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Genetic features of *P. falciparum* parasites collected in 2012-2016 and anti-malaria resistance along China-Myanmar border

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Abstracts

Backgrounds The therapeutic efficacy study (TES) of Dihydroartemisinin-Piperaquine (DHA-PIP) for uncomplicated *P.falciparum* patients had implemented during 2012-2016 along China (Yunnan province) -Myanmar border. The study focusing on the genetic features of falciparum parasites based on *in vivo* parasite clearance time (PCT) was investigated to explore if they had produced potential resistance to DHA and PIP at molecular level.

Methods The genetic features were invested based on K13 propeller genotypes, Copy numbers of genes PfPM2 and Pfmdr1 and 9 microsatellite loci (Short Tandem Repeats, STR) flanking the K13 gene on chromosome 13. The (PCTs) , copy numbers, expected heterozygosity (*He*), coefficient of gene differentiation (F*st*) were calculated, compared and analyzed in Graph Prim5.0 and SPSS 23 with method of One-way Anova (Post Hoc test:LSD) or Nonparametric tests. Genetic diversity (*He* and F*st*) of parasites was estimated in Excel 2016 with method of GenAlEx 6.501.

Results In NW (North-West Yunnan province bordering with Myanmar) area, F446I prevalence was 58.96% (79/134) and no significant different PCT50 presented between any two K13 groups (Chi-Square=2.35, P=0.31, df=2). But the PCT50 of parasite isolates in this area (12.10, 10.72-13.47, n=136 hrs) was significantly shorter than that in SW (South-West Yunnan province bordering with Myanmar) area (P=0.036, t=-2.11, df=174) where isolates all showed wild K13 genotype. For the copy numbers of Pfmdr1 gene, 14.63% (18/123) and 62.5% (10/16) parasite isolates showed amplification (≥ 1.6) in NW and SW areas, but for those of PfM2 gene, none of them did. Based on STR data, *He* values of parasite isolates of 4 groups lined as ML group (Menglian County in SW), F446I group, Others (Non-F446I K13 mutation) group and W (wild

K13 genotype) group from low to high. All of them showed significant with one another (Chi-Square=25.20, df=3, P=0.000). The mean F*st* value of 4 groups at 9 different loci (0.410±0.067) were significant higher than that of 3 NW groups (0.238±0.041) (Paired-T Test: t=-3.220, df=8, P=0.009). Highest mean F*st* (0.320±0.053) presented between ML group and W group when all mean F*st* values between W group and each of other 3 groups were compared.

Conclusions According to our study, we are inclined to conclude that no confirmed resistance of *P.falciparum* parasites to DHA and PIP occurred, although parasite isolates with prolonged PCTs were observed. Potentially resistant parasite isolates are widely spread along the Yunnan-Myanmar border based on the genetic index of K13, PfM2/3 and Pfmdr1, along with great genetic differentiation based on different areas and K13 mutations. F446I is the domain K13 mutation allele in NW area and seem to have emerged newly in those years.

Key words: Therapeutic Efficacy Study; Dihydroartemisinin-piperaquine (DHA-PIP); Anti-malaria resistance; China-Myanmar border, K13

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Backgrounds

In response to the widespread Chloroquine (CQ) resistance, the use of Artemisinin-based Combination Therapy (ACT)-the use of a potent short-acting artemisinin and a less-potent, long-acting partner drug was recommended world widely for the treatment of uncomplicated *Plasmodium falciparum* malaria [1-3]. One of the ACTs Dihydroartemisinin-Piperaquine (DHA-PIP), was started to be wildly applied in Southeast Asia as the first-line antimalarial

treatment for *P.falciparum* malaria since 2000s [1-2]. Recently, it was also applied in Africa, where *P. falciparum* malaria is hyper-endemic [4-10].

However, following the widespread resistance to CQ and other antimalarial drugs, *P.falciparum* parasites produced resistance to DHA-PIP too, shortly after it was widely used in Southeast Asia, initially showing prolonged parasite clearance time (PCT) and subsequently treatment failures as the partner drug also failed [11-24]. The epicenter of the resistance was Cambodia where CQ resistance had also been first emerged [1-2, 11-20]. This resistance rapidly spread to other neighboring countries, such as Thailand, Vietnam, laos, PDR and Myanmar, which all are part of the Greater Mekong sub-region (GMS) countries [25-28].

A few studies revealed that the decreasing efficiency of DHA-PIP in the GMS involved resistance to both DHA and PIP [11-21]. Based on the single nucleotide polymorphisms (SNPs) examination and correction analysis with genomic tools, mutant K13-propeller alleles, such as C580Y, R539T, Y493H, I543T, were verified to be correlated with the spread of resistance in west Cambodia and other GMS countries[29]. This finding alerted close attention to K13 mutation of *P.falciparum* parasites world widely. Furthermore, because the resistance of *P.falciparum* to the fast-acting artemtisinin component meaning longer exposure of the parasite to the long-acting partner drugs, the more chances of parasite survival [11-21], the resistance to PIP appeared too in the same place where artemisinin-resistant parasites were first reported. Genome-wide association studies revealing that amplification of two protease genes plasmepsin 2/3 (PfPM2/3) was associated with clinical resistance to PIP in Cambodia, established makers for resistance of parasites to PIP [30-31].

In China, since 1979, PIP had been used extensively as a replacement drug of CQ [32-33]. Also, artemisinin drugs had been deployed mostly as monotherapies prior to 2005 [32]. In 1990s, a series of *in vitro* microtechnique assays showed that resistance to multiply antimalarial drugs (Pfmdr) was present in South-west Yunnan Province bordering with Myanmar, including both PIP and some artemisinin drugs [34-37]. After 2005, the national drug policy had changed first-line anti-malaria drugs to ACTs, such as DHA-PIP, Amodiaquine artesunate, artemisinin naphthoquine, artemisinin piperaquine. DHA-PIP was mostly applied in most of China [32].

The surveillance study on efficacy and safety of anti-malarial drugs for the treatment of uncomplicated malaria (TES) was carried out and supported by WHO since 2008 [38-39]. Overall, the clinical follow-ups showed that DHA-PIP in 2007-2013 remained highly efficacious for treating uncomplicated *P. falciparum* in China-Myanmar border [39]. In this study, genetic features of *P. falciparum* isolates collected during 2012 -2016 would be analyzed to explore their potential threat and the resistance situation in this region.

Method

Study sites

From 2012 to 2016, malaria patients were enrolled from 4 surveillance sites, Menglian county, Tengchong county, Yingjiang county, and Ruili city which are all bordering with Myanmar. Among them, Menglian county belongs to Pu'er prefectures located in South-west (SW) Yunnan province. The other 3 sites are part of Baoshan and and Dehong Prefectures respectively

and located in North-west (NW) Yunnan province (See Fig1).

Study population

People infected with uncomplicated *P. falciparum* malaria, satisfying the inclusion and exclusion criteria, attending the study health clinic, aged between 6 months and 60 years old, except unmarried women 12-18 years old, were enrolled in the TES, treated on site with 3-day DHA-PIP and monitored weekly for 42 days. The follow-up consisted of the fixed schedule of check-up visits and corresponding clinical and laboratory examinations according to the WHO protocol. All adult patients signed an informed consent form for participation. Parents or guardians gave informed consent on behalf of children. Children over 12 years of age signed an informed assent form.

Treatment

For falciparum patients were treated with DHA-PIP once daily for 3 consecutive days with a dose of 2mg/kg/day DHA and 16mg/kg/day PIP. One tablet of DHA-PIP contained 40mg of DHA and 320 mg PIP according to the therapeutic dosage recommended by WHO. DHA-PIP came from WHO.

All doses of medicine were supervised and patients were observed for 30 min after medicine administration for adverse reactions. The person giving treatment signed at the treatment sheet of the Case Report Form after every drug administration.

Microscopic blood examination and blood collection

Thick and thin films of blood were made on slides on D0, D1, D2, D3, D7, D14, D28, D35, D42. Two to three drops of blood were collected on filter paper on D0 and the day of failure.

Parasite Clearance time (PCTs)

Based on parasitemia data collected during following-up each case, PCTs for each case was calculated using WHO parasite clearance estimator WHOApplication-6-18. Data of Slope of Half life (H) and duration that 50%, 75%, 90%, 95% and 99% parasites was cleared (PCT 50, PCT 75, PCT 90, PCT 95 and PCT 99) were collected. Only PCT 50 data were used in comparison based on different genetic types and sites.

Sequencing the K13-propeller domain

The K13-propeller domain was amplified according to the description of Ariey, et al [32]. The high-fidelity Taq DNA polymerase (Takara 9158A) was pursed from Takara Biomedical Technology (Da Lian) Co., Ltd. The PCR products were sequenced by Map Biotech Co, Ltd (Shanghai) and the sequences were aligned with that of wild Pf37D to confirm the single nucleotide polymorphisms (SNP).

All parasite isolates with completed following-up data were divided into different groups according to their K13 genotypes.

Estimation of copy number PfPM2 and Pfmdr1

Sequences of PfPM2 and Pfmdr1 were amplified according to the methods described by Witkowski, et al [31]. The TB Green™ *Premix Ex Taq*™ II (Tli RNaseH Plus) used in test was pursed from Takara Biomedical Technology (Da Lian) Co., Ltd. The copy numbers of PfPM2 were estimated by the equitation of Y=0.4583x+0.7109, ($x=2^{-\Delta Ct}$, ΔCt=Ct *PfPM2* - Ct *Pfβ-tubulin*). The copy numbers of Pfmdr1 were estimated by the equitation of Y=0.4497x+0.8976, ($x=2^{-\Delta Ct}$, ΔCt=Ct *Pfmdr1* - Ct *Pfβ-tubulin*).

Microsatellite loci genotyping

Nine microsatellite loci (-56.0Kb, -50Kb, -6.36Kb, 1.7Kb, -0.15Kb, 8.6Kb, 11Kb, 15.1Kb, 31.5Kb) flanking the K13 gene on chromosome 13 were tested according to the method described by Talundzic, et al [1] and analyzed in Excel2016 with method of GenAlEx 6501. Genetic diversity was estimated using expected heterozygosity (*He*) and coefficient of gene differentiation (*Fst*).

Data analyses

All the data were collected in Excel 2016. They were calculated, compared and analyzed in software GraphPad Prism 5.1 and SPSS 23. The mean values and 95% Confidence Interval (CI) of mean values were calculated with Column statistics method in Column analyses. Significance difference（$P < 0.05$, significant; $P \geqq 0.05$, no significant）was evaluated by One-Way-ANOVA(Post-Hoc Multiple Comparisions: LSD) in SPSS 23. When the $P < 0.05$ in the Test of Homogeneity of Variances, Independent-Samples-T-Test would be used for further analysis. If $P < 0.05$ in Levene's Test for Equality of Variances presented, Nonparametric Tests (2 Independent-Samples-Tests, Test type: Mann-Whiteney-U) would applied instead of T-Test.

Results

General briefing of samples testing

In NW area, 176 patients were CFU. Among them, 4.55% (8/176) patients showed D3 positive, while 2.50% (1/40) in SW area.

Genotypes of K13 mutations

Among 174 parasite isolates which obtained K13 genotypes, 46.90% (99/174) present K13 mutations (See Table 1) and all of them were from NW area. In SW area all 40 isolates showed K13 wild genotype.

In NW area, the total K13 mutation rate was 73.88% (99/134) and the F446I prevalence was 58.96% (79/134). Most of the isolates in NW area were collected from Yingjiang county (59.20%, 103/174), with a K13 mutation prevalence of 85.44% (88/103) there. Also in NW area, prevalence of K13 mutations in different years showed dramatically increase from 2012 (49.09%, 27/55) to 2013 (95.74%, 45/47) and 2014 (92.31%, 24/26).

The composition structure of all K13 mutational isolates was shown in Fig 2. A total of 11 mutation alleles were found, in which most of them were F446I (80%, 79/99) and followed by P574L (7%, 7/99). The other mutations alleles showed very low prevalence of 1-2%, such as C469Y (2%, 2/99), C580Y (2%, 2/99), N458Y (2%, 2/99). Most of the mutation alleles were found in Yingjiang County. In Tengchong county and Ruili city, only 2 K13 mutations alleles were detected in each site (Table 2).

Based on the K13 genotypes, 129 isolates with CFU data in NW area were divided into 3 groups. In details, 34 isolates with K13 wild genotype were classified as W group, 75 with F446I as F446I group, 20 with other K13 mutational genotypes as Others group. Forty isolates with K13 wild genotype in Menglian county in SW area were defined as ML group.

Comparison of PCT50 based on K13 genotypes

The average H, PCT50 and PCT99 in NW area were 4.96 (4.46-5.46, N=129), 12.85 (11.54-

14.16, N=129) and 34.22 (31.75-36.69, N=129) hrs respectively. The PCTs data of 4 groups were shown in Table 3. The average H of W and F446I groups were 4.26 (3.06-5.47, N=34) hrs and 4.51 (3.87-5.14, N=71) hrs respectively, while those of Others and ML groups were a little more than 5 hrs. However, all of the average PCT99 were less than 49 hrs.

The PCT50 of different groups were compared and the results are shown in Fig 3. The mean PCT50 of 3 groups in NW area were 10.36 (7.69-13.03, N=34) hrs in W group, 12.63(10.94-14.3, N=75) hrs in F446I group, and 13.98 (8.65-19.31, N=20) hrs in Others group. No significant different PCT50 presented between any two groups in this area (Chi-Square=2.35, P=0.31, df=2). For the parasite isolates in ML group, their mean PCT50 was 15.41 hrs (12.02-18.80, N=40) and significantly higher than those in W group and F446I group. (Z=-3.09, P=0.002; Z=-2.24, P=0.025).

Comparison of PCT50 based on time

Details of K13 mutations of isolates collected in 2012 and 2013-2014 were shown in Table 4. Among parasites collected in 2012, 48.15% (26/54) showed K13 mutations and 57.69% (15/26) of the mutations were F446I. The total F446I prevalence were 27.78% (15/54) in this year. For total isolates in 2013 and 2014 (2013-2014 isolate), 95.65% (66/69) of them showed K13 mutation and 86.36% (57/66) of the mutations were F446I. The total F446I prevalence were 82.61% (57/69) in these 2 years.

The comparison of mean PCT50 between isolates of 2012 (11.43, 9.44-13.41, N=54) and 2013-2014 (13.61, 11.53-15.70, N=69) in NW area showed no difference (P=0.14, t=-1.49, df=121).

Comparison of PCT50 based on sites

The PCT50 of parasite isolates in NW area (12.10, 10.72-13.47, n=136) hrs was significantly shorter than that in SW (ML group) area (P=0.036, t=-2.11, df=174). The mean PCT50 of isolates in Tengchong and Yingjiang were 11.87 (9.31-14.43, N=28) and 12.32 (10.69-13.95, N=102) separately. However, no significant difference presented between each of these 3 surveillance sites (F=2.11, P=0.13). Data of those in Ruili site were not analyzed because only 6 cases were included.

Amplification of PfM2 and Pfmdr1 gene

For the PfM2 gene, no amplification (copy number<1.6) was present in all isolates, though the mean copy number of isolates in NW area (0.95, 0.93-0.96, N=126) was significantly lower than that in SW area (1.08, 1.00-1.16, N=15) (Z=-3.36, P=0.001) (See Fig 4).

For the copy numbers of Pfmdr1 gene, 14.63% (18/123) isolates in NW area and 62.5% (10/16) isolates in SW area showed more than 1.6, with mean values of 1.39 (1.35-1.42, N=123) and 1.63 (1.48-1.77, N=15) separately. The difference between their average numbers was significant (P<0.001, t=4.05, df=137). The mean copy numbers of Pfmdr1 gene based on K13 genotypes were also calculated and compared (see Fig 5). They were 1.41 (1.34-1.48, N=31) in W group, 1.37 (1.31-1.42, N=69) in F446I group and 1.40 (1.34-1.47, N=20) in Others group, with 19.35% (6/31), 13.04% (9/69) and 10% (2/20) isolates showing copy numbers of more than 1.6 in each group. There were no significant difference between every two groups in NW area (F=0.61, P=0.55, df=2), but they were significant lower than that in SW area (Z=-3.21, P=0.001).

There was no significant difference on PCT50 between isolates with single and double Pfmdr1 copy numbers (P= 0.50, t=-0.67, df=137).

Correlation coefficient (r value) between PCT50 and copy numbers of Pfmdr1 gene was estimated. No strong correlations presented (r=0.056, P=0.52).

Reduced genetic *He* of the mutational K13 propeller allele and increased F*st* between parasites of ML group and W group

He values of parasite isolates were calculated basing on STR data and grouped according to different K13 genotypes and areas were shown in Fig 6. Their mean *He* values were lined as ML group, F446I group, Others group and W group from low to high. In ML group from SW area, *He* values of parasites at all loci sites was 0 (N=8). In NW area, average *He* value of F446I isolates was 0.15 (0.014-0.28, N=71) with each individual value of less than 0.30 at all loci except of one which was 0.52 and located at upstream -56Kb of K13 gene. The *He* values in Others group were different greatly from 0 to 0.65 with a mean value of 0.39 (0.20-0.58, n=7). The mean *He* value in W group was the highest among 4 groups which was 0.65 (0.52-0.78, n=34). All the mean *He*s showed significant difference (Chi-Square=25.20, df=3, P=0.000) with one another.

F*st* values of parasite isolates were also calculated basing on STR data and grouped according to different K13 genotypes and areas. Nearly all F*st* values at 9 different loci (See Table 5[40]) between different groups were higher than 0.05, except of that at the site of -56Kb, which presented 0.048 between Others group and W group. The mean F*st* values of 4 groups at 9 different loci (0.410±0.067) were significant higher than that of 3 NW groups (0.238±0.041) (Paired-T Test: t=-3.220， df=8， P=0.009). The highest difference between them presented at the sites of -0.15Kb (0.743 and 0.143) and 1.7Kb (0.474 and 0.213) which were closest to K13 genes among 9 loci along chromosome 13. The mean F*st* values between W and each of other

3 groups at 9 loci were also showed in Table 5. That between ML and W groups were significantly higher than other 2 pairs (Paired-T Test: t=-2.659, df=8, P=0.029; t=-4.966, df=8, P=0.001). However, F*st* of F446I group and W group at site of 1.7 kb closing to K13 gene did not show lower value than that of ML group and W group.

Discussion

Following the spreading pattern of resistance to chloroquine and sulfadoxine/pyrimethamine which firstly emerged in the South East Asia region in 1970s and then expanded to Africa [29,33,41], ACTs resistance appeared in Cambodia and then spread to nearly the whole GMS [16, 25-28, 42-43].

Among neighboring countries, drug policies may differ considerably. So, parasites populations at borders might experience divergent drug selection pressures, favoring the emergence of multiple drug resistant parasites [23, 26-27, 44-48]. Yunnan province of China is one part of the GMS. Higher malaria prevalence along the China-Myanmar border was reported than along China-Vietnam and China-Laos border [49], and this area was also the main source of imported malaria in Yunnan Province. Thus, surveillance sites along the China-Myanmar border were strategically chosen to carry out TES studies in 2008-2016 in China [38-39,50]. In contrast to the other GMS countries where artemisinin partial resistance is present, the *in vivo* results showed that DHA-PIP was still highly efficacious in China [39]. To survey the spread and development of malaria resistance, a few molecular studies on antimalarial drugs resistance along China-Myanmar border were reported sequentially in

recent years, shortly after the K13 allele was identified as the marker for *P. falciparum* isolates resistance to artemisine. All results verified an outstanding genetic features of *P. falciparum* isolates in NW area, that F446I was the domain K13 mutation allele, but no F446I were found in SW area [33, 51]. Our findings in NW falciparum isolates agreed with these findings. This was distinctly different from some other GMS countries, such as in Cambodia and Thailand whose dominant K13 mutation was C580Y, though F446I allele was sporadically reported there. This difference might be explained by the parasites' accumulative adaptations to their sustained different host environments [52]

The parasite lineages with C580Y allele along the Thai-Cambodia and Thai-Myanmar borders were observed to have originally evolved independently [20]. In our study, mean *He* value of 0.15 (0.014-0.28, N=71) in F446I isolates was lowest among 3 NW groups and also lower than that of C580Y isolates (mean *He* = 0.3526 ± 0.08, N = 26) [1]. Low *He* value meant low diversity within isolate, which might indicate that F446I isolates had experienced shortest selection pressure or least gene exchanges with other genotypes since they appeared. Furthermore, mean F*st* value of 0.161 (±0.032) at 9 microsatellite loci implied great differentiation had produced between F446I group and W group. Thus F446I isolates might be inferred to emerge newly in those years in the process of adapting to its environmental pressures such as from antimalarial drugs and then spread along NW Yunnan-Myanmar border as the C580Y isolate had did along Thai-Cambodia and Thai-Myanmar borders [20]. Otherwise, parasite isolates in W group with a little higher than moderate variations (mean *He*=0.65, 0.52-0.78), were the most polymorphisms among all 4 groups which was similar with other reports [1]. The mono-morphisms (*He*=0) in ML group was in accord with

previous genotype analysis which were conducted based on investigation of the three polymorphic genetic markers msp1, msp2, and glurp and also were consistent with their background of a *P. falciparum* outbreak[53]. However, higher F*st* value (mean =0.320±0.053) between ML and W groups than between other pairs hinted parasites in this group had differentiate greatly from W isolate than other K13 mutational isolates did. This increased F*st* values happened to correspond to the higher PCTs in this group than in other 3 groups.

Some field studies showed that the efficacy of DHA-PIP decreased with the increasing of prevalence of mutant kelch13 alleles [16, 20, 25, 54]. For example, in Pursat, Cambodia, where the prevalence of mutant kelch13 alleles had increased from 40% in 2003–2004, to 77% in 2012–2013, the efficacy of DNA-PIP had decreased from 98% in 2005, to 63% in 2012–2013[16]. In our study, great increase of prevalence of mutant K13 alleles was also observed from 2012 to 2013-2014. This increase was mainly due to the spread of F446I prevalence. However, no corresponding decrease of the efficacy of DHA-PIP was observed in 2 different periods. This might be because the limited patient data and short interval.

The dominant K13 mutation, C580Y, had been accepted as artemisinin resistance-conferring mutation and many DHA-PIP treatment failures were widely believed to be due to it [11-17, 25-28]. In our study, falciparum parasites in F446I group showed higher H value (4.51, 3.87-5.14 hrs) than those reported in Tanzania isolates (< 3 hrs) and Uganda isolates (2.15, 1.64-2.61 hrs) [55-56] , but less than those isolates such as in Central Vietnam (6.2 hrs) [57]. Inferring from the PCT99 data of F446I isolates (34.12 hrs, 30.71-37.53 hrs), F446I isolate might show higher PCT100 than that in Uganda (17.55 hrs, 14.66–20.66 hrs) [56], but less than those in Cambodia-Thailand border (65.6 ± 27.3 hrs) [58]and some areas in Vietnam

(>60 hrs) [28, 57]. On account of above comparison, it might deduced that though F446I isolates had showed prolonged PCTs, they were still relatively more susceptible to DHA than C580Y isolates did in some GMS countries. Following this point, the D3 positive rate (4.82%, 8/166) in NW area and in vitro results of F446I, C580Y isolates to DHA under same condition were reviewed. Their results both supported the point in some way [4, 8, 10, 28, 55, 57, 59]. In this study, F446I isolates did not show significant longer PCTs than those in W group, this meant mutation of F446I along China-Myanmar border was not confirmed as the resistant marker. This view was contrary to other research results [50, 59-60]. Bases on careful comparison, the conflict was attributed to the fact that this study was *in vivo* test and the internal period that the protozoemia was checked was every 24 hrs in this study, which was different from the *in vitro* tests and 8 hrs internal period in other studies. Additionally, the coexistence of greatest prolonged PCTs and highest differentiation in ML isolates from W parasites showed that resistance of parasites to artemisinin drugs might be influenced by polygenic loci beside of K13 gene. However, samples and STR data were limited in this study. Only 20 Non-F446I K13 mutational isolates were collected and they had to be gathered in Others group and analyzed together. STR data of Ohters group and ML group only based on 7 and 8 isolates respectively might influence the results in some degree. So more studies were required to confirm the points.

Some reports inferred that through the exposure of greater parasite biomasses to ACTs *in vivo*, artemisinin resistance promoted the evolution of resistance to partner drugs such as PIP [16, 28, 31,]. So, following the K13 detection, copy numbers of PfPM2 and Pfmdr genes which

indicate PIP and multiply resistance were also estimated in our study. However, our results were contrary to or did not supported previous findings.

In some reports, PfM2/3 copy number accounts for bimodal PIP resistance among Cambodian *P. falciparum* isolate [15, 30-31, 61]. Oppositely, no amplifications of PfM2 (1.08 ±0.080) were found in all 4 groups in our study though the copy numbers in ML group were significantly higher than in NW area. So evidence in this study did not revealed the probability that artemisinin resistance promoted the evolution of resistance to partner drugs such as PIP in China-Myanmar border.

However, impulsive conclusions that PIP resistance had disappeared along the China-Myanmar border could not be made based only on this limited data, because there were quite a few conflicting reports about the amplification of PfM2 and PIP resistance [62-63]. For example, overexpression of PfM2/3 did not directly cause reduction in *P. falciparum* sensitivity to artesunate and piperaquine according to *ex vivo* assays [62]. Furthermore, cross-resistance between PIP and CQ might be another clue. There was a study which ascribed the recent treatment failure of DHA-PIP to the existence of parasites with reduced PIP susceptibility because PIP monotherapy was widely used in Cambodia in 1990s [64]. This condition was same with the history in China. PIP monotherapy was also widely applied and resistance were reported in Yunnan province in China in 1990s [32-33,35]. At that time, the rapid emergence of PIP resistance in China was explained by the cross-resistance between PIP and CQ [41, 63].

In our study, amplification of Pfmdr1 were found in both NW and SW areas with percentage at 14.63% (18/123) and 62.5% (10/16) which implied that Pfmdr could be widely spread in

these two areas. The significant higher mean PCTs and higher mean Pfmdr1 copy number than in NW isolates concurrently presented in SW parasite isolates just catered to a point that the increase of copy number of Pfmdr1 gene was related to the resistance of artemisinin and its derivatives revealed by a series of studies in laboratory and field [65-67]. However, the fact that no difference of PCT50s presented between single Pfmdr1 isolates and multiple ones, and no correlation presented between PCT50 and copy numbers of Pfmdr1 gene did not give more support to this point. So close surveillance and further study are required to clarify this complex problem.

Conclusions

According to our study, no confirmed resistance of *P.falciparum* parasites to DHA and PIP had occurred, although parasite isolates with prolonged PCTs were observed. The prolonged PCTs seemed to relate to Pfmdr1and polygenetic sites beside of K13 gene. However, our data did not give full support. So more evidence is required. Potentially resistant parasite isolates are widely spread along the Yunnan-Myanmar border based on the genetic index of K13, PfM2/3 and Pfmdr1, along with great genetic differentiation bases on different areas and K13 mutations. F446I was the domain K13 mutation allele in NW area but not in SW area. The F446I isolates seem to have emerged newly in those years but have not produced strong resistance to DHA based on *in vivo* data. Close genetic surveillance is still warranted especially on imported Pf malaria cases along the Yunnan-Myanmar border.

Competing interests

The authors declare that they have no competing interests.

Ethical approval

According to the Helsinki Declaration, ethical approval for the study was granted by the Ethics Committee of National Institute of Parasitic Diseases, China CDC. The purpose of the study was explained and then approval was sought from patients and their caretakers. Informed written consent was obtained from patient or carers of Child patients. All results were kept confidential and were unlinked to any identifying information.

Consent for publication

All authors have read and agreed to publish this article.

Availability of data and material

The data and material in this file were available by contacting Dr Mei Li, li_mei76@163.com.

Authors' contributions

Li M, Liu H, Tang LH and Yang HL designed the study and developed the protocol. Li M processed all the samples and analyzed the data, and wrote the first draft of the paper. Tu H helped to analyze the data and draw the map. Liu H, Tang LH and Maria Dorina Geluz Bustos revised the paper and ensured quality TES implementation. All authors read and approved the final manuscript.

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Figures

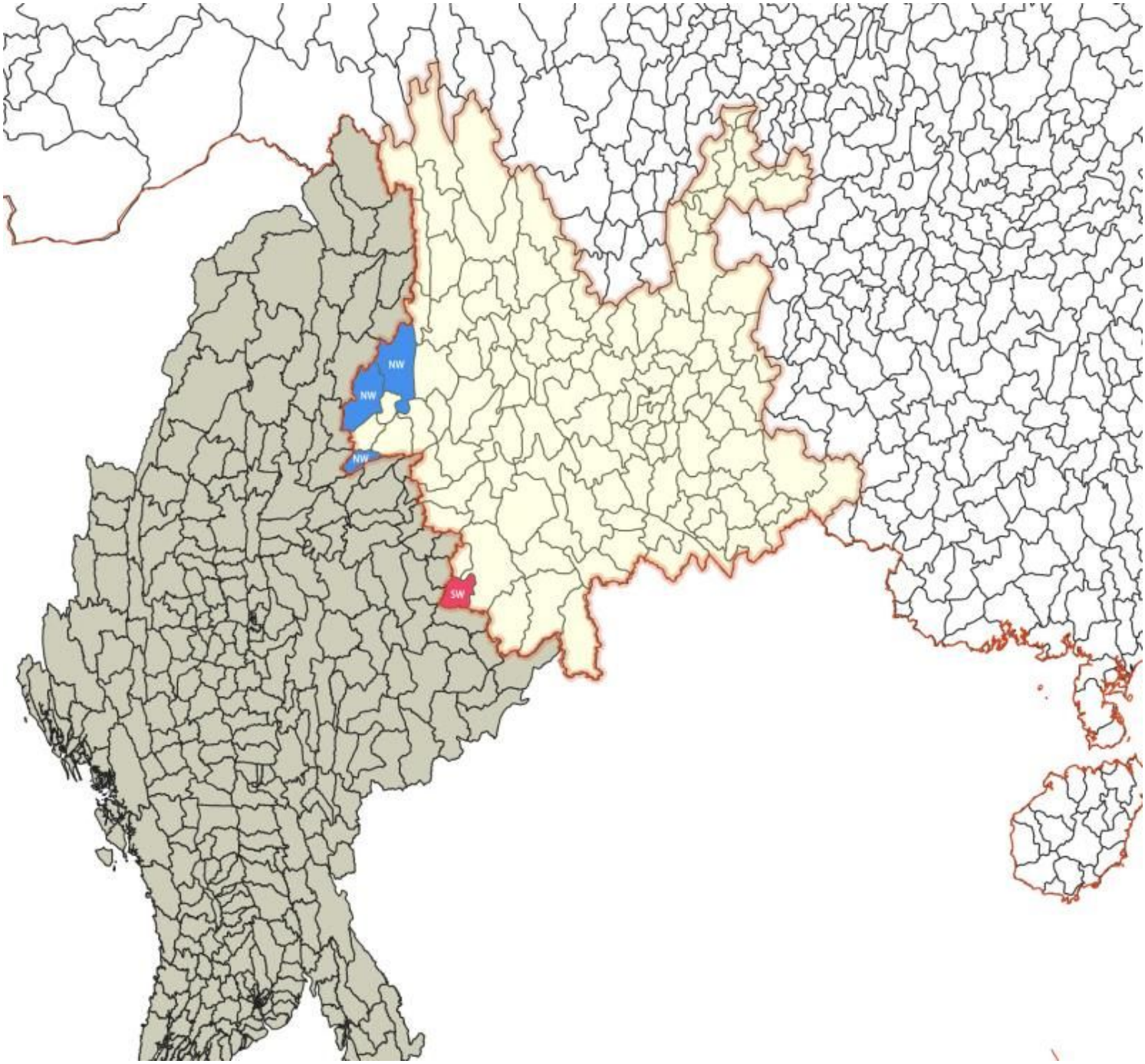

Figure 1

Study sites; Blue area: North-west Yunnan province including 3 surveillance sites; Red area: South-west Yunnan province including 1 surveillances site. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

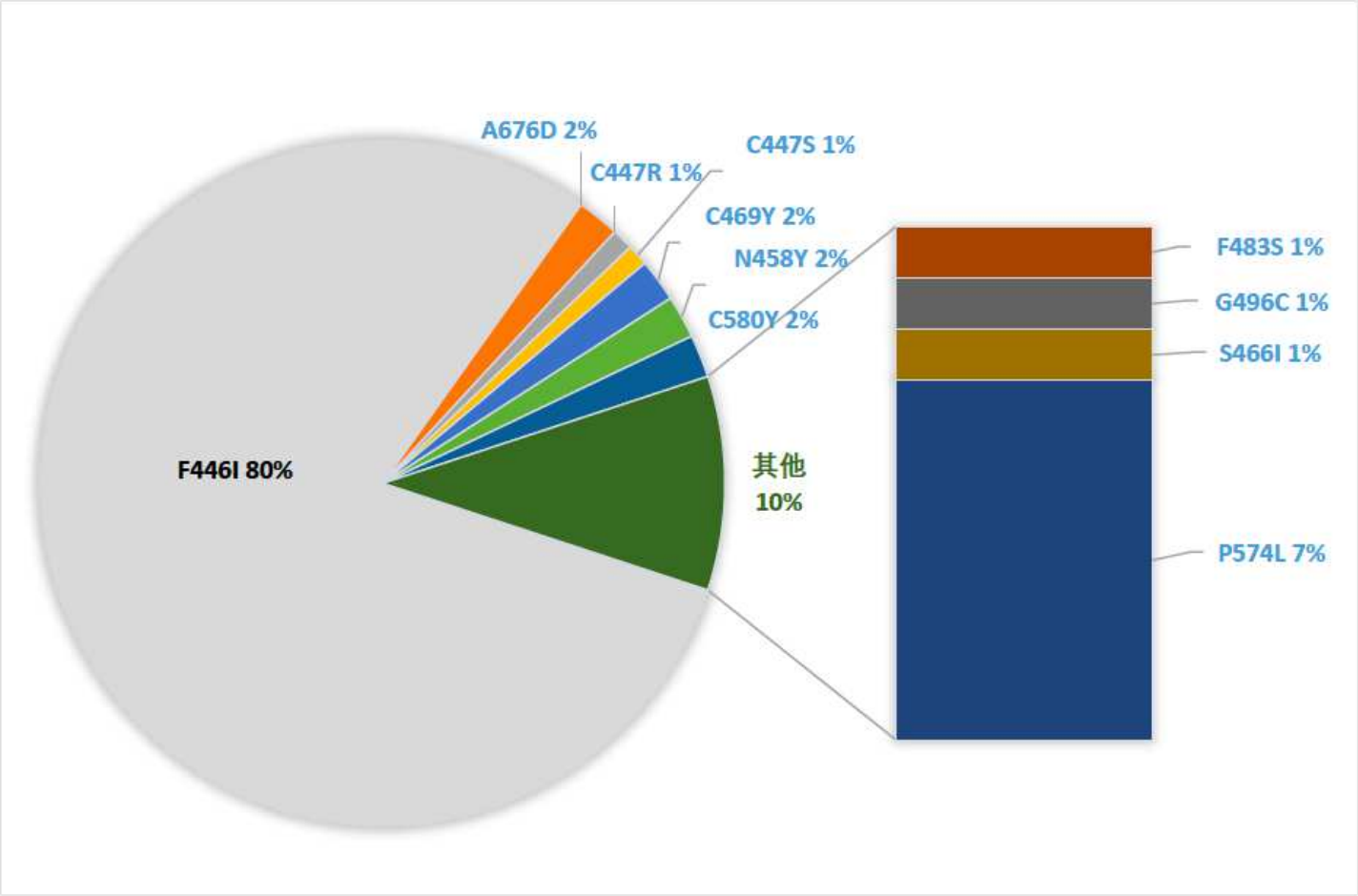

Figure 2

Composition structure of different K13 mutational genotypes along China-Myanmar border

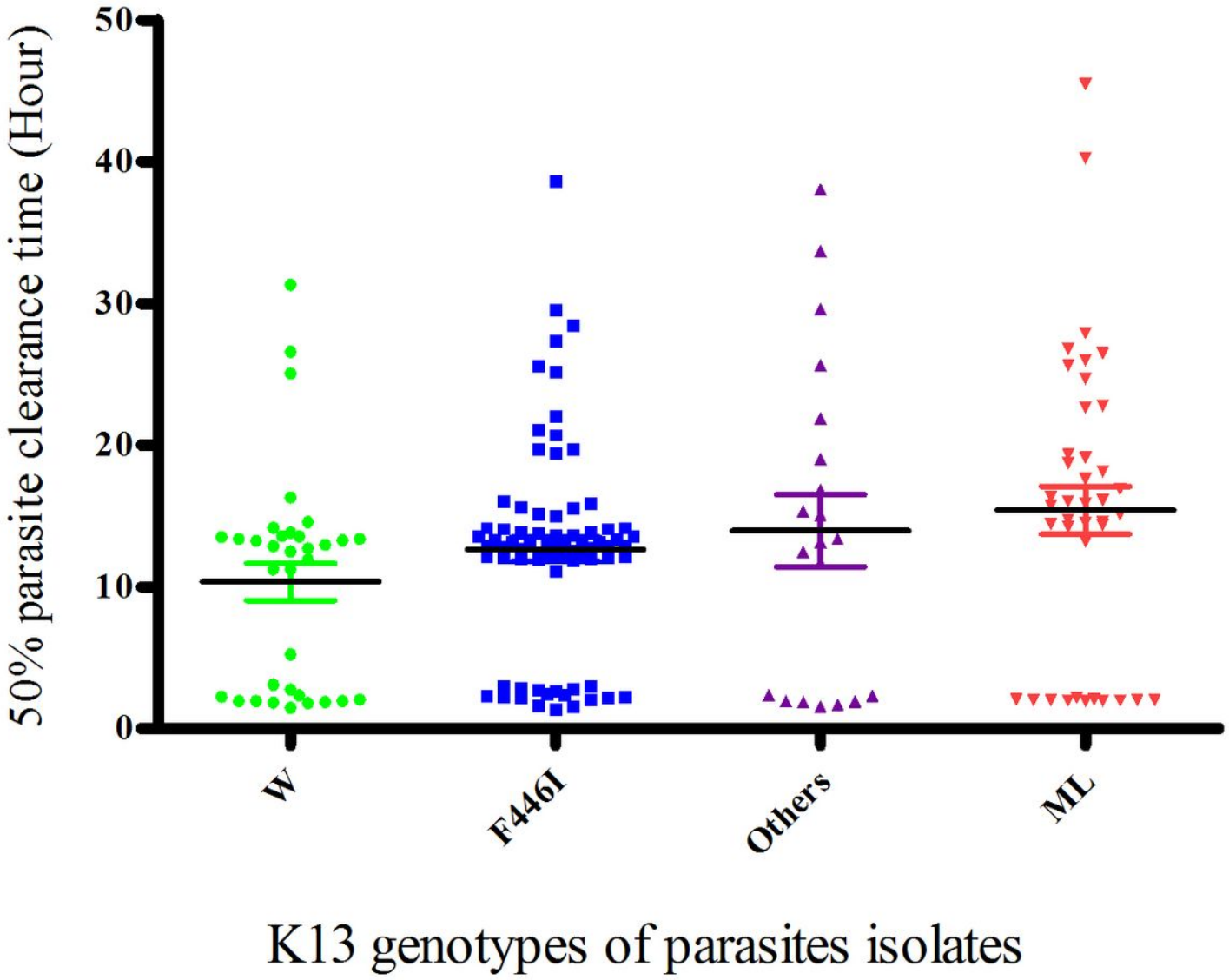

Figure 3

PCT50 variation based on K13 genotypes

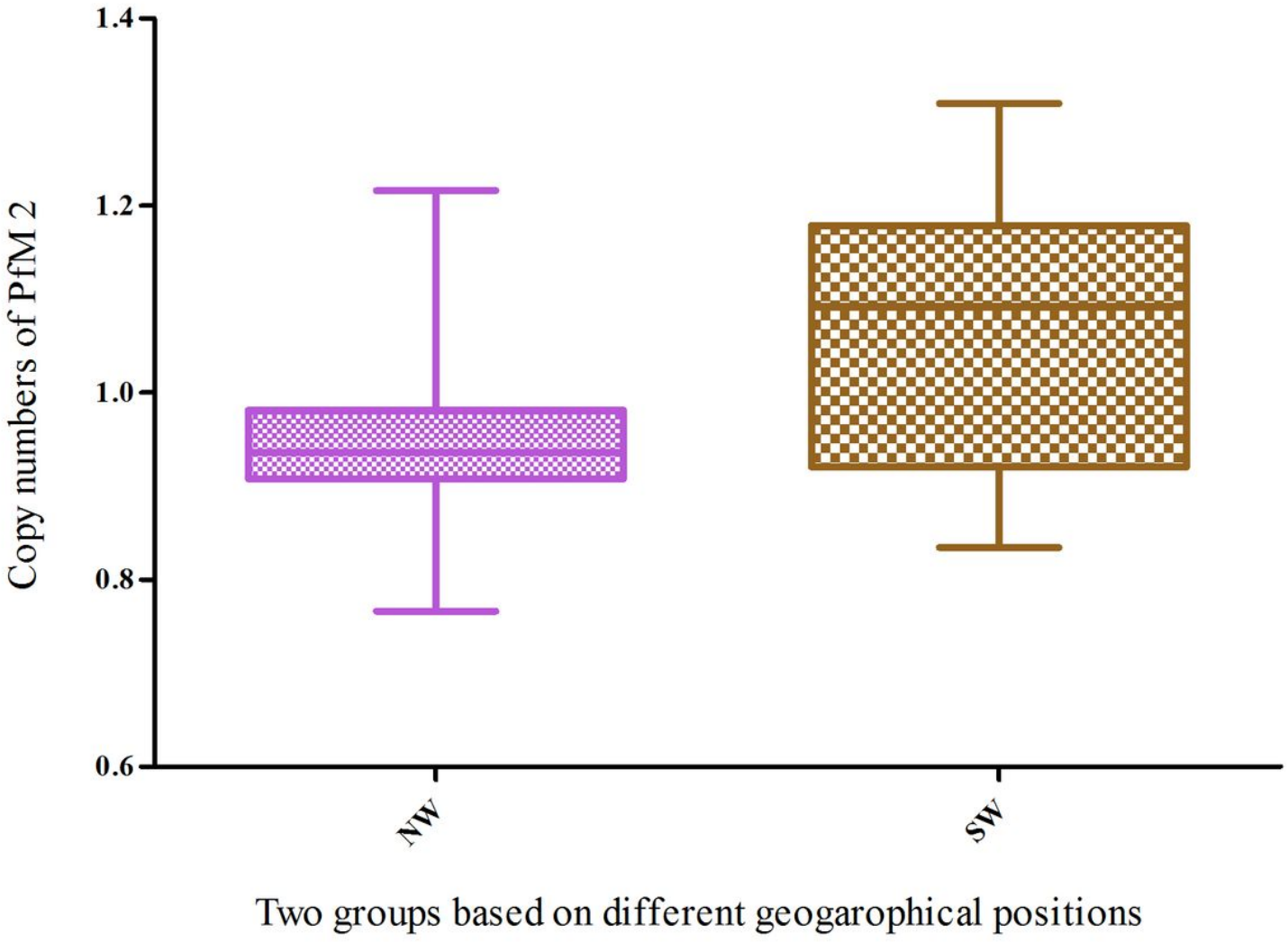

Figure 4

Copy numbers difference of PfM 2 gene based on geographical positions along China-Myanmar border

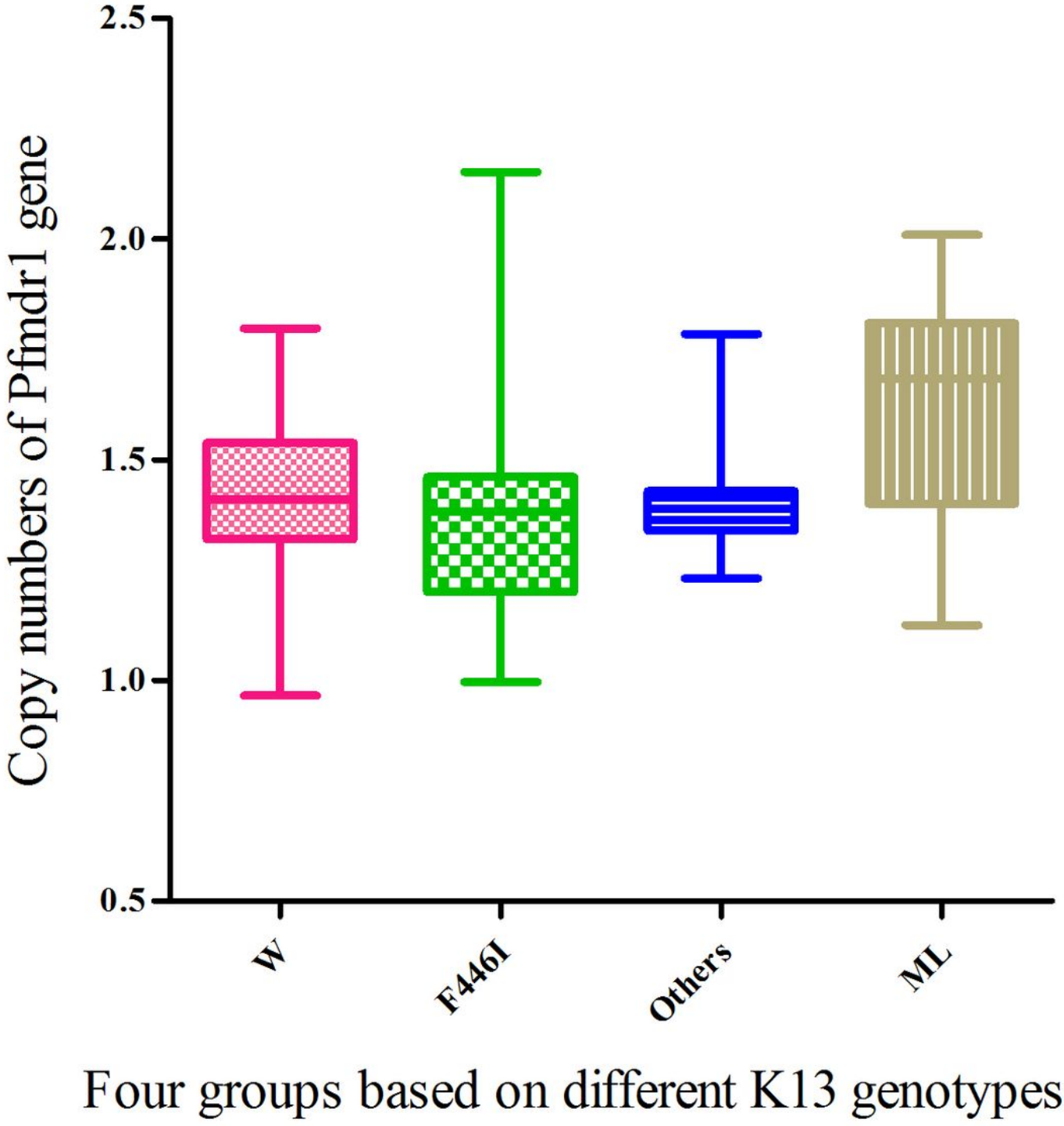

Figure 5

Copy numbers difference of Pfmdr1 gene based on K13 genotypes along China-Myanmar border

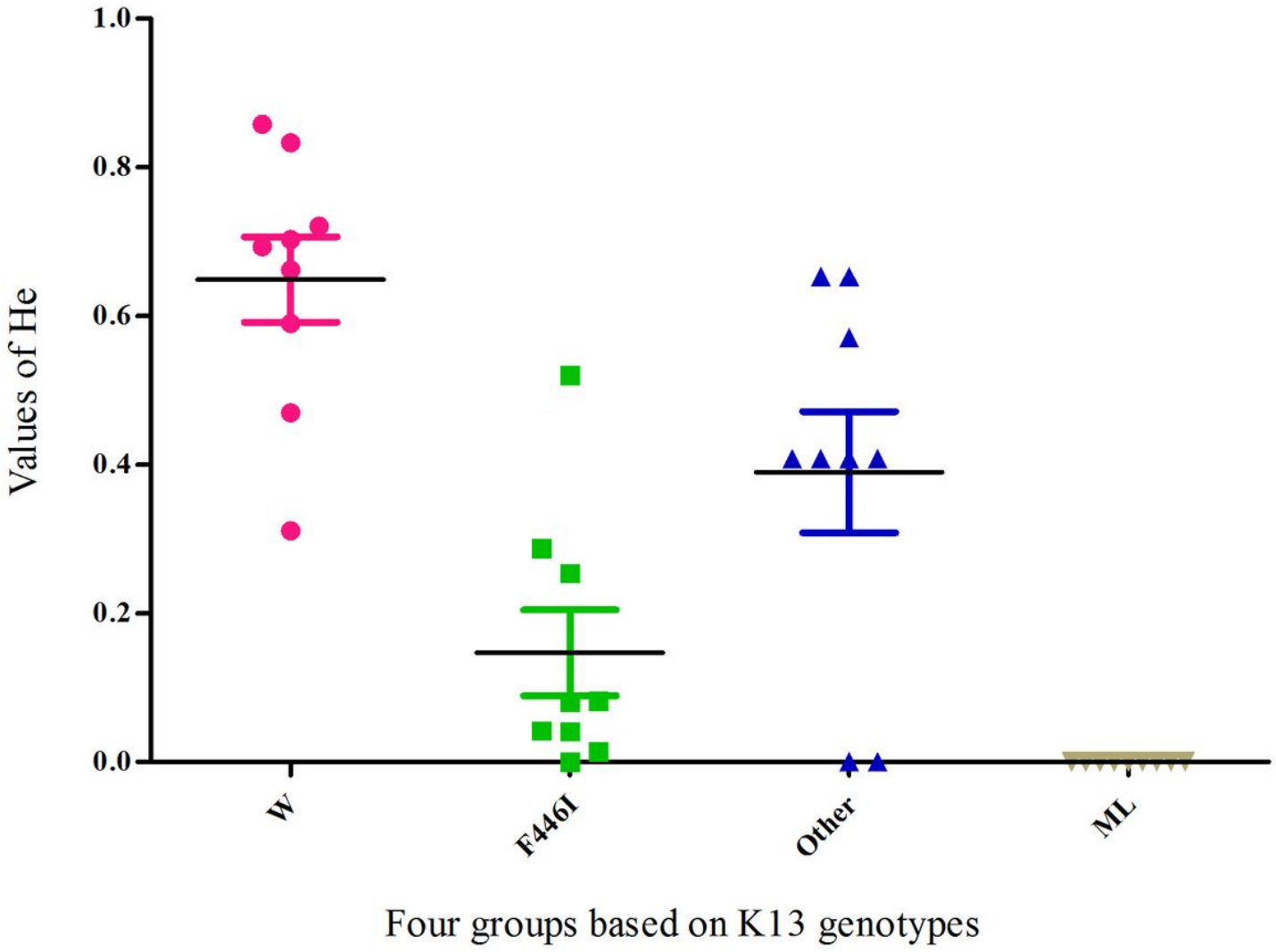

Figure 6

He variation of different groups based on K13 genotypes