Polymorphism in coding region of human CYP2D6 gene contributes to failed radical cure of *vivax malaria* using primaquine

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Abstract

Background: Recent studies found that damaged enzyme activity of cytochrome P450 isoenzyme 2D6 (CYP2D6) can lead to compromised efficacy of primaquine in killing *Plasmodium* during liver dormancy and consequently relapsed malaria. Therefore, WHO has listed the decrease enzyme activity of CYP2D6 as one of the four scenarios that are not suitable for primaquine therapy. Based on analysis of CYP2D6 gene polymorphisms in relapsed *vivax malaria* patients, this study preliminarily revealed the genetic association between human CYP2D6 genotype and the declined efficacy of primaquine treatment in vivax malaria.

Methods: Blood samples of vivax malaria cases treated with "chloroquine/primaquine eight-day therapy" from 2014 to 2018 in Yunnan Province were collected, and the vivax malaria relapsed cases after clinical curing were determined by identifying the infected *Plasmodium* isolates through gene traceability analysis. Human genomic DNA was extracted from blood samples, and 9 exons of CYP2D6 gene were amplified by PCR and then sequenced. The CDS of CYP2D6 gene was obtained by alignment and splicing with the wild-type reference sequence. The mutation types of coding DNA sequences and their association with vivax malaria relapse were analyzed, and the activity phenotype of CYP2D6 isozyme was predicted by its allelic form.

Results: One hundred and fifty-six blood samples from 75 relapsed cases of vivax malaria and the same number of 75 non-relapsed vivax malaria cases were collected for PCR amplification. Two amplification products (2411bp and 2388bp) containing exon1-4 and exon5-9 of CYP2D6 gene were obtained from every sample. The 1491-bp-length CDS chains of CYP2D6 gene were obtained from those 150 samples, and were defined as 24 haplotypes (Hap_1~Hap_24). 17 haplotypes were determined from the sequences of vivax malaria relapsed patients and 15 haplotypes were from those of non-relapsed patients. Hap_6 sequencing showed G>C, C>T and G>C base substitutions at three loci of c.408, c.886 and c.1457, resulting in V136V invariance and R296C and S486T variation at 136th, 296th and 486th amino acid, and the odds ratio of Hap_6 to vivax malaria relapse was 5.615 ($P=0.05$). The diploids of CYP2D6 gene in relapsed cases were 100% mutation homozygous (C/C, 10/10) at c.408, 80% (8/10) at c.1457 and 20% (2/10) at c.886, respectively. However, the coding DNA sequences from non-relapsed cases only showed mutation heterozygote (C/T, 2/2) at c.886 locus. The allelic form of relapsed patients was CYP2D6 *2, and diploid score was used to indirectly predict the CYP2D6 enzyme activity of Hap_6, and the relapsed patients belong to normal metabolizer (NM).

Conclusion: Among the numerous mutations of CYP2D6 gene, the joint mutations at three loci (c.408, c.886, c.1457) are most closely related to the damaged CYP2D6 enzyme activity. Whether c.886 locus mutation plays a critical role contributing to such damage warrants further verification with expanded sample size.

Background

In recent years, the scope of global malaria epidemic has gradually narrowed down, yet the death tolls of malaria deaths remain high, and the incidence of malaria has been increasing in certain areas [1]. Technological limitations may pose daunting challenges to the elimination of malaria, such as the difficulty in identifying low-density *Plasmodium* infection [2], the population expansion of drug resistant *Plasmodium* [3–4], and the lack of antimalarial drug reserves [5]. No local cases of malaria have been reported in China

since 2016 [6]. However, Yunnan Province, as a traditional malaria endemic area in China, still bears the burden of hundreds of cases of malaria imported from abroad every year. From 2011 to 2018, 50.7% of the imported malaria cases in Yunnan province accounted for *P. vivax* [7]. Therefore, the effective eradication of *P. vivax* calls for eliminating the source of infection and blocking its transmission in Yunnan.

So far, primaquine is the only drug that has been reported effective to kill *P. vivax* hypnozoite. WHO recommended the use of primaquine as the only anti-relapsing drug for treating malaria [8]. However, as the active component that kills *P. vivax* hypnozoite, 5-hydroxy-primaquine requires the catalytic effect of CYP2D6 enzyme in human hepatocytes [9–11]. CYP2D6, also known as debrisoquine 4-hydroxylase, is an isozyme belonging to the CYP450 superfamily and an important phase I drug metabolizing enzyme in the human body. Its catalytic functional group is a protein containing ferroheme-mercaptide. Moreover, CYP2D6 is also the only enzyme in the P450 family that cannot be induced. It features low capacity and high affinity. CYP2D6 only accounts for 4% of the total P450 enzyme protein in the liver, guiding the metabolism of ~30% of commonly used clinical drugs, including primaquine [11–13]. However, studies have found that the heterogeneity of CYP2D6 activity is mostly controlled by its genetic variation of various degrees [14], and that the damaged CYP2D6 isoenzyme activity caused by genetic polymorphism of CYP2D6 gene has become an adverse factor aggravating the risk of primaquine use. The undermined CYP2D6 enzyme activity would obstruct the generation of 5-hydroxy-primaquine, making large doses or repeated use of primaquine a common remedy for the declined efficacy of primaquine in the treatment. Meanwhile, patients with G6PD deficiency may experience dual hazards of primaquine exposure [8, 15–16]. The more dismal reality is that geographic distribution of damaged CYP2D6 enzyme activity is highly overlapped with G6PD deficiency, which is in the endemic area of *P. vivax* [17–18]. Hence, it is necessary to investigate not only the physical type of G6PD but also the activity of CYP2D6 isoenzyme in the treatment of vivax malaria [15, 18]. WHO defines that G6PD deficiency, impaired CYP2D6 function, pregnant or lactating women, and infants are not suitable for the use of primaquine [11, 15].

The measurement methods of CYP2D6 enzyme activity include phenotype observation and genotype analysis prediction. Compared with phenotype measurement, the prediction method based on CYP2D6 genotype has the following advantages [19–20]. Firstly, genotypes do not change in one's life, and is not affected by environment or biological factors [20–21]. Secondly, genotyping only requires one specimen and can be completed before medication treatment of the patients and therefore has superior prospective [22]. In addition, human CYP2D6 genes can be easily sequenced to determine the combinations of CYP2D6 alleles [23–24]. Therefore, it is more stable and convenient for batch operation to predict the phenotype of CYP2D6 enzyme activity by genotyping [19, 25–29]. The prediction is based on the locus status of CYP2D6 gene [14], or the allelic combination form of zygote [30–31]. The identified genotypes are no more than alleles with complete function, reduced function or loss of function. The indicators of CYP2D6 enzyme activity include Poor metabolizer (PM), Intermediate metabolizer (IM), Normal metabolizer (NM) and Ultra-rapid metabolizer (UM), which cover various levels of CYP2D6 enzymes from ultra-fast acting to non-metabolic activity [29, 31]. However, the number of alleles variants in CYP2D6 gene has reached 63 excluding a series of sub-variants (<http://www.cypalleles.ki.se/>) [29]. Genotype analysis and prediction method calls for further precision and comprehensive optimization [32].

Gene mutations in enzymes related to drug metabolism not only affect drug efficacy, but can even lead to toxic effect of drug reversal. Therefore, understanding the specific CYP2D6 enzyme activity in different individuals [15] is the prerequisite to ensure radical treatment of vivax malaria with personalized primaquine dose [29, 33]. However, despite the frequent use of primaquine in malaria areas in Yunnan, the exposure risk of primaquine pertaining to G6PD gene has not been fully understood, nor has the polymorphic investigation into CYP2D6 mutation been conducted, nor has the appropriate population for safe use of primaquine been clarified. To improve gene prediction method of CYP2D6 and provide safety cure of *P. vivax* by using primaquine at the molecular level, this study intends to investigate the genetic relationship between human CYP2D6 genotype and the declined efficacy of primaquine in the radical cure of *P. vivax*.

Materials And Methods

Subjects and blood sample

Blood samples of vivax malaria cases diagnosed by Yunnan Province (double parasitology diagnosis of light microscopy and *Plasmodium* gene detection) and reported from 2014 and 2018 were collected. 0.6ml venous blood filter sample was used for gene analysis. The cases were divided into two groups: relapsed and non-relapsed. The relapsed cases consisted of patients who underwent "chloroquine/primaquine eight-day therapy" yet relapsed, and that their genetic tracing of *pvcsp* gene showed that the infection was caused by the same *Plasmodium* isolates. Non-relapsed cases were randomly selected from vivax malaria cases from 2014 to 2018. The two groups had equal number of cases. Malaria infection sites were identified by epidemiological investigation. Informed consent was obtained from all the subjects for collecting blood samples and its purposes.

Extraction of human genomic DNA

Three filter paper blood membranes with a diameter of 5 mm were used and human genomic DNA was extracted using QIAgen Mini Kit kit (Germany, QIAamp Company's DNA Mini Kit), according to the manufacture's instruction, and was stored at -20 ° C prior to use.

PCR amplification of CYP2D6 gene fragment

The PCR amplification primers of CYP2D6 gene were designed by using GenBank (<https://www.ncbi.nlm.nih.gov/gene/>), reference sequence (ID#NC_000022.11) was used as template, following the reaction conditions in previous references [26,34], The primers for first-round nested PCR covering the coding region of CYP2D6 gene exons1-4 is: 5'-CCAGTGACAGATAAGGGTGC-3' and 5'-GACGTGGATAGGAGGTACAGAG-3'; the primers for second-round nested PCR is: 5'-GGTGACTTCTCCGACCAGG-3' and 5'-TTCCCAAACCCATCTATGC-3'. The amplification region was 42131088~42128678, and the expected fragment length of the product was 2411 bp. The primers that cover exons5 ~ 9 upstream or downstream in the coding region are: 5'-GCCGACTGAGCCCTGGGAGGTAGGTA-3' and

5'-GCTGGGGCCTGAGACTT-3. The amplification area was 42126035 ~ 42128422, and the amplification products had an expected fragment length of 2388 bp. For all the PCR reaction systems, we used 2.6µl DNA template, 14.0µl 2 × Taq PCR hybrid system (QIAGEN, Germany), 0.7µl upstream primer, 0.7µl downstream primers (20µmol / L). The total volume was made up to 25.0µl with ddH₂O. PCR reaction conditions were clarified as follows: 92 to 94 ° C for 2-5 mins; 92 to 94 ° C for 10-30s, 50 to 56 ° C for 15-30s, 68 to 72 ° C for 2 to 3 min, 35 cycles; 68 to 72 ° C for 7 mins. Each fragment was subjected to 3 amplifications in parallel. The amplified products were observed using 1.5% agarose gel electrophoresis. The positive products were sent to Shanghai Meiji Biomedical Technology Co, Ltd. for bi-directional Sanger sequencing. At least two identical sequences were used for subsequent analysis.

Mutation analysis of CYP2D6 coding region

The sequencing results were trimmed using DNASTar v5.10 and BioEdit v7.0.9.0 software. The obtained DNA sequence was compared with wild-type CYP2D6 gene (GenBank: NC_000022.11) by sequence alignment to confirm exons 1-9 in the DNA sequence of CYP2D6 gene. The DNA sequences of these 9 exons were spliced from exon 1 to exon 9 into the CDS of CYP2D6 gene. MEGA v5.04 software was used to align the CDS strand with the wild-type sequence to confirm the loci of missense mutation and synonymous mutation. DnaSP v6.11.01 software was used to identify missense mutation and synonymous mutation locus of CDS chain, and the parameters of DNA diversity index (π) and expected heterozygosity (H_e) were calculated [35]. The allele form of CYP2D6 gene coding region was determined by reference [26-28].

SPSS software (version 21.0; IBN; Chicago; IL) was used to conduct chi-square test on the frequency of haplotype co-existing in relapsed and non-relapsed patients. The OR value of haplotypes with statistical significance and the relapse rate of *P. vivax* were calculated. Significant level was set at $\alpha = 0.05$. Value ≤ 1 indicates protective factor and > 1 means risk factor.

Prediction of CYP2D6 enzyme activity

The mutation loci of CYP2D6 gene were used to directly predict the enzymatic activity as one of four phenotypes, including PM, IM, NM and UM in accordance with previously introduced methods [19-20]. The allelic form of the coding region of CYP2D6 gene was identified according to the methods described by Gaedigk et al [21], Sistonen et al [26] and Zanger et al [28]. Among them, genotype * 1 is wild-type allele; * 2 has base substitution at c. 408, c. 886 and c. 1457. Nevertheless, both two are of the same functional normal type allele. * 10 and * 41 are the allele of decreased enzyme activity caused by C > T and G > A base substitution at c.100 and c.1023+36, respectively.

Subsequently, we referred to previously described methods [29,32] to assign the activity score of the allelic form of CYP2D6 gene. The score of wild-type allele, c. 100 alleles and intron c. 1023+36 alleles at each locus were 1.0, 0.25 and 0.5 respectively. The wild homozygotes of the two loci were 2.0, and the scores of mutant homozygotes and mutant heterozygotes were 0.5, 1.0 and 1.25, 1.5, respectively. In the event of multiple loci

in the genotype, the enzyme activity score is calculated as the sum of the scores of the zygotes included. When the diploid score x falls within the interval of $0.0, 0 < x < 1.25, 1.25 \leq x \leq 2.25, > 2.25$, then it indicates that CYP2D6 isozyme activity has the four phenotypes of PM, IM, NM and UM, respectively.

Results

Sample information and PCR amplification of human CYP2D6 gene

A total of 75 relapsed cases of *P. vivax* were enrolled. Among them 71 cases, 2 cases and 2 cases had one, two and three relapse events, respectively. The basic information of 75 non-relapsed cases is listed in Table 1. The mean age of the 150 cases was 33 years, male-female ratio was 2.66:1, and the proportions of vivax infections in Myanmar, Africa, Laos and Yunnan were 96.7% (145/150), 1.3% (2/150), 0.7% (1/150) and 1.3% (2/150), respectively.

Table 1. Information of 150 vivax malaria cases with successful amplified CYP2D6 gene exon1-9 fragments

Years information	2014	2015	2016	2017	2018	Total
Number	18	27	20	10	75	150
1.Relapse times						
0	0	0	0	0	75	75
1	17	25	19	10	0	71
2	0	1	1	0	0	2
3	1	1	0	0	0	2
2.Age ^a						
Maximum	62	52	56	62	69	60
minimum	21	7	4	2	1	7
Average	36	35	29	33	33	33
3.Gender ^b						
Male	16	22	14	6	51	109
Female	2	5	6	4	24	41
4.Infection source ^c						
Myanmar	16	26	18	10	75	145
Africa	1	0	1	0	0	2
Laos	0	1	0	0	0	1
Yunnan indigenous	1	0	1	0	0	2

^a Years old, ^b Numbers, ^c Identified by epidemiological investigation

156 blood samples of relapsed cases in total were collected, and PCR amplification of two segments of human CYP2D6 gene including exons1-4 and exons5-9 was performed, along with 75 blood samples of non-relapsed cases. The amplification products went through electrophoretic and the clear target bands were observed at 2411 bp and 2388 bp, respectively (Fig.1).

Fig.1 PCR products of CYP2D6 gene fragment. **a** M: DNA marker; lane 1: blank control of PCR for the first round; lane 2: blank control of PCR for the second round; lane 3-6: The amplification products of exon 1-4 in

CYP2D6 gene. **b** M: DNA marker; lane 1: blank control of PCR; lane 2-5: The amplification products of exon 5-9 in CYP2D6 gene;

Polymorphism analysis of CYP2D6 gene coding region

Locus polymorphism of CDS chain

The PCR amplification products of CYP2D6 gene from the relapsed cases and non-relapsed cases were trimmed, and the CDS chains containing complete exon1-9 (total length = 1491bp) were obtained from all samples. A total of 150 CDS chains were selected from each of the 75 relapsed case and 75 non-relapsed cases, and were compared with wild-type sequence (NC:000022.11). Base replacement at 12 loci, including c.31 and c100 was found (Table 2). The proportion of the third and the first base replacement of the codon triplet was 41.7% (5/12), and the proportion of the second base replacement was 16.6% (2/12). 7 missense mutation loci and 5 synonymous mutation loci were determined. 91.7% (11/12) mutated loci were found in the sequence of relapsed cases, and 66.7% (8/12) mutated loci were determined in the sequence of non-relapsed cases (Table 2).

Table 2. Comparison of loci polymorphisms in 1staa-497thaa coding region of CYP2D6 gene

Relapse cases					Non-relapse cases				
Orders	Loci	BS	Codon change	Variation	Orders	Loci	BS	Codon change	Variation
1	c.31	G A	GTG ATG	V11M	1	–	–	–	–
2	c.100	C T	CCA TCA	P34S	2	c.100	C T	CCA TCA	P34S
3	c.271	C A	CTG ATG	L91M	3	c.271	C T	CTG TTG	L91L
4	c.281	A G	CAC CGC	H94R	4	–	–	–	–
5	c.294	C G	ACC ACG	T98T	5	c.294	C G	ACC ACG	T98T
6	c.297	C T	GCC GCT	A99A	6	–	–	–	–
7	c.336	C T	TTC TTT	F112F	7	c.336	C T	TTC TTT	F112F
8	c.408	G C	GTC GTG	V136V	8	c.408	G C	GTC GTG	V136V
9	c.505	G A	GGT AGT	G169S	9	–	–	–	–
10	–	–	–	–	10	c.801	C A	CCC CCA	P267P
11	c.886	C T	CGC TGC	R296C	11	c.886	C T	CGC TGC	R296C
12	c.1457	G C	AGC ACC	S486T	12	c.1457	G C	AGC ACC	S486T

Abbreviations: BS, Base substitution

Polymorphism of haplotypes

150 CDS chains of CYP2D6 gene were clustered into 24 haplotypes(Hap_1–Hap_24), as shown in Fig. 2. Among them, Hap_3 is wild-type sequence, and all the rest are mutant sequence. Hap_2 accounted for the largest proportion of 36.6% (55/150), followed by 15.3% for Hap_3(23/150), 8.6% for Hap_4 (13/150), 6.6% for Hap_5 (10/150), 8.0% for Hap_6 (12/150), 6.6% for Hap_7 (10/150) and 4.6% for Hap_21 (7/150). The

proportions of Hap_1, Hap_8, Hap_9, Hap_10, Hap_12, Hap_13, Hap_15, Hap_16, Hap_18, Hap_19, Hap_20, Hap_22, Hap_23, and Hap_24 were as low as 0.7% (1/150). The proportions of Hap_11, Hap_14 and Hap_17 haplotypes were 1.3% (2/150). Haplotypes with one locus mutation included Hap_7, Hap_14, and Hap_15. The two mutations were Hap_5, Hap_11, Hap_16 and Hap_17. The three loci mutation were Hap_4, Hap_6, Hap_10, Hap_18, Hap_19, Hap_22, and Hap_24. The four loci mutation were Hap_1, Hap_2, Hap_8, Hap_20, Hap_21, and Hap_23. Hap_13 showed five loci mutations; Hap_12 had six loci mutations; Hap_9 had seven loci mutations. There are 17 haplotypes in the sequence of relapsed patients, π and H_e equal to 0.0015 and 0.8191, respectively. There were 15 haplotypes in the sequence of non-relapsed patients ($\pi = 0.0014$ and $H_e = 0.8065$). There are eight haplotypes in the sequence of relapsed and non-relapsed patients, including Hap_2, Hap_3, Hap_4, Hap_5, Hap_6, Hap_7, Hap_14 and Hap_17 (Fig. 2).

Fig2. The 9 exons are expressed by numbered boxes with DNA polymorphisms indicated on the top. The predicted amino acid changes are indicated below. No synonymous mutation was found. R represented Relapsed cases, while N indicates Non-relapsed cases.

Haplotypes of CYP2D6 gene coding region and their association with relapse

The odds ratio of genotype to relapse was calculated by selecting six haplotype sequences with sequence number > 1 that simultaneously existed in relapsed patients and non-relapsed patients and could be used for analysis (Table 3). Among them, the OR values of Hap_4 and Hap_6 were 0.159 (95% CI: 0.746 ~ 0.034) and 5.615 (95% CI: 26.577 ~ 1.186), respectively. These were the only two haplotypes that showed statistical significance ($P \leq 0.05$), which demonstrated that Hap_4 indicates reduced relapse risk. By contrast, the risk of relapse for Hap_6 was about 5.615-fold higher than that of other haplotypes. The OR values of Hap_2, Hap_3, Hap_5 and Hap_7 showed no statistical significance ($P > 0.05$) (Table 3).

Table 3. Analysis of the association between haplotypes and relapse

Hap	Relapse cases(n)	Non-relapse cases(n)	OR	95%CI		P
				Upper limit	Low limit	
Hap_2	28	27	1.059	2.058	0.545	0.865 ^a (NS)
Hap_3	8	15	0.478	1.206	1.189	0.113 ^a (NS)
Hap_4	2	11	0.159	0.746	0.034	0.009 ^a (S)
Hap_5	8	2	4.358	21.258	0.894	0.050 ^a (NS)
Hap_6	10	2	5.615	26.577	1.186	0.016 ^a (S)
Hap_7	7	3	2.471	9.944	0.614	0.190 ^a (NS)

Abbreviations: n, number of cases; NS, not significant; S, significant. ^a Chi-square test

Enzyme activity prediction of significant haplotypes and their diploids

Hap_6 and Hap_4 were verified to exert effect on relapse based on the prediction of enzyme activity. Among them, there were 12 CDS chains in Hap_6 (Table 4) from 10 relapsed patients and 2 non-relapsed patients. The diploids in relapsed cases presented 4 genotypes, including *1/*2, *2/*2, *2/*41 and *1/*41. Among the 4 scoring loci of each genotype, including c.408, c.886, c.1023+36 and c.1457, mutation alleles were found only in the diploid loci of c.886 and c.1023+36. Regardless of the diploid of c.886 being mutant heterozygosity (C/T) or mutant homozygosity (T/T), as long as it co-exists with the mutant heterozygosity of c.1023+36, the genotype score of CYP2D6 cannot reach the full score of 2.0. In this case, the score is 1.5. The enzyme activity of CYP2D6 was as "NM" in accordance with the score (Table 4). Two genotypes of *1/*2 and *1/*41 were found in the non-relapsed cases. By contrast, *1/*41 was also determined in the relapsed cases and reached the genotype score of 1.5. It was predicted to be at "NM" level in terms of enzyme activity, along with the *1/*2 genotype showing mutant heterozygous (C/T) only at c.886 and its genotype score reaching the full score of 2.0 (Table 4).

Table 4. Prediction of Hap-6 and Hap-4 and their enzyme activities

A. Hap_6								
Genotypes	Relapse times	No. of samples	Scored loci and their diploid				Caudle's method	
			c.408	c.886	c.1023+36 ^a	c.1457	Score of diploid	Degree
NC_000022.11	–	–	G/G	C/C	G/G	G/G	2	NM
*1/*2	0	1	C/C	C/ <i>T</i>	G/G	C/C	2	NM
*1/*41	0	1	C/C	C/ <i>T</i>	G/ <i>A</i>	C/C	1.5	NM
*1/*2	1	1	C/C	C/ <i>T</i>	G/G	C/C	2	NM
*2/*2	1	5	C/C	<i>T/T</i>	G/G	C/C	2	NM
*2/*41	1	3	C/C	<i>T/T</i>	G/ <i>A</i>	C/C	1.5	NM
*1/*41	1	1	C/C	C/ <i>T</i>	G/ <i>A</i>	C/C	1.5	NM
B. Hap_4								
Genotypes	Relapse times	No. of samples	Scored loci and their diploid				Caudle's method	
			c.100	c.408	–	c.1457	Score of diploid	Degree
NC_000022.11	–	–	C/C	G/G	–	G/G	2	NM
*10/*10	0	2	<i>T/T</i>	<i>C/C</i>	–	<i>C/C</i>	0.5	IM
*1/*10-1	0	3	C/ <i>T</i>	<i>C/C</i>	–	G/ <i>C</i>	1.25	NM
*1/*10-2	0	6	C/ <i>T</i>	<i>C/C</i>	–	<i>C/C</i>	1.25	NM
*1/*10-2	1	2	C/ <i>T</i>	<i>C/C</i>	–	<i>C/C</i>	1.25	NM

^a Intronic position c.1023+36 (Intron 2988) has been considered as defining allele *41 because the predictability of this mutation for impaired enzyme function has been demonstrated recently

The 13 CDS chains belonging to Hap_4 was found in 2 relapsed and 11 non-relapsed patients (Table 4). In the diploid of relapsed cases, only one genotype was found, which is *1/*10-2. Its diploid expression at the three scoring loci c.100, c.408 and c.1457 showed mutant heterozygosity (C/T), mutant homozygosity (C/C) and mutant homozygosity (C/C), respectively. Such pattern resembled the genotype of 54.6% (6/11) of non-relapsed cases, and reached the genotype score of 1.25. The CYP2D6 enzyme activity based on this score was predicted at "NM" level (Table 4). The remaining 5 non-relapsed cases were classified as two genotypes of *10/*10 and *1/*10-1. The diploids of genotypes *10/*10 at the three scoring loci of c.100, c.408 and c.1457 were all mutant homozygous (T/T), (C/C) and (C/C), with the lowest genotype score being 0.5. In 3

cases of *1/*10-1 genotype, mutation heterozygosity (C/T) was found at the c.100 scoring locus, the genotype score was 1.25, and its CYP2D6 enzyme activity was predicted as "NM" level (Table 4).

Discussion

The 4383-bp-length CYP2D6 gene is located on chromosome 22q13.1 and consists of 8 ~ 9 exons and 7 ~ 8 introns. The length of its CDS is 1338 ~ 1491 bp in length, and encodes 446 and 497 amino acids. The heterogeneity of CYP2D6 monooxygenase activity from complete dysfunction to ultra-rapid enzyme metabolism can be attributed to certain genotypes of CYP2D6. Among the 150 identified alleles, multiple mutations, such as CYP2D6*3, CYP2D6*4, CYP2D6*5 and CYP2D6*6, are considered to be associated with compromised CYP2D6 enzyme activity [34, 36].

To our knowledge, the current study is the first one that attempts to analyze mutation polymorphism in the whole gene coding region of human CYP2D6 gene, thereby verifying its possible associated with impaired CYP2D6 enzyme activity in patients with relapsed vivax malaria events after primaquine treatment in Yunnan Province. Among the 150 CDS chains of CYP2D6 genes, 12 base substitutions were detected and were identified as 7 missense mutation loci and 5 synonymous mutation loci. Among them, 91.7% (11/12) mutation locus was found in the sequence of relapsed patients, and 66.7% (8/12) mutation locus was found in the sequence of non-relapsed patients. Among the 24 haplotypes defined, Hap_2 makes up the largest proportion of the sequences, reaching as high as 36.6% (55/150). The haplotype has multiple mutations at four loci, including c.100C > T, c.336C > T, c.408G > C, c.1457G > C. These mutations are disparate from previous studies which reported that CYP2D6*10 allele are easy to detected in Asian populations [34, 37]. CYP2D6*10 allele has multiple mutations at c. 100C > T, c. 408G > C and c. 1457G > C, which are less at c. 336C > T than Hap_2. This gap might be resulted from the fact that the sequencing method of the whole gene coding region is easier to detect all base substitutions. However, whether the differences in common alleles of CYP2D6 are worthy of in-depth research is subject to different populations. The population studied in this paper is not random, but those who reside in disease endemic areas and showed 50% chances of recurrent vivax malaria patient (75/150). Thusly, the genotypes of such population may show the screening outcome of human defective genes by malaria epidemic in an accurate manner [38].

Although it is speculated that the enzyme activity damage in the heterogeneity of CYP2D6 may be related to the failed therapy of primaquine to kill hypnozoite of *P. vivax* [8, 18, 39–41], the correlation between different genotypes and the relapse of *P. vivax* is poorly understood. In this study, we found that the genotypes with combined mutations at c. 408, c. 886 and c. 1457 were most closely related to the relapse of vivax malaria (OR = 5.615, $P < 0.05$) (Table 3). Mutations at c. 408 and c. 1457, and the individuals with homozygous mutations at c. 886 had relapse phenotype (Table 4). Only 1/2 of mutant heterozygotes indicate relapse, suggesting that among the genotypes with multiple locus mutations such as c. 408, c. 886 and c. 1457, the key to prevent vivax malaria relapse may lie in mutation at c. 886 locus. Such findings is not consistent with that of Silvino et al [41], who believes that c. 100, c. 321 and other locus mutations are more closely related to vivax malaria relapse. Such disparity might be attributed to the difference between the two methods used for detecting gene mutation loci. Silvino et al. employed the method of restriction enzyme digestion for individual loci, such as c.100 and c.321, while the whole gene coding region sequencing method used in this study was

easier to find locus polymorphism in the gene. Of note, in determining the close association of multiple mutations at c. 408, c. 886 and c. 1457 with the relapse of vivax malaria, intron c. 1023 + 36 mutation may play a role in aggravating the damage of CYP2D6 enzyme activity. In 4 relapsed cases of c. 886 mutation heterozygosity (C/T) or homozygous mutation (T/T), mutations were found at c. 1023 + 36, which was only the genetic background of mutant heterozygotes. This finding concurs with c. 1023 + 36 mutations found by Silvino et al [41] and Raimundo et al [42] in patients who suffered from failed cure of vivax malaria. On the other hand, the multiple mutation genotypes at c. 100, c. 408 and c. 1457 loci found in this study depicted a unique phenomenon of reducing the relapse risk of vivax malaria (OR = 0.159, $P < 0.05$), and warrants the necessity of further analysis.

At present, the main methods for predicting CYP2D6 enzyme activity based on gene information include the CYP2D6-locus-mutation-polymorphism-based direct prediction method summarized by Zanger et al [28] and indirect prediction method of genotype score recommended by Gaedigk et al [29] and Caudle et al [32]. Because the latter presents the advantage of quantifying the degree of gene mutation and more abundant genotypic criteria, we used this method to predict and analyze the activity of CYP2D6 enzymes in this study. However, regardless of Hap_6 indicating risk status or Hap_4 serving as protective factors, all enzyme activity phenotyping were predicted to be above IM grade. The allele for most of the relapsed patients was CYP2D6*2, and multi-locus genotypes had scores of greater than 1.5. By comparison, most of the non-relapsed patients had CYP2D6*10 allele, and their multi-locus genotypes had a score of less than 1.5. Except two cases belonging to IM phenotype, the others belonged to NM phenotype. This result is not in line with that of Brasil et al [40], which reported that most of the alleles in relapsed patients are CYP2D6*10, CYP2D6*39 and so forth. Moreover, it contradicts the clinical observation of relapsed patients in this study. Such conflict might be justified by the reason that CYP2D6 enzyme activity prediction method used in this study to obtain whole gene coding region information takes into the account of more alleles and thereby leads to enhanced genotype score. In contrast, Brasil et al. detected only a few mutation loci and therefore could miss out the reverse effects of other locus mutations. CYP2D6*2 allele showing heterogeneous phenotypes with normal and abnormal enzyme activity [43] suggests that identifying heterogeneous CYP2D6 enzyme activity with similar genotypes is the key [21, 29], no matter what strategy is used to predict CYP2D6 enzyme activity. Meanwhile, the prediction method needs to be further optimized. This study predicted the unconventional phenotype of CYP2D6 enzyme activity in patients with relapsed vivax malaria on the basis of genetic information, and therefore did not rule out the relapsed vivax malaria cases which acquired primaquine resistance after treatment. Chen et al. [44] observed the resistance of Plasmodium to primaquine in relapsed vivax malaria cases in the Australian Defense Force, therefore it is essential to eliminate the influence of interfering factors in genetic studies. This is another aspect that deserves improved prediction method to analyze CYP2D6 enzyme activity.

In this study, although only Sanger sequencing method was used to analyze the sequence polymorphism in the whole coding region of CYP2D6 gene, the strategy of three parallel repeats was adopted for each PCR reaction and the sequencing of its products. Such effort helped eliminate the error rate in single PCR direct sequencing to a certain extent. Meanwhile, the cost-effective sequencing analysis method is more suitable for molecular epidemiological cross-sectional investigation of CYP2D6 gene mutation polymorphism in the endemic areas where primaquine is used to treat vivax malaria. However, there are still some shortcomings in

this study. Firstly, the sample size is not large enough, and the low-frequency alleles of CYP2D6 genes detected in the study should be further verified. Secondly, in the study of genetic association between CYP2D6 genotypes and relapsed *P. vivax*, although the identification of relapsed cases had achieved the genetic origin of infected strains, it did not rule out the confounding factors such as the possible resistance of *Plasmodium* to primaquine, hence the identification of relapsed vivax malaria patients may have been enlarged. Finally, although the CYP2D6 enzyme activity was analyzed by using the method of zygotic allelic to assign the activity score recently recommended by Caudle et al [32], this study came to define more CYP2D6 gene locus polymorphisms. Since there is no benchmark standard to assign the activity scores to these loci polymorphisms, the predicted CYP2D6 enzyme activity in this study may not be completely homogenous with the findings of Caudle et al.

Conclusion

In conclusion, the CYP2D6 enzyme activity prediction method based on the sequencing of whole gene coding region of CYP2D6 preliminarily revealed the CYP2D6 gene mutation polymorphisms in relapsed *P. vivax* patients who showed poor curative effect of primaquine in Yunnan. This study ushers in the analysis of genetic correlation factors in relapsed *P. vivax* patients in China. The important findings are as follows: (1) Among the multiple mutations of CYP2D6 gene, the joint mutations at three loci, including c.408, c.886 and c.1457, are most closely related to damaged CYP2D6 enzyme activity. The mutation of c.408 and c.1457 can give rise to compromised CYP2D6 enzyme activity, yet whether such damage is aggravated by mutation of c.886 requires a larger sample size for further validation;. (2) The key to predict CYP2D6 enzyme activity by genotype is to define the genotype accurately and accordingly, to study the genetic correlation between genotype and enzyme activity phenotype with reasonable sample size. Because the whole gene sequencing method can fully identify the mutation loci, it is promising as a molecular epidemiological investigation method for CYP2D6 gene mutation polymorphism.

Abbreviations

CYP2D6: Cytochrome P450, family 2, subfamily D, polypeptide 6; CDS: coding DNA sequence;

vivax: *Plasmodium vivax*; PM: Poor metabolizer; IM: Intermediate metabolizer; NM: Normal metabolizer; UM: Ultra-rapid metabolizer; *pvcsp*: *Plasmodium vivax* circumsporozoite protein; Hap: haplotype; π : Diversity index; He: Expected heterozygosity; OR: odds ratio;

Declarations

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Authors' contributions

HH was responsible for the gene testing, study design, statistics and analysis of the data, and wrote the manuscript; YDong performed coordination of all project, study design, analysis of the data and the manuscript guidance and revision. SL participated in the study design; YX, YL, YDeng, MC, CZ performed the collection blood samples and microscopy examination. All authors read and approved the final draft of the manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The study was approved by Yunnan Institute of Parasitic Diseases and by the Ethical Committee. Genetic testing experiment, etc. were performed on stored blood samples obtained as part of routine diagnostic work-up patients with fever suspected of malaria. Although the absence of risk and the anonymous data processing are ensured among the study, consent from potential malaria patients need to be obtained during collecting blood samples. Demographic, clinical and epidemiological information of each fever patient will be collected. Database access will be restricted by password, and Yunnan Institute Parasitic Diseases will not allow retrieving and saving the personal identification information into the project database. It is committed not to provide information about the patient to any person unrelated to the study.

Consent for publication

All authors provided their consent for the publication of this report.

Competing interests

The authors declare that they have no competing interests.

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References

1. WHO. World malaria report 2018. Geneva: World Health Organization; 2018.
<https://www.who.int/malaria/publications/world-malaria-report-2018/en/>. Accessed 10 Mar 2020.
2. Recker M, Bull PC. Recent advances in the molecular epidemiology of clinical malaria. *F1000Res*. 2018;7.
3. Thuy-Nhien N, Tuyen NK, Tong NT, Vy NT, Thanh NV, Van HT, et al. K13 Propeller Mutations in *Plasmodium falciparum* Populations in Regions of Malaria Endemicity in Vietnam from 2009 to 2016. *Antimicrob Agents Chemother*. 2017; 61.
4. Dong Y, Wang J, Sun A, Deng Y, Chen M, Xu Y, et al. Genetic association between the Pfk13 gene mutation and artemisinin resistance phenotype in *Plasmodium falciparum* isolates from Yunnan Province, China. *Malar J*. 2018; 17:478.
5. WHO Malaria Policy Advisory Committee and Secretarial. Malaria Policy Advisory Committee to the WHO: conclusions and recommendations of September 2012 meeting. *Malar J*. 2012; 11:424.
6. Feng J, Zhang L, Huang F, Yin ZH, Tu H, Xia ZG, et al. Ready for malaria elimination: zero indigenous case reported in the People's Republic of China. *Malar J*. 2018; 17:315.
7. Zhang L, Feng J, Tu H, Xia ZG, Zhou SS. Challenges in malaria elimination the epidemiological characteristics of *Plasmodium vivax* in China from 2011 to 2018. *Chin J Parasitol Parasit Dis*. 2019; 37:532-538.
8. Bennett JW, Pybus BS, Yadava A, Tosh D, Sousa JC, McCarthy WF, et al. Primaquine failure and cytochrome P-450 2D6 in *Plasmodium vivax* malaria. *N Engl J Med*. 2013; 369:1381–2.
9. Marcsisin SR, Reichard G, Pybus BS. Primaquine pharmacology in the context of CYP2D6 pharmacogenomics: Current state of the art. *Pharmacol Ther*. 2016; 161:1-10.
10. Vale N, Moreira R, Gomes P. Primaquine revisited six decades after its discovery. *Eur J Med Chem*. 2009; 44:937-53.
11. Pybus BS, Sousa JC, Jin X, Ferguson JA, Christian RE, Barnhart R, et al. CYP450 phenotyping and accurate mass identification of metabolites of the 8-aminoquinoline, anti-malarial drug primaquine. *Malar J*. 2012; 11:259.

12. Wen ZG, An L, Lin F, Wang QZ. Advance in clinical application of CYP2D6 genetic polymorphisms. *J Mol Diagn Ther.* 2013; 67-72.
13. Zeng R, Mao XB, Liu X, Huang J. Analysis of CYP2D6 gene polymorphism of Chinese population in Hubei. *Int J Lab Med.* 2018; 39:1540-1542+1546.
14. Twist GP, Gaedigk A, Miller NA, Farrow EG, Willig LK, Dinwiddie DL, et al. Constellation: a tool for rapid, automated phenotype assignment of a highly polymorphic pharmacogene, CYP2D6, from whole-genome sequences. *NPJ Genom Med.* 2016; 1:15007.
15. Baird JK, Battle KE, Howes RE. Primaquine ineligibility in anti-relapse therapy of *Plasmodium vivax* malaria: the problem of G6PD deficiency and cytochrome P-450 2D6 polymorphisms. *Malar J.* 2018; 17:42.
16. Nelwan EJ, Ekawati LL, Tjahjono B, Setiabudy R, Sutanto I, Chand K, et al. Randomized trial of primaquine hypnozoitocidal efficacy when administered with artemisinin-combined blood schizontocides for radical cure of *Plasmodium vivax* in Indonesia. *BMC Med.* 2015; 13:294.
17. Sutanto I, Tjahjono B, Basri H, Taylor WR, Putri FA, Meilia RA, et al. Randomized, open-label trial of primaquine against vivax malaria relapse in Indonesia. *Antimicrob Agents Chemother.* 2013; 57:1128–35.
18. Baird JK, Louisa M, Noviyanti R, Ekawati L, Elyazar I, Subekti, et al. Association of Impaired Cytochrome P450 2D6 Activity Genotype and Phenotype With Therapeutic Efficacy of Primaquine Treatment for Latent *Plasmodium vivax* Malaria. *JAMA Netw Open.* 2018; 1: e181449.
19. Griesse EU, Zanger UM, Brudermanns U, Gaedigk A, Mikus G, Morike K, et al. Assessment of the predictive power of genotypes for the in-vivo catalytic function of CYP2D6 in a German population. *Pharmacogenetics.* 1998; 8:15–26.
20. McElroy S, Sachse C, Brockmoller J, Richmond J, Lira M, Friedman D, et al. CYP2D6 genotyping as an alternative to phenotyping for determination of metabolic status in a clinical trial setting. *AAPS PharmSci.* 2000; 2: E33.
21. Gaedigk A, Bradford LD, Marcucci KA, Leeder JS. Unique CYP2D6 activity distribution and genotype-phenotype discordance in black Americans. *Clin Pharmacol Ther.* 2002; 72:76-89.
22. Lindpaintner K. Pharmacogenetics and the future of medical practice. *Br J Clin Pharmacol.* 2002; 54:221–30.
23. Crews KR, Gaedigk A, Dunnenberger HM, Klein TE, Shen DD, Callaghan JT, et al. Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines for codeine therapy in the context of cytochrome P450 2D6 (CYP2D6) genotype. *Clin Pharmacol Ther.* 2012; 91:321-326.
24. Crews KR, Gaedigk A, Dunnenberger HM, Leeder JS, Klein TE, Caudle KE, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for cytochrome P450 2D6 genotype and codeine therapy: 2014 update. *Clin Pharmacol Ther.* 2014; 95:376-382.
25. De Gregori M, Allegri M, De Gregori S, Garbin G, Tinelli C, Regazzi M, et al. How and why to screen for CYP2D6 interindividual variability in patients under pharmacological treatments. *Curr Drug Metab.* 2010; 11:276-82.

26. Sistonen J, Fuselli S, Levo A, Sajantila A. CYP2D6 genotyping by a multiplex primer extension reaction. Clin Chem. 2005; 51:1291-1295.
27. Sistonen J, Sajantila A, Lao O, Corander J, Barbujani G, Fuselli S. CYP2D6 worldwide genetic variation shows high frequency of altered activity variants and no continental structure. Pharmacogenet Genomics. 2007; 17:93-101.
28. Zanger UM, Raimundo S, Eichelbaum M. Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. Naunyn Schmiedebergs Arch Pharmacol. 2004; 369:23-37.
29. Gaedigk A, Simon SD, Pearce RE, Bradford LD, Kennedy MJ, Leeder JS. The CYP2D6 activity score: translating genotype information into a qualitative measure of phenotype. Clin Pharmacol Ther. 2008; 83:234-42.
30. Kamenski G, Ayazseven S, Berndt A, Fink W, Kamenski L, Zehetmayer S, et al. Clinical Relevance of CYP2D6 Polymorphisms in Patients of an Austrian Medical Practice: A Family Practice-Based Observational Study. Drugs Real World Outcomes. 2019. doi: 10.1007/s40801-019-00177-4
31. Gaedigk A, Sangkuhl K, Whirl-Carrillo M, Klein T, Leeder JS. Prediction of CYP2D6 phenotype from genotype across world populations. Genet Med. 2016. doi: 10.1038/gim.2016.80
32. Caudle KE, Sangkuhl K, Whirl-Carrillo M, Swen JJ, Haidar CE, Klein TE, et al. Standardizing CYP2D6 genotype to phenotype translation: consensus recommendations from the clinical pharmacogenetics implementation consortium and dutch pharmacogenetics Working Group. Clin Transl Sci. 2019. doi: 10.1111/cts.12692
33. Zhang RY, Yang Y, Zou LY, Pei KL, Jing SC, Wang RF, et al. The Study on CYP2D6,CYP3A5,CYP1A2 Genetic Polymorphism in Chinese Han Population. Journal of Psychiatry. 2018; 31:129-132.
34. Swadi AA, Mohammad BI, Hadi NR. Correlation of CYP2D6 allelic polymorphism to outcome of acute coronary syndrome in mid-Euphrates Iraqi patients on metoprolol therapy. Gene. 2019; 703:112-119.
35. Dong Y, Deng Y, Xu YC, Chen MN, Mao XH, Sun AM, et al. Analysis of genes associated with antifolate drug resistance in *Plasmodium vivax* from different infection sources. Chin J Parasitol Parasit Dis. 2018; 36:203-11.
36. Gaedigk A, Ingelman-Sundberg M, Miller NA, Leeder JS, Whirl-Carrillo M, Klein TE, et al. The Pharmacogene Variation (PharmVar) Consortium: Incorporation of the Human Cytochrome P450 (CYP) Allele Nomenclature Database. Clin Pharmacol Ther. 2018; 103:399-401.
37. Dong AN, Ahemad N, Pan Y, Palanisamy UD, Yiap BC, Ong CE. Functional and structural characterisation of common cytochrome P450 2D6 allelic variants-roles of Pro34 and Thr107 in catalysis and inhibition. Naunyn Schmiedebergs Arch Pharmacol. 2019; 392:1015-1029.
38. Dong Y, Liu SP, Xu YC, Liu Y, Deng Y, Chen MN. Mutations and predicted structure change of G6PD isolated from a patient with primaquine-induced hemolysis in Yunnan Province. Chin J Parasitol Parasit Dis. 2019; 37:399-405.
39. St Jean PL, Xue Z, Carter N, Koh GC, Duparc S, Taylor M, et al. Tafenoquine treatment of *Plasmodium vivax* malaria: suggestive evidence that CYP2D6 reduced metabolism is not associated with relapse in the Phase 2b DETECTIVE trial. Malar J. 2016; 15:97.

40. Brasil LW, Rodrigues-Soares F, Santoro AB, Almeida ACG, Kuhn A, Ramasawmy R, et al. CYP2D6 activity and the risk of recurrence of *Plasmodium vivax* malaria in the Brazilian Amazon: a prospective cohort study. *Malar J.* 2018; 17:57.
41. Silvino AC, Costa GL, Araujo FC, Ascher DB, Pires DE, Fontes CJ, et al. Variation in human cytochrome P-450 drug-metabolism genes: a gateway to the understanding of *Plasmodium vivax* relapses. *PLoS ONE.* 2016; 11: e0160172.
42. Raimundo S, Toscano C, Klein K, Fischer J, Griesse EU, Eichelbaum M, et al. A novel intronic mutation, 2988GA, with high predictivity for impaired function of cytochrome P450 2D6 in white subjects. *Clin Pharmacol Ther.* 2004; 76:128-38.
43. Wang D, Poi MJ, Sun X, Gaedigk A, Leeder JS, Sadee W. Common CYP2D6 polymorphisms affecting alternative splicing and transcription: long-range haplotypes with two regulatory variants modulate CYP2D6 activity. *Hum Mol Genet.* 2014; 23:268-278.
44. Chen N, Dowd S, Gatton ML, Aulif A, Edstein MD, Cheng Q. Cytochrome P450 2D6 profiles and their relationship with outcomes of primaquine anti-relapse therapy in Australian Defence Force personnel deployed to Papua New Guinea and East Timor. *Malar J.* 2019; 18:140.

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Figures

Figure 1

PCR products of CYP2D6 gene fragment. a M: DNA marker; lane 1: blank control of PCR for the first round; lane 2: blank control of PCR for the second round; lane 3-6: The amplification products of exon 1-4 in CYP2D6 gene. b M: DNA marker; lane 1: blank control of PCR; lane 2-5: The amplification products of exon 5-9 in CYP2D6 gene;

Figure 2

The 9 exons are expressed by numbered boxes with DNA polymorphisms indicated on the top. The predicted amino acid changes are indicated below. No synonymous mutation was found. R represented Relapsed cases, while N indicates Non-relapsed cases.

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