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Genome-wide scanning for genetic markers associated with anti-malarial drugs sensitivity of *Plasmodium falciparum* isolates from the China-Myanmar border region

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Abstract

Background Understanding the emergence and spread of anti-malarial resistance, particularly to artemisinin and its partner drugs, is essential for eradicating malaria in worldwide. To identify genetic markers associated with susceptibility to common anti-malarial drugs, the in vitro sensitivities of anti-malarial drugs were evaluated, and a genome-wide association study of *Plasmodium falciparum* susceptibility in vitro to multiple anti-malarial drugs was conducted.

Methods Genomic DNA from 34 samples of *P. falciparum* collected between 2007 and 2010 in the Nabang-Lazan Valley along the China-Myanmar border was extracted and subjected to whole-genome sequencing. The standard SYBR Green I-based fluorescence assay and RSA assay were used to evaluated the in vitro sensitivities of anti-malarial drugs. Plink v1.90 was used to investigate the associations of genome-wide SNP with in vitro sensitivities to anti-malarial drugs.

Results The proportion of isolates showed reduced-susceptible to CQ,SP,QN,PPQ and PND were 88.24%,92.59% ,8.82%,8.82%,5.88%, respectively. 93.54% of isolates showed high level of the IC_{50} values of CQ have a *pfcrt* CIETS mutations. The isolates with *pfdhfr* IRNI, NRNL and IRNL mutations showed high SP IC_{50} values. SNPs on *pfhsp90* and *pfevp1* showed significant association with IC_{50} values of CQ. Of particular interest is the significant association found between a locus on chromosome 13 and the sensitivity to dihydroartemisinin. This locus is situated within the gene encoding the inner membrane complex protein 1F (*IMC1F*),which has been found to be associated with the *kelch13* compartment in schizont stages of *P. falciparum*.

Conclusions Multiple genetic markers correlating with anti-malarial drug susceptibility were identified in the study, which provide a reference for further investigations into the association between oxidative stress-mediated activity and anti-malarial drugs susceptibility.

Keywords Antimalarial drugs, Resistance, Genetic markers, Plasmodium falciparum

Background

According to the world malaria report, 249 million new cases and 608,000 deaths were reported in 2022 alone [1], indicating the emergency to eradicate malaria worldwide. The widespread resistance to anti-malarial drugs has been a major obstacle to

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malaria treatment and drug development. The malaria parasite *Plasmodium falciparum* is resistant to many anti-malarial drugs, including chloroquine (CQ), sulfadoxine-pyrimethamine (SP) [2]. The current main treatment for *P. falciparum* malaria is artemisinin-based combination therapy (ACT), which combine a fast-acting artemisinin derivative with a long-acting partner drug. The use of ACT has achieved extraordinary success in malaria treatment, with the global burden showing a 37% reduction from 2000 to 2015 [3]. Unfortunately, resistance to first-line ACT in *P. falciparum* has emerged and spread in Southeast Asia, and is now threatening sub-Saharan Africa [4, 5]. The Greater Mekong Sub-region (GMS) of Southeast Asia is an epicentre of anti-malarial drug resistance, where parasites initially showed resistance to chloroquine, mefloquine and sulfadoxine-pyrimethamine [6, 7], and where resistance to ACT first emerged [8]. It is imperative to understand the genetic factors associated with emergence and spread of drug resistance, and to develop an effective strategy to combat drug resistance in GMS. Multiple SNPs have been reported to be associated with drug resistance in *P. falciparum* from GMS, especially Cambodia [9].

On the China-Myanmar border, chloroquine has been used since 1960 to treat falciparum malaria in this region. After 20 years of use, it was gradually replaced by artemisinin drugs as the majority of *P. falciparum* in the region developed resistance to chloroquine. Pyrimethamine and sulfadoxine were used as a combination strategy (Anti malaria Tablet No. 2) since 1970s and stopped in the late 1990s. *Plasmodium falciparum* isolates from China-Myanmar border still exhibit high drug resistance to both CQ and SP. Piperaquine (PPQ) has been used as the first-line treatment for CQ-resistant *P. falciparum* since the early 1970s in the China-Myanmar border area[10].Within the GMS, the history of anti-malarial drug usage and drug sensitivity in the China-Myanmar border region differ significantly from the rest of the GMS, leading to speculation that there may be novel mechanisms of resistance to dihydroartemisinin (DHA) and other anti-malarials in this region[11, 12]. Therefore, it is essential to monitor the prevalence of known genetic resistance markers and identify new drug targets in malaria parasites in this region.

34 *P. falciparum* samples were collected from the China-Myanmar border region at different times for in vitro sensitivity analysis of commonly used anti-malarial drugs.The in vitro drug assays and genome-wide association studies (GWAS) were combined to characterize the regional distribution of anti-malarial drug sensitivity and identify *P. falciparum* genetic markers potentially associated with resistance to multiple anti-malarial drugs.

Methods

Sample collection and culture adaption

The isolates were collected from symptomatic malaria patients diagnosed by microscopic examination from 2007 to 2010. The collecting site is located on the China-Myanmar border (CMB) named Lazan valley. Parasites were cultured in O+human red blood cells with complete RPMI 1640 medium under an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 in vitro. Extraction of Genomic DNA and sequencing were described as previous study [13]. Scientific and ethical clearance of the study was obtained from the Internal Review Board of Naval Medical University. Written informed consent for study participation was obtained from all patients.

Drug assay

The standard SYBR Green I-based fluorescence assay was used to assess parasite sensitivities to 5 commonly used anti-malarial drugs: CQ, SP, PPQ, quinine (QN), and pyronaridine (PND).Three biological replicates were tested for each isolate and laboratory isolate FCC1/HN was always included as a reference. Growth inhibition of parasite cultures at 1% packed cell volume and 1% parasitaemia was determined on 96-well plates by exposure to serial dilutions of 5 drugs. DNA were released from cultured parasites and stained with SYBR Green I dye after incubation at 37 °C for 72 h. The plate was kept in dark for 30 min and signals were read in a FluoStar microplate reader (BMG Labtech, Germany). Half-maximal inhibitory concentrations (IC_{50}) were estimated by non-linear regression using GraphPad Prism 5. RSA was performed as previously described [15]. Briefly, the highly synchronous 0–3 h post-invasion parasites were exposed to 700 nM DHA or 0.1% dimethyl sulfoxide (DMSO) as the control for 6 h. They were then washed off with RPMI 1640 and cultivated for additional 66 h. Survival rates were measured microscopically by Giemsa-stained thin smears by counting the viable parasites surviving in DHA-treated versus DMSO-treated cultures.

Genome-wide association

To detect genetic marker associated with altered sensitivities to CQ,SP,PPQ,QN,PND and DHA, GWAS was conducted between the drug value and SNPs from whole genome by PLINK v1.90 [14]. Data for parasites' ring-survival rate(RSA) of DHA were generated in a previous study [15]. From SNPs of the 34 *P. falciparum* isolates detected in previous study [13], SNP set with minor allele frequency (MAF) > 0.05 was selected for

further GWAS analysis. Multi-drugs IC_{50} value converted to logarithm transformation was used as the continuous dependent variable for CQ,SP,PPQ,QN and PND, the original value of RSA for DHA was used because RSA values of most parasites were lower than 10%. Bonferroni correction was applied to define a significance threshold of p-value of 3.09×10^{-6} or less for GWAS analysis and a suggestive threshold of p-value of 10^{-4} or less to identify relatively high-ranking loci.

Statistical analysis

All statistical analyses were performed with R4.2.1 software. IC_{50} values were reported in Geometric Mean and standard deviation. Mann–Whitney U test was conducted to compare differences in IC_{50} among different sample years. Cross-susceptibility was analysed using the Spearman's correlation (r). A two-sided p value ≤ 0.05 was set as the significance threshold.

Results

In vitro sensitivity of field isolates to anti-malarial drugs

Previous work reported the continuous cultivation and genome sequencing of 34 *P. falciparum* isolates from Nabang-Lazan valley in China-Myanmar border (Fig. S1, Table S1) [13]. Building on this foundation, the present study assessed the susceptibility of these isolates to six regionally relevant anti-malarial drugs, including CQ, SP, PPQ, QN, PND and DHA. Data for parasite ring survival rate (RSA) of DHA were generated in a previous study [15]. The results for these five anti-malarial drugs are shown in Fig. 1 and Table 1. Overall, most of the tested isolates had a high IC_{50} to both CQ and SP, with a median IC_{50} value of 492.5 nM (range 20.8 to 1089.1 nM) and

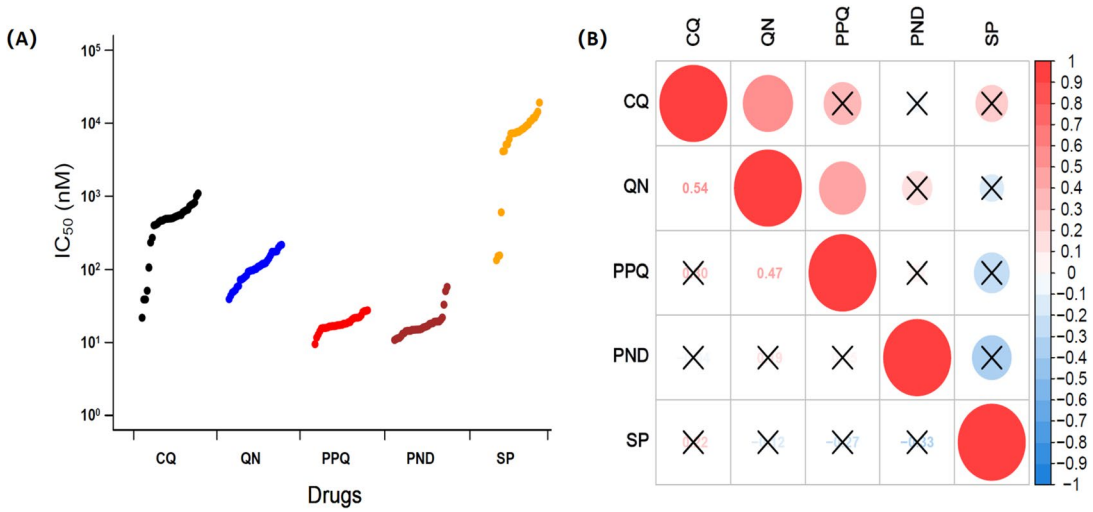

Fig. 1 **a** Parasite sensitivities to 5 anti-malarial drugs in vitro. IC_{50} values to CQ, SP, PPQ, QN and PND were sorted from the lowest to the highest values. IC_{50} values are shown in nM **b** Correlations between sensitivities of isolates to paired drugs analyzed by Spearman's test. The degree of correlation between sensitivities of paired drugs is color-coded. × indicates that correlations is not significant(P > 0.05)

Table 1 In vitro sensitivity of 34 *Plasmodium falciparum* isolates from China-Myanmar to different anti-malarial drugs

Drugs	No	Geometric Mean	SD	Range	Median	FCC1/HN (Mean ± SD)	Pvalue	Cutoff(nM)	Isolates above cutoff(N(%))
CQ	34	386.87	246.19	20.8–1089.1	492.5	22.16 ± 4.75	< 0.001	100#	30(88.24%)
SP	27	30844.45	29834.53	410.65–137792.5	45519.0	633.71 ± 152.75	0.001	2000#	25(92.59%)
PPQ	34	31.34	9.27	13.85–52.46	29.9	24.67 ± 6.84	0.017	49.88	3(8.82%)
PND	34	28.47	23.84	16.39–128.75	24.8	24.84 ± 6.09	0.384	76.15	2(5.88%)
QN	34	249.31	154.27	80.75–630	254.4	60.36 ± 12.64	< 0.001	557.85	3(8.82%)

Cutoffs for resistance are based on earlier report in previous study [29]. #. The rest of the cutoff values were based on calculated based on mean + 2 × SD of IC_{50}s from the field isolates

SD standard deviation

* P values are from Mann–Whitney U test for comparison between field isolates and FCC1/HN

45,519.0 nM (range 410.7 to 137792.5 nM), respectively. The ranges of CQ IC_{50} and SP IC_{50} were 52.4 folds and 335.5 folds of the minimum $IC_{50,}$ respectively. While the ranges of the IC_{50} values to the other three anti-malarial drugs were relatively narrow (3.8 folds for PPQ; 7.8 folds for QN and 7.9 folds for PND).

The *P. falciparum* FCC1/HN strain was isolated from Hainan Island of China, and has been adapted to in vitro continuous cultivation for several decades and was sensitive to common anti-malarial drugs. This parasite is used as reference sensitive strain for the in vitro drug sensitivity assay. The in vitro sensitivity of the FCC1/HN line to the anti-malarial drugs was assessed in parallel, with the IC_{50} values determined for each tested drug (Table 1). Compared with the control strain, the tested isolates had significantly higher mean IC_{50} values to CQ (386.87 nM, $P<0.001$) and SP(30844.45 nM, $P<0.01$). According to the IC_{50} values of the parasites, only 4 of 34 tested isolates were sensitive to CQ and their IC_{50} values were lower than 100 nM and similar to that of the FCC1/HN line (Table 1). Only 2 of 27 isolates were sensitive to SP. Given that there were no threshold values defined for resistance to PPQ, PND, and QN, we used the mean value plus 2 standard deviations (SD) as arbitrary cutoff values for potential resistance [16].The geometric mean IC_{50} values of QN,PPQ and PND were lower than the cutoff value of 557.85 nM,49.88 nM, 76.15 nM, with 8.82%,8.82%,5.88% of the isolates exceeding these values.

Spearman's correlation analysis was performed on IC_{50} values of anti-malarial drug pairs to assess potential correlations in parasite sensitivities.. Pairwise comparison (Fig. 1) showed that there were highly significant, positive correlations between sensitivities to CQ and QN($r=0.54$, $P=0.001$). Similarly, QN showed significant correlations with PPQ ($r=0.47$, $P=0.005$).

There appeared to be two types of temporal trends of parasites sensitivities to the 6 anti-malarials tested (Fig. 2). The in vitro IC_{50} of the parasites to the 3 aminoalcohol drugs CQ, SP, and QN showed a similar trend of a significant increasing in 2007, 2008 and 2009–2010. Among them, parasites showed a significant higher IC_{50} to CQ and SP in 2009–2010 than 2007 ($P<0.05$, Mann–Whitney U test) (Fig. 2). For QN, there was a significantly continuous increase in mean IC_{50} values in 2007–2010 ($P<0.05$, Mann–Whitney U test). For PPQ and PND, there was no significant change on IC_{50} values during 2009–2010.For DHA, although the changes of RSA were not significant, the RSA values to DHA showed an upward annual trend.

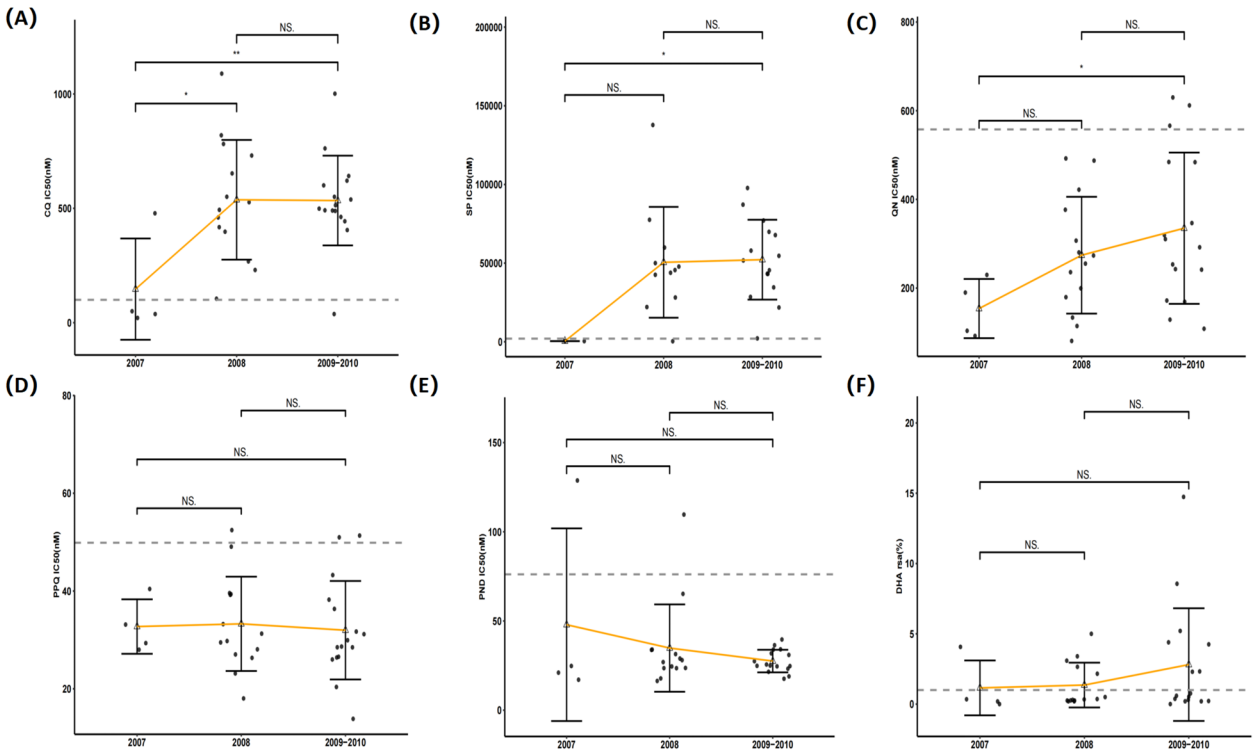

Fig. 2 In vitro sensitivities of culture-adapted parasite isolates to six anti-malarial drugs. The dot plot shows that the median and interquartile range. Gray dotted lines indicate the cutoff values used to define reduced sensitivity: CQ, 100 nM; SP, 2000 nM;PPQ:49.88 nM; QN, 557.85 nM;PND,76.15 nM;DHA,1%. *, ** indicate significance at $P<0.05$, 0.01, respectively. NS indicate no significance at $P<0.05$

Correlation of the IC_{50} values with substitutions in the resistant genes of the isolates

Since the resistant genes of *pfcrt, pfdhfr* and *pfdhps, pfmdr1, pfmrp1, pfnhe* were associated with CQ, SP and QN response, respectively, subsequent analysis focused on SNPs within the above resistance-associated genes, utilizing genome sequencing data from previously reported isolates. As shown in Table 2, for *pfcrt*, M74I, N75E, K76T and A220S all reached fixation in the parasite population of this region, mutation rates at these positions occurred at 100% during 2008–2010. For *pfdhfr* that confers resistance to SP, S108N mutation rate approached fixation (100%) and C59R mutation rate appeared to have increased and reached 100% through the years. The I164L mutation rate was also highly prevalent at 78.53%.

Among the mutations in *pfdhps* that were associated with the resistance to sulfadoxine, the S436A mutation rate was 57.14%. The K540E mutation rate was relatively low at 8.82%. For *pfmdr1*, there was no mutation detected in the N86Y and N1042D of the parasite population, whereas the Y184F mutation rate reached 35.29% and appeared to have increased through the years.

For *pfmrp1*, H191Y, I876V and S437A exceeded 60% in the parasite population. For *pfphe* gene that associated with altered sensitivity to QN, H869Y mutation rate approached fixation (100%) and N894K mutation rate was 52.94%, there was no mutation detected in the V950G of the parasite population.

As shown in Table 3, of 31 isolates that have available *pfcrt* gene sequence in the dataset, 29 showing high level of the IC_{50} values(over 10 folds) have a *pfcrt* C**IETS** haplotype that has been reported to be associated with resistant to CQ[17], while 2 isolates that are sensitive to CQ have the wild type of *pfcrt* CVMNK haplotype (Table 3). Similarly, the isolates revealing high level of SP IC_{50} values harbored *pfdhfr* triple (**IRN**I and N**RNL**) or quadruple (**IRNL**) mutations, while the isolates with lower SP IC_{50} values have a single(NC**N**I) mutation in the *pfdhfr* gene (Table 3). These data indicated the sensitivities of the isolates to both CQ and SP were associated with the resistant-conferring mutations

Table 2 The prevalence of mutations in genes associated with drug resistance in different years

Gene	Mutation	2007(n = 4)	2008(n = 14)	2009(n = 14)	2010(n = 2)	Total
pfcrt[#]	C72S	0	0	0	0	0
	M74I	33.33	100	100	100	93.55
	N75E	33.33	100	100	100	93.55
	K76T	33.33	100	100	100	93.55
	A220S	33.33	100	100	100	93.55
pfdhfr	N51I	50	78.57	50	100	64.71
	C59R	50	92.86	100	100	91.18
	S108N	100	100	100	100	100.00
	I164L	50	78.57	78.57	50	73.53
pfdhps	S436A	50	57.14	57.14	100	58.82
	G437A	50	7.14	7.14	0	11.76
	K540E	25	0	14.29	0	8.82
	A581G	0	28.57	0	0	11.76
	A613S	0	0	0	0	0.00
pfmdr1	N86Y	0	0	0	0	0.00
	Y184F	25	35.71	35.71	50	35.29
	N1042D	0	0	0	0	0.00
pfmrp1	H191Y	75	64.29	78.57	100	73.53
	N325S	0	14.29	21.43	0	14.71
	S437A	75	64.29	78.57	0	67.65
	I876V	75	50	64.29	0	55.88
	F1390I	50	14.29	7.14	0	14.71
	K1466R	0	0	0	0	0.00
pfnhe	H869Y	100	100	100	100	100.00
	N894K	0	71.43	57.14	0	52.94
	V950G	0	0	0	0	0.00

[#] In the years 2007, 2008, and 2009, there was a loss of information at the *pfcrt* locus for one parasite isolate each year

Table 3 Comparison of IC_{50} values and substitutions in the *Plasmodium falciparum* resistant genes of the isolates

Isolates	CQ		SP			QN			
	IC_{50}	Hap in *pfcrt*	IC_{50}	Hap in *pfdhfr*	Hap in *pfdhps*	IC_{50}	Hap in *pfmdr1*	Hap in *pfmrp1*	Hap in *pfnhe*
X08.71yuan	436.75	CIETS	44697	IRNI	**A**GKAA	114.5	NYN	HNSIFK	**Y**NV
DS_10	819.45	CIETS	45582	IRNL	SGK**G**A	273.55	NYN	HNSIFK	**Y**NV
DS_11	513.55	–	2160.5	N**RN**I	S**A**KAA	483.9	NYN	**Y**N**AV**FK	**Y**NV
DS_14	491.5	CIETS	21796	IRNL	SGK**G**A	171.85	N**F**N	**YSA**IFK	YKV
DS_15	488.75	CIETS	45455.5	NRNL	**A**GKAA	310.6	NYN	**Y**N**AV**FK	**Y**NV
DS_16	37.815	CIETS	–	NRNL	SGK**G**A	169.1	N**F**N	**Y**N**AV**FK	YKV
DS_3	498.75	CIETS	69880	IRNI	SGK**G**A	292	NYN	HNSIFK	YKV
DS_4	490.3	CIETS	51679	IRNL	**A**GKAA	566.15	NYN	HNS**VI**K	YKV
DS_7	462.4	CIETS	28497.5	IRNI	**A**GKAA	630	NYN	**Y**N**AV**FK	**Y**NV
DS_8	538.65	CIETS	34570.5	IRNL	**A**GKAA	484.3	N**F**N	**Y**N**A**IFK	**Y**NV
DS_9	1089.1	CIETS	47784.5	IRNL	**A**GKAA	280.4	NYN	**Y**N**AV**FK	**Y**NV
F07.18	50.15	CMNKA	421.05	NC**N**I	S**A**KAA	92.09	NYN	YNAVIK	**Y**NV
F07.40	37.765	–	–	IRNL	**A**GKAA	103.7	NYN	HNSIFK	**Y**NV
F07.50	478.35	CIETS	–	IRNL	**A**G**E**AA	229.7	N**F**N	**Y**N**AV**FK	**Y**NV
F07.59	20.8	CMNKA	410.65	NC**N**I	S**A**KAA	189.8	NYN	YNAVIK	**Y**NV
F08B.17	230.45	CIETS	–	IRNL	**A**GKAA	133.7	NFD	HNSIFK	YKV
F08B.23	460.2	CIETS	137792.5	IRNL	SGK**G**A	80.745	N**F**N	**Y**N**AV**FK	YKV
F08B.33	397.75	CIETS	–	NRNL	SGK**G**A	179.5	N**F**N	HNSIFK	**Y**NV
F08B.39	267.95	CIETS	59891	IRNL	**A**GKAA	255.15	NYN	**Y**N**AV**FK	YKV
F08B.42	493.45	CIETS	22078.5	IRNL	**A**GKAA	492.55	NYN	**YSA**IFK	**Y**NV
F08B.49	417.7	CIETS	–	IRNL	SGKAA	306.9	N**F**N	**Y**N**AV**FK	**Y**NV
F08B.51	526.9	–	–	NC**N**I	S**A**KAA	376.6	NYN	YNAVIK	**Y**NV
F08B.52	550.55	CIETS	50019.5	IRNL	SGK**G**A	235.85	NYN	**Y**N**AV**FK	YKV
F08B.66	652.75	CIETS	28146	NRNL	**A**GKAA	487.35	NYN	YNAVIK	**Y**NV
F08B.74	781.5	CIETS	43863.5	IRNI	**A**GKAA	421.8	N**F**N	HNSIFK	**Y**NV
F09A.16	405.15	CIETS	67815.5	IRNL	SGK**G**A	108.27	N**F**N	HNSIFK	**Y**NV
F09A.54	620.85	CIETS	43152	N**RN**L	SGK**G**A	612.05	N**F**N	**Y**N**AV**FK	**Y**NV
F09M.1	600.45	CIETS	87157.5	IRNL	**A**GKAA	319.35	NYN	**Y**N**AV**FK	YKV
F09M.14	550.35	CIETS	77063.5	N**RN**L	**A**GKAA	242.75	NYN	**Y**N**AV**FK	**Y**NV
F09M.4	443.55	CIETS	54628.5	IRNL	**A**GKAA	128.95	NYN	**Y**N**AV**FK	**Y**NV
F09N.60	762	CIETS	43224	IRNL	**A**GKAA	346.6	NYN	**Y**N**AV**FK	**Y**NV
F09P.13	641.5	CIETS	57921	N**RN**L	**A**G**E**AA	241.4	NYN	**YSA**IFK	YKV
F09P.15	1001.3	CIETS	97734.5	N**RN**I	**A**G**E**AA	253.55	N**F**N	**YSA**IFK	**Y**NV
HDW_24	730.95	CIETS	42578.5	IRNL	**A**GKAA	199.65	NYN	**YSA**IFK	**Y**NV

a Haplotypes were based on amino acids at the positions *pfcrt* (72, 74, 75, 76, 220); *pfdhfr*(51, 59, 108, 164), *pfdhps*(436, 437, 540, 581,613);*pfmdr1*(86, 184,1042); *pfmrp1*(191, 325, 437, 876, 1390,1446);and *pfnhe*(869, 894, 950). Mutant residues are in bold

B"-" represent that genotype was missing in this parasite

in these resistant genes. Meanwhile, there was no significantly association among IC_{50} values of QN and haplotype of *pfmdr1, pfmrp1*, *pfnhe.*

Genetic marker associated with altered parasite sensitivities to anti-malarial drugs

To detect genetic marker associated with altered sensitivities to CQ,SP,PPQ,QN,PND and DHA, GWAS was conducted between the log-transformed IC_{50} value and SNPs from whole genome. A dataset of 15,701 SNPs with minor allele frequency (MAF) > 0.05 was derived from *P. falciparum* isolates (n = 34) previously characterized [13], enabling subsequent GWAS analysis. The association between SNP genotypes and log-transformed IC_{50} values (treated as a continuous dependent variable) or original RSA values were analysed by PLINK.

The principal gene *pfcrt* associated with CQ resistance has been well characterized and is often used as the proof of principle in GWAS. To internally validate

GWAS, associations with the established CQ resistance marker in the *pfcrt* gene were prioritized for analysis. GWAS with log-transformed IC_{50} identified 28 SNPs that were significantly associated with the response to CQ, 4 of which located in the CQ resistant *pfcrt* gene. As shown in Fig. 3 and Table S2, *pfcg1* and *pfcg2* were also significantly associated with the reduced sensitivity to CQ in the study parasite population. In addition to *pfcrt* and its flanking genes, one SNP in PF3D7_0708400 (*pfhsp90*) and one SNP in PF3D70410000 (*pfevp1*) also had significant association with IC_{50} values of CQ. For SP, one SNP located in *dhfr* was significantly ($P=3.332\times10^{-5}$) associated with the reduced sensitivity to SP. 54 SNPs were also significantly identified by GWAS, which located in 35 genes including *eba175, pfcrt, pfcg1* (Fig. 3, Table S2). For in vitro sensitivities to PND, significant associations were identified with 3 SNPs, one of which was located in *sf1*. However, no SNP was found to be significantly associated with reduced sensitivity of PPQ and QN.

GWAS with RSA value of DHA identified 30 SNPs that were significantly associated with the RSA values. Among these SNPs, 3 were located in a region of 85 kb ~ 335 kb around *kelch13*, one of which was located in inner membrane complex protein gene *imc1f*.This protein IMC1F has been found association with the *kelch13* compartment in schizont and merozoite stages of *P. falciparum* [18]. Meanwhile, 2 SNPs were located in *myod* and *ccp4* gene, respectively. One member of the ApiAP2 transcription factor family (PF3D7_0613800) also showed a strong association with RSA values of the parasites.

Discussion

In the study, the in vitro sensitivities of isolates to anti-malarial drugs and GWAS analysis were conducted to investigate the association between SNPs and sensitivities of anti-malarial drugs. Compared with other region of the GMS such as Thailand and Cambodia, China-Myanmar border has a different history of anti-malarial drug usage. In our study, *P. falciparum* isolates from this region still exhibited high drug resistance to both CQ and SP. Consistent with previous reports [19], the mutation frequency in *pfcrt, pfdhfr* and *pfdhps* genes were very high, which may probably be due to the widespread use of CQ to treat sympatric *P. vivax* infections and the use of antifolate drugs for treating bacterial infections. According to the results, the proportion of the isolates that showed reduced sensitivity to PPQ in vitro assays was 8.82% in the China-Myanmar border area, similar to the results (6.5% and 5.1%) that have been reported [19, 20]. Although the parasites showed reduced sensitivity to QN in the China-Myanmar border area, there was no significant association between QN IC_{50} value and haplotype of *pfmrp1, pfmdr1* and *pfnhe*.

The principal gene *pfcrt* associated with resistance to CQ have been well characterized and are often used as the proof of principle in GWAS. The association between *pfcrt* and IC_{50} values of CQ was significantly detected in the study. In addition, *cg1* and *cg2* genes were identified associated with CQ resistance, which were previously identified to be linked to the CQR phenotype [21]. One SNP on PF3D7_0708400 (*pfhsp90*) also showed significant association with IC_{50} values of CQ. A recent study uncovered that the molecular chaperone *pfhsp90* regulates the abundance and activity of the histone-deacetylase *pfsir2*, a prominent regulator of *Plasmodium* epigenome [22]. Apart from the above genes, association between mutation of *pfevp1* and IC_{50} value of CQ was also identified in the study. It has been found that the expression of EVP1 results in a change in TVN-mediated lipid import at the host membrane and that it is required for intracellular parasite growth, but not invasion [23].GWAS was also used to discover the PPQ resistance-associated mutations, while no significant association was found between SNP set and in vitro IC_{50} values of PPQ, one reason maybe that the IC_{50} data are inadequate to assess piperaquine resistance. Previous study showed that only 5.6% parasite isolates displaying PSA values of > 10% in China-Myanmar border area [24]. Artemisinin resistance is associated with mutations in the propeller domain of the Kelch family protein K13 [25, 26]. Meanwhile, more and more non-K13-mediated resistance mechanisms have been discovered [27]. *Plasmodium falciparum* on the China-Myanmar border had distinctive origin of artemisinin-resistant. It is of great concern to find SNPs associated with artemisinin resistance here. GWAS of RSA data revealed significant association of 3 SNPs within an 85–335 kb genomic region flanking the *K13* locus, including one SNP localized in inner membrane complex protein gene *imc1f*. This protein IMC1F has been found to be associated with the *kelch13* compartment in schizont and merozoite stages of *P. falciparum* [19].

Meanwhile, 3 loci located in vacuolar protein sorting-associated protein (VPS11,VPS35) were suggestively significant. These proteins are crucially involved in protein trafficking, trans-membrane, vesicular transport and parasite survival, and have been found participating in oxidative stress-mediated anti-malarial activity of plakortin [28].

The study has certain limitations due to its small sample size and the relatively early collection period. For instance, the small sample size may have precluded the detection of certain variations. To reduce the impact of sample size and temporal constraints on SNP

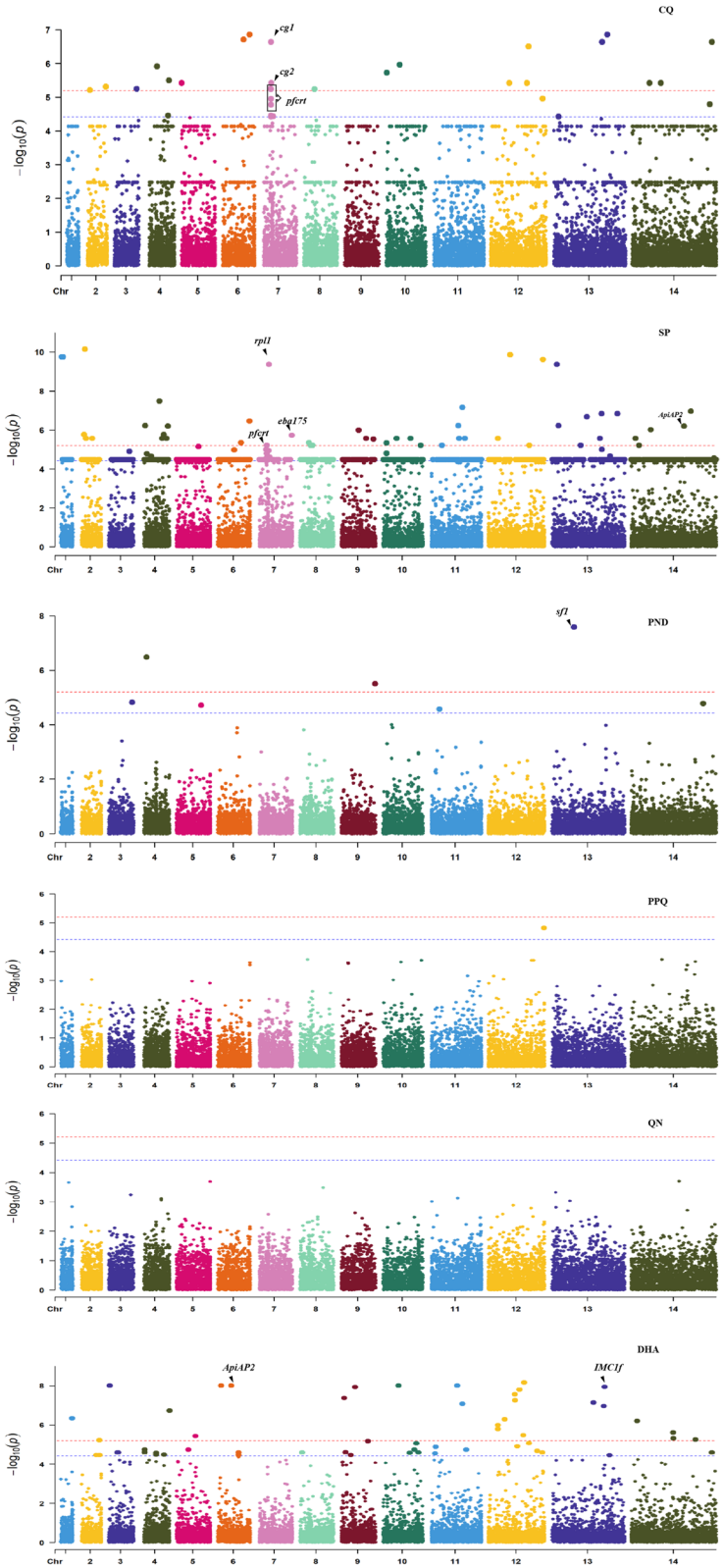

Fig. 3 A summary representation of genome wide association results of the P. falciparum to 6 anti-malarial drugs. Each point represents 1 of 15,701 SNPs with MAF > 0.05 in a set of 34 isolates. The dashed red horizontal line indicates the significance threshold of a *P value* of 6.37×10^{-6} after Bonferroni correction and the blue one indicates the suggestive threshold of a *P value* of 3.82×10^{-5}. GWAS analysis with log-transformed phenotype data by plink. *CQ* chloroquine, *SP* sulfadoxine- pyrimethamine, *PPQ* piperaquine, *PND* pyronaridine, *QN* quinine, *DHA* dihydroartemisinin

detection, future studies should consider expanding the sample size and extending the sample collection time frame. Despite the above limitations, the study still offers important insights into the research topic. Some potential markers that may modulate susceptibility to key anti-malarials were identified. Although these genetic markers were identified in isolates from over a decade ago, they still possess significant referential value for current drug resistance surveillance. These historical data serve as a foundational baseline, facilitating an enhanced understanding of the evolutionary trajectories and dissemination pathways of resistance mechanisms. Consequently, these historical data may offer a preliminary scientific foundation for resistance surveillance and management.

Conclusions

The history of anti-malarial drug usage is complicated in the China-Myanmar border region and the *P. falciparum* isolates had different genetic backgrounds in that region. In the study, multiple candidate SNPs associated with drug-resistance were identified, especially for CQ and artemisinin resistance. The association between oxidative stress-mediated activity and anti-malarial drug susceptibility has been identified and requires further validation.

Abbreviations

CQ	Chloroquine
SP	Sulfadoxine-pyrimethamine
PY	Pyrimethamine
PPQ	Piperaquine
QN	Quinine
PND	Pyronaridine
ACT	Artemisinin-based combination therapy
GMS	Greater Mekong Subregion
SNPs	Single nucleotide polymorphisms
DHA	Dihydroartemisinin
CMB	China-Myanmar Border
GWAS	Genome Wide association study
RSA	Ring-survival rate
IC_{50}	Half maximal inhibitory concentration
HN	Hainan Island of China
VSP	Erythrocyte vesicle protein
TVN	Tubovesicular network

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05319-4.

Additional file 1: Fig. S1. The sampling site map for *P. falciparum* isolates utilized in the study.

Additional file 2: Table S1. Details for number of studied samples and year of collection

Additional file 3: Table S2. Significant SNP set associated with anti-malarial drugs

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Author contributions

YNT and DMZ conceived and designed this study. RY and YFH performed the experiments. YNT analyzed the data. YNT, RY, and DMZ drafted the manuscript. All authors reviewed the manuscript.

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Availability of data and materials

Illumina sequencing reads from this study are available in the European Nucleotide Archive (PRJEB32255). The SNP data are available in the European Variation Archive (PRJEB34415).

Declarations

Ethical approval and consent to participate

Scientific and ethical clearance of the study was obtained from the Internal Review Board of Naval Medical University. Written informed consent for study participation was obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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