 2020). These mutations, while potentially differing in their specific impacts on the protein's function, share a common narrative in the broader scenario of malaria treatment and drug resistance (Boonyalai *et al.,* 2020;Xie *et al.,* 2020). Firstly, both the C580Y and E415G mutations likely originated through random genetic variation, which is a standard part of the evolutionary process of any organism, including *P. falciparum (White 2014)*. These mutations are not directly caused by the presence of ART; instead, they occur spontaneously due to errors in DNA replication or repair mechanisms (Coppée *et al.,* 2019;White 2014). However, the presence of ART in the treatment landscape creates a selective pressure that influences which mutations persist and spread in the parasite population (Noreen *et al.,* 2021). Additionally, the C580Y mutation is one of the most documented mutations associated with ART resistance, found in various regions and closely linked to a reduction in the parasite's susceptibility to artemisinin (Mairet-Khedim *et al.,* 2021;Pau *et al.,* 2019;Zaw *et al.,* 2020). The E415G mutation, while less thoroughly researched, may also contribute to resistance, though its specific role and prevalence require more investigation.

In summary, both the C580Y and E415G mutations likely appeared randomly, but their spread and prevalence in certain geographical regions were driven by the selective pressure of ART use. This pattern illustrates how drug resistance can emerge and propagate in response to the environmental pressures exerted by widespread drug use, a critical factor in planning strategies for malaria treatment and prevention.

CONCLUSION

Our study implies the critical role of the C580Y mutation in the *pfK13* gene in ART resistance and its impact on the efficacy of ACTs in several southern provinces of Vietnam. The findings highlight the necessity of monitoring these mutations as molecular markers for drug resistance and call for the exploration of alternative treatment strategies in the face of evolving antimalarial drug resistance. This study contributes valuable insights to the molecular epidemiology of malaria in Vietnam and emphasizes the urgency of addressing ART resistance in the global fight against malaria.

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