Genetic Polymorphism of human CYP2D6 gene coding region results in failed radical cure of vivax malaria using primaquine

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Abstract

Background: Primaquine is the traditional medicine could effectively eliminate the hypnozoites of *P. vivax*, and therefore has been recommended by World Health Organization (WHO) as the anti-relapsing drug for the treatment of vivax malaria patient. However, 5-hydroxy-primaquine, the active component in primaquine to kill *P. vivax* hypnozoite has to be catalyzed by Cytochrome P450, family 2, subfamily D, polypeptide 6 (CYP2D6) enzyme in human hepatocytes. Nevertheless, suppressed CYP2D6 enzyme activity caused by CYP2D6 gene mutation has been considered as a risk factor for the efficacy of primaquine. Based on the analysis of CYP2D6 gene polymorphism in relapsed vivax malaria patients, the current study aims to preliminarily reveal the genetic association between human CYP2D6 genotype and the curative effect of primaquine in vivax malaria.

Methods: Blood samples of vivax malaria cases received "chloroquine/primaquine eight-day therapy" from 2014 to 2018 in Yunnan Province were collected. The relapsed cases were determined by identifying the homogeneity of *P. vivax* circumsporozoite protein (*pvcsp*) gene sequences between the *Plasmodiums* isolated from patients' infected blood samples. Human genomic DNA was extracted from the blood samples, and 9 exons of CYP2D6 gene were amplified by polymerase chain reaction (PCR) and were then sequenced. The coding DNA sequence (CDS) of CYP2D6 gene was obtained by splicing and aligning with the wild-type reference sequence. The mutation types of CDS and their association with vivax malaria relapse were analyzed, and the activity phenotype of CYP2D6 isozyme was predicted by the allelic form of CYP2D6 gene.

Results: A total of 156 blood samples from 75 relapsed cases of vivax malaria and 75 non-relapsed cases were collected for nested-PCR amplification. Two nested-PCR products, the CYP2D6 gene fragments containing exon1-4 and exon5-9 (2411bp and 2388bp), were obtained from the sample. After splicing and combining these two amplification products, we found that the CDS of CYP2D6 gene in each sample was 1491bp in length. Moreover, we identified 24 haplotypes (Hap_1~Hap_24) in the Clustered CYP2D6 full-CDS of 150 patients, including 17 haplotypes in relapsed patients and 15 haplotypes in non-relapsed patients (8 haplotypes co-existed in two groups of patients). Hap_6 showed G>C, C>T and G>C base substitutions at c.408, c.886 and c.1457, resulting in V136V invariance, and variations of R296C and S486T at 136th, 296th and 486th amino acid sites, respectively. The risk of relapsing vivax malaria in Hap_6 was about 5.615-fold higher than in other haplotypes. In the relapsed cases, c.408 locus mutation of CYP2D6 gene was 100% homozygous allele (C/C, 10/10), whereas c.886 locus mutation was 80% homozygous allele (T/T, 8/10) and 20% heterozygous allele (C/T, 2/2). However, the non-relapsed cases only showed mutation heterozygote allele (C/T, 2/2) at c.886 locus. Moreover, most of mutant allele types in relapsed patients were CYP2D6 *2, and the CYP2D6 enzyme activity was predicted as normal metabolizer (NM) according to the scoring of diploid allele of Hap_6 sequence.

Conclusion: Among the numerous mutations of CYP2D6 gene, triple mutations at three loci (c.408, c.886, c.1457) are most closely related to impaired CYP2D6 enzyme activity, and greater sample size should be applied to ascertain whether c.886 locus mutation plays a critical role.

Background

In recent years, the global malaria epidemic has gradually scaled down, yet the death tolls of malaria remain high, and the number of malaria cases in certain areas has rebounded [1]. Meanwhile, multiple technological limitations 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 variety in antimalarial drug [5]. Although Chinese local cases (without a travel history to malaria epidemic areas outside China within the last 30 days before the onset of malaria) of malaria have not been reported since 2016 [6], China still need to cope with a large number of imported malaria cases every year. Located in southwest China bordering Myanmar, Laos and other Southeast Asian countries (Fig.1), Yunnan Province had experienced large-scale malaria epidemic and was once the hardest-hit area of malaria. As long as Africa and Southeast Asia are the main sources of imported malaria cases in China [7], Yunnan Province is still facing a great risk of imported malaria, especially vivax malaria. From 2011 to 2018, 50.7% of the imported malaria cases in Yunnan Province are caused by *P. vivax* [8]. Therefore, the effective elimination of malaria in Yunnan Province calls for the strict enforcement to block the transmission of *P. vivax*.

Fig 1. The map of China highlighting the geographic location of Yunnan Province. Different colors represent different administrative areas, and green labels indicate the main source of infection in areas with imported cases.

Primaquine is the traditional medication proved to be effective in eradicating the hypnozoites of *P. vivax*, and the World Health Organization (WHO) recommended it as the available antimalarial drug for the treatment of relapsed malaria (except for the new drug Tafenoquine) [9]. However, the active component in primaquine for killing *P. vivax* hypnozoite, 5-hydroxy-primaquine, requires the catalysis of Cytochrome P450, family 2, subfamily D, polypeptide 6 (CYP2D6) enzyme in human hepatocytes [10-12]. CYP2D6, also known as debrisoquine-4-hydroxylase, is an isozyme belonging to the Cytochrome P450 (CYP450) superfamily and an important phase I drug-metabolizing enzyme in the human system. CYP2D6 is the only enzyme in the CYP450 superfamily that cannot be created through inducer in the environment and it has low capacity and high affinity. Even though CYP2D6 only accounts for 4% of the total P450 enzyme protein in the liver, it dominates the metabolism of ~30% commonly used drugs, including primaquine [12]. Studies have found that the heterogeneity of CYP2D6 activity is predominantly governed by its genetic variation [13], and that decreased CYP2D6 isoenzyme activity caused by genetic polymorphism would obstruct the generation of 5-hydroxy-primaquine, making large doses or repeated use of primaquine a common remedy to compensate for the declined efficacy of primaquine in the treatment. Meanwhile, primaquine can cause life threatening hemolysis in patients with Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency, subsequently inflicting dual hazards, including low efficacy of anti-relapse treatment of primaquine and the continuously hemolysis, on the patients [9,14-15]. Owing to the dismal fact that the distribution of patients with defective CYP2D6 is highly coincident with those with G6PD-deficiency in the endemic area of vivax malaria [16-17], the patient's G6PD gene type and CYP2D6 enzyme activity should be identified by care workers prior to receiving anti-relapse treatment of vivax malaria [14,17]. WHO defines that G6PD deficiency, impaired CYP2D6 function, pregnant or lactating women, and young infants are not suitable for primaquine treatment [12,14].

CYP2D6 enzyme activity is measured mainly through phenotype observation and genotype analysis prediction. Compared with phenotype measurement, prediction method based on CYP2D6 genotype is characterized by the following advantages [18-19]. Firstly, genotypes do not change throughout one's life, and are not affected by environment or physical factors [19-20]. Secondly, genotyping only requires one specimen and can be completed before medication treatment [21]. In addition, human CYP2D6 genes can be easily sequenced to determine the mutation types of CYP2D6 alleles [22-23]. Therefore, the use of genotyping method can create more stable results in predicting the phenotype of CYP2D6 enzyme activity, and easy-to-handle for related research [18,24-28]. The prediction is based on the locus status of CYP2D6 gene [13], or the allelic combination form of zygote [29-30]. The identified genotypes consist of alleles with complete function, reduced function or loss of function; the indications of CYP2D6 enzyme activity include Poor metabolizer (PM), Intermediate metabolizer (IM), Normal metabolizer (NM) and Ultra-rapid metabolizer (UM), covering various active levels of CYP2D6 enzymes from ultra-fast to non-metabolic[28,30]. However, more than 80 CYP2D6 alleles have been defined by the Cytochrome P450 Nomenclature Committee at <http://www.cypalleles.ki.se> [23]. The prediction of CYP2D6 enzyme activity by performing genotype analysis needs to be further optimized to enhance accuracy and systematicness [31].

Gene mutations of enzymes related to drug metabolism not only affect the clinical consequences of drug metabolism, but can even lead to individual adverse event [13]. Therefore, understanding the specific CYP2D6 enzyme activity in different individual is the prerequisite to ensure radical treatment of vivax malaria based on personalized primaquine dose [28]. Despite the frequent use of primaquine in malaria endemic areas of Yunnan, no systematic survey about genetic polymorphisms of CYP2D6 has been conducted. Therefore, the appropriate population for primaquine treatment is yet to be clarified. In order to identify the unsuitable population for primaquine use and to understand the CYP2D6 enzyme activity level of the population in malaria endemic areas of Yunnan Province. This study intends to investigate the genetic association between human CYP2D6 genotype and declined efficacy of primaquine in the radical cure of *P. vivax*.

Materials And Methods

Subjects and blood sample

Blood samples of diagnosed and reported vivax malaria cases were consecutively collected in Yunnan Province from 2014 to 2018. Samples used for reexamination were provided by the Centers for Disease Control and Prevention in Yunnan Province. The mono-infection of *P. vivax* requires dual parasitically confirmation by both microscopy examination and *Plasmodium* 18S rRNA gene detection (PCR) by Yunnan Province Reference Laboratory (YNRL). Dried blood spots samples for genetic analysis were derived from 0.6 ml of venous blood, which were then put in a drying tube and stored at -80 °C before DNA extraction. The cases were divided into two groups: relapsed group and non-relapsed group. All vivax malaria patients spent three days in the hospital and underwent three-days oral Chloroquine therapy (total 1550 mg), followed by an 8-day course of Primaquine (22.5 mg/day). The relapsed cases consisted of patients who relapsed after receiving above-mentioned treatment, and their infection was caused by the same *Plasmodium* in two samples detected by sequencing of *P. vivax* circumsporozoite protein (*pvcsp*). A total of 75 relapsed cases

were confirmed in Yunnan Province from 2014 to 2018. Non-relapsed cases were randomly selected from vivax malaria cases of 2018. To avoid bias caused by different sample size, the two groups had equal number of cases, and the total number of experimental samples was set as 150. The infection sources of vivax malaria cases were confirmed according to epidemiological investigation: those without a travel history to epidemic areas outside Yunnan Province within the last 30 days before the onset of malaria were defined as local cases; those have history of travelling to epidemic regions, such as Myanmar and Africa, were regarded as imported cases.

Extraction of human genomic DNA

Three dried blood spots (diameter = 5 mm) were used, and human genomic DNA was extracted by using QIAgen Mini Kit kit (Germany, QIAamp Company's DNA Mini Kit), according to the manufacture's instruction. The extracted DNA was stored at -20 °C for the subsequent assay.

PCR amplification of CYP2D6 gene fragment

The primers of CYP2D6 for polymerase chain reaction (PCR) amplification 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 condition in previous studies [25,32]. The primers for first-round nested PCR covering the coding region of CYP2D6 gene exons1~4 were: 5'-CCAGTGACAGATAAGGGTGC-3' and 5'-GACGTGGATAGGAGGTACAGAG-3'; the primers for second-round nested PCR were: 5'-GGTGACTTCTCCGACCAGG-3' and 5'-TTCCCAAACCCATCTATGC-3'. The final amplification region was 42131088 ~ 42128678, and the expected fragment length of the product was 2411 bp. The primers that cover exons 5~9 of CYP2D6 gene were: 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 (20umol / L), 0.7µl downstream primers (20umol / L). The total volume was adjusted 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. The triplicated parallel repetition was adopted for each PCR reaction. The amplified products were observed using 1.5% agarose gel electrophoresis. The positive amplification products were sent to Shanghai Meiji Biomedical Technology Co, Ltd. for sequencing by using the dideoxy chain-termination method. Only the sequences that showed identical results in at least two tests were used for subsequent analysis.

Mutation analysis of CYP2D6 coding region

The sequencing results were aligned by using DNASTar v5.10 and BioEdit v7.0.9.0 software. The obtained all DNA sequences were assessed by using the Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) in the NCBI platform. Sequences with identifications equals 100% and Query cover above 99% were considered as CYP2D6 gene sequence of human. The DNA sequence of exon 1~9 region of CYP2D6 gene was determined and spliced into CYP2D6 CDS in the order from exon1 to exon9. MEGA v5.04 software was used to align the CDS of each case 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 in each CDS, and the parameters of DNA diversity index (π) and expected heterozygosity (H_e) were calculated. The allele form of CYP2D6 gene coding region was determined by using the method described in previous studies [25-27].

SPSS software (version 21.0; IBN; Chicago; IL) was used to conduct chi-square test on the frequency of haplotype co-existed in relapsed and non-relapsed patients. The odds ratio (OR) value of haplotypes with statistical significance were calculated by comparing to the relapse rate of *P. vivax*. The significance level was set as $P < 0.05$; OR ≥ 1 indicates that the haplotype is a protective factor, otherwise a risk factor.

Prediction of CYP2D6 enzyme activity

The mutation loci (base substitution loci) of CYP2D6 gene were used to directly predict the enzymatic activity as one of the following four phenotypes, PM, IM, NM or UM. The allelic form of the coding region of CYP2D6 gene was identified according to the methods described by Gaedigk et al [20], Sistonen et al [25] and Zanger et al [27]. Among them, CYP2D6* 1 is wild-type allele; CYP2D6* 2 showed base substitutions at c. 408, c. 886 and c. 1457. Nevertheless, both two have the same functional normal allele type. CYP2D6 * 10 and CYP2D6* 41 are the alleles of decreased enzyme activity caused by C > T and G >A base substitution at c.100 and c.1023+36, respectively.

Subsequently, we assigned the activity score of the allelic form of CYP2D6 gene according to previously described methods [28,31]. It was found that the mutations at c. 408, c. 886 and c. 1457 are not implicated in impaired enzyme activity, therefore these three loci cannot be included in the activity score system. By comparison, the wild type alleles of c.100 and c.1023+36 had the assigned score of 1.0. The mutant alleles of c.100 and intron c.1023+36 scored 0.25 and 0.5, respectively. For the event of multiple loci in the genotype, the enzyme activity score was calculated as the sum of the scores of the included zygotes. The intervals of the diploid score within 0.0; $0 < x < 1.25$; $1.25 \leq x \leq 2.25$; $x > 2.25$ indicate the PM, IM, NM and UM phenotype of CYP2D6 isozyme activity, respectively.

Results

Sample information and PCR amplification of human CYP2D6 gene

A total of 75 relapsed cases of *P. vivax* were enrolled, including 71 cases of one relapsed event, 2 cases of two relapse events, and two case of three relapsed events. The geographic information of 75 non-relapsed cases is listed in Table 1. The male-female ratio was 2.66:1, and the proportions of cases imported from

Myanmar, Africa countries, Laos and Yunnan were 96.7% (145/150), 1.3% (2/150), 0.7% (1/150) and 1.3% (2/150), respectively. The odds ratio of relapsed was calculated in different age groups and genders. Statistical significance was determined in age groups of 5-20 years (OR = 0.380; 95% CI: 0.986~0.146) and 21-60 years (OR = 2.471; 95% CI: 5.417~1.127). Thusly, the risk of relapsed of vivax malaria in patients aged 5-20 years was significantly lower, whereas the risk of relapsed in the patients aged 21-60 was 2.471 times higher than that of other groups. No significant correlation between other age groups or gender and the relapsed of vivax malaria was determined.

Table 1. Information of 150 vivax malaria cases for amplification of CYP2D6 gene exon1-9 fragments

Characteristic	Years					Total
	2014	2015	2016	2017	2018	
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						
0-4	0	0	1	2	1	4
5-20	0	2	4	1	16	23
21-60	17	25	15	6	51	114
above 60	1	0	0	1	7	9
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

PCR amplification of two segments of human CYP2D6 gene, including exons1-4 and exons5-9, was performed by using 156 blood samples of relapsed cases and 75 blood samples of non-relapsed cases. The amplification products showed clear bands at 2000bp, which were considered as the target bands (Fig 2.).

a

b

Fig 2. PCR products of CYP2D6 gene fragment. **a** M: DNA marker; 1: blank control of PCR for the first round; 2: blank control of PCR for the second round; 3-6: The amplification products of exon 1-4 in CYP2D6 gene; **b** M: DNA marker; 1: blank control of PCR; 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 the complete exon1-9 (total length = 1491bp) were obtained from all samples. A total of 150 CDS chains (Genbank accession number: MT339044-MT339193) were selected from the 75 relapsed case and 75 non-relapsed cases, and were then compared with wild-type sequence (NC: 000022.11). Base substitutions at 12 loci, such as c.31 and c.100, were determined (Table 2). The proportions of third-base and first-base substitution in the codon triplet were 41.7% (5/12), and the proportion of second-base substitution was 16.6% (2/12). 7 missense mutation loci and 5 synonymous mutation loci were determined. The mutation loci of the relapsed cases accounted for 91.7% (11/12), whereas the mutation loci of non-relapsed cases accounted for 66.7% (8/12) (Table 2)

Table 2. Polymorphism Comparison of Relapse cases and Non-relapse cases in the coding domain of CYP2D6 Genes from 1st aa to 497th aa

Relapse cases			Non-relapse cases		
Loci	Codon change ^a	Amino acid change	Loci	Codon change ^a	Amino acid change
c.31	GTG → ATG	V11M	–	–	–
c.100	CCA → TCA	P34S	c.100	CCA → TCA	P34S
c.271	CTG → ATG	L91M	c.271	CTG → TTG	L91L
c.281	CAC → CGC	H94R	–	–	–
c.294	ACC → ACG	T98T	c.294	ACC → ACG	T98T
c.297	GCC → GCT	A99A	–	–	–
c.336	TTC → TTT	F112F	c.336	TTC → TTT	F112F
c.408	GTC → GTG	V136V	c.408	GTC → GTG	V136V
c.505	GGT → AGT	G169S	–	–	–
–	–	–	c.801	CCC → CCA	P267P
c.886	CGC → TGC	R296C	c.886	CGC → TGC	R296C
c.1457	AGC → ACC	S486T	c.1457	AGC → ACC	S486T

^a DNA base highlighted in bold indicates the occurrence of SNP.

Polymorphism of haplotypes

A total of 24 CYP2D6 haplotypes (Hap_1–Hap_24) were clustered amongst the 150 vivax malaria samples; 17 haplotypes were observed in the sequences of relapsed patients ($\pi=0.0015$, $He=0.8191$). 15 haplotypes were observed in the sequences of non-relapsed patients ($\pi=0.0014$ and $He=0.8065$). 8 haplotypes, including Hap_2, Hap_3, Hap_4, Hap_5, Hap_6, Hap_7, Hap_14 and Hap_17, were found in both relapsed and non-relapsed patients. Haplotype and frequency were shown in Fig. 3. Hap_3 was wild-type sequence, and the rest were 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).

Fig 3. 9 exons are indicated by numbered boxes with DNA polymorphisms indicated on top. Predicted amino acid

changes are indicated below; no change is synonymous mutation. R: Relapsed cases; N: Non-relapsed cases.

Haplotypes of CYP2D6 gene coding region and their association with relapse

We selected the Hap_2 ~ Hap_6 haplotype sequences which had more than one CDS and were found in relapsed and non-relapsed patients to calculate the odds ratio (Table3). Among them, only two haplotypes, Hap_4 (OR=0.159; 95% CI: 0.746 ~ 0.034) and Hap_6 (OR=5.615; 95% CI: 26.577 ~ 1.186) showed statistical significance ($P \leq 0.05$). Hence, Hap_4 indicates reduced risk of relapsing. By contrast, Hap_6 suggests 5.615-fold higher risk of relapse than other haplotypes. The OR values of Hap_2, Hap_3, Hap_5 and Hap_7 showed no statistically significant difference ($P > 0.05$) (Table 3).

Table 3 Analysis of the relationship 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)

n: number of cases; NS: not significant; S: significant; a: Chi-square test

Enzyme activity prediction of significant haplotypes and their diploids

The enzyme activity of Hap_6 and Hap_4 genotypes, which were validated to implicate in recurrence of vivax malaria, was predicted. As for the 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. Of the c.408, c.886, c.1023+36 and c.1457 mutations, only c.886 and c.1023+36 were diploid alleles. Whether the diploid of c.886 showed mutant heterozygosity (C/T) or mutant homozygosity (T/T), as long as it co-existed with mutant heterozygosity of c.1023+36, the genotype score of CYP2D6 couldn't reach 2.0. In this case, the score was 1.5 and the enzyme activity of CYP2D6 belonged to "NM" (Table 4). The genotypes of *1/*2 and *1/*41 were found in the non-relapsed cases. However, *1/*41 was also determined in the relapsed cases and reached 1.5 in genotype score. Hence its enzyme activity was predicted as "NM" level. Moreover, *1/*2 genotype which showed mutant heterozygous (C/T) only at c.886 reached the peak genotype score of 2.0 and was therefore predicted as "NM" level (Table 4).

Table 4 Prediction of enzyme activities of Hap-6 and Hap-4 genotypes

A. Hap_6								
Genotypes	Relapse times	No. of samples	Mutation 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	Mutation 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 predictivity of this mutation for impaired enzyme function has been demonstrated recently

13 CDS chains in Hap_4 was found in 2 relapsed and 11 non-relapsed patients (Table 4). In the relapsed cases, *1/*10-2 was the only diploid genotype. Mutations of *1/*10-2 at loci c.100, c.408 and c.1457 were heterozygosity (C/T), homozygosity (C/C) and homozygosity (C/C), respectively. Such pattern resembled the genotype of 54.6% (6/11) of the non-relapsed cases, and reached the genotype score of 1.25, which indicates "NM" level for CYP2D6 enzyme activity (Table 4). The remaining 5 non-relapsed cases were classified as genotype *10/*10 and *1/*10-1. The diploids of genotypes *10/*10 at c.100, c.408 and c.1457 loci were mutant homozygous (*T/T*), (*C/C*) and (*C/C*), respectively, reaching the lowest genotype score of 0.5. It was predicted that the enzyme activity of CYP2D6 belongs to "IM" level (Table 4). In 3 cases of *1/*10-1 genotype,

mutation heterozygosity (C/T) was found at c.100 loci; its genotype score of 1.25 suggests "NM" level of CYP2D6 enzyme activity (Table 4).

Discussion

Located on chromosome 22q13.1, CYP2D6 gene (4383bp in length) consists of 8~9 exons and 7~8 introns. Its CDS is 1338~1491bp in length and encodes 446~497 amino acids. The heterogeneity of CYP2D6 monooxygenase activity varies from complete dysfunction to ultra-rapid enzyme metabolism, depending on the various genotypes of CYP2D6 [14]. Among the 150 identified alleles, multiple mutations such as CYP2D6*3, CYP2D6*4, CYP2D6*5 and CYP2D6*6 are considered to contribute to compromised CYP2D6 enzyme activity [32,33].

To our knowledge, the current study is the first one that attempts to analyze mutation polymorphism in the whole coding region of human CYP2D6 gene, thereby verifying its possible association with thwarted CYP2D6 enzyme activity in relapsed vivax malaria patients after primaquine treatment. Among the 24 haplotypes defined, Hap_2 makes up the largest proportion of the sequences, reaching as high as 36.6% (55/150). This haplotype shows multiple mutations at four loci, including c.100C > T, c.336C > T, c.408G > C, c.1457G > C. These mutations are different from previous studies which reported that CYP2D6*10 allele is frequently detected in Asian populations [32, 34]. CYP2D6*10 allele has multiple mutations at c. 100(C > T), c. 408(G > C) and c. 1457(G > C), yet is devoid of c. 336C > T mutation as opposed to Hap_2. Such disparity might be attributed to fact that the sequencing of the whole gene coding region can easily detect all base substitutions. However, the factor of different population should be taken into account to determine whether such differences in common alleles of CYP2D6 gene are worthy of in-depth research. The population studied in this paper is not randomly selected, but is composed of vivax malaria patients residing in endemic areas, and 50% of them (75/150) had relapsed malaria. Thusly, the genotypes of such population may show the accurately selective outcome of human defective genes after the screening of malaria epidemic.

Although decreased enzyme activity in the heterogeneity of CYP2D6 is speculated to lead to failed therapy of primaquine to kill hypnozoite of *P. vivax* [9,17,35-37], 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), and that mutation at c. 886 locus is the key indicator that determines vivax malaria relapse. Such findings are not consistent with that of Silvino et al [37], which 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 different methods used for detecting gene mutation loci. Silvino et al. employed restriction enzyme digestion analysis to identify individual mutation locus, such as c.100 and c.321. Nevertheless, we applied whole gene coding region sequencing method to find locus polymorphism. Of note, in determining the close association between multiple mutations at c. 408, c. 886 and c. 1457 with the relapse of vivax malaria, we found that intron c.1023+36 mutation may play a critical role in aggravating damaged CYP2D6 enzyme activity. In the 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 mutant heterozygotes. This finding concurs with c. 1023+36 mutations found by Silvino et al [37] and Raimundo et al [38] in patients suffering from failed treatment of vivax malaria. Additionally, the multiple mutation genotypes at c.100, c.408 and c.1457 loci determined in the preset study depicted a unique phenomenon of reduced risk of vivax malaria relapse (OR = 0.159, $P < 0.05$), and such findings warrants further analysis and confirmation.

To date, direct prediction method based on CYP2D6 locus mutation polymorphism, which was summarized by Zanger et al [27] and indirect prediction method of genotype scoring, which was recommended by Gaedigk et al [28] and Caudle et al [31], are applied to predict CYP2D6 enzyme activity based on genetic information. Since the genotype scoring method can quantify the degree of gene mutation and abides by more abundant genotypic criteria, this method was used in the study. However, no matter Hap_6 indicates high risk of relapse or Hap_4 functions as the protective factors, we predicted that all the genotypes are above IM level. CYP2D6*2 is the allele type that indicates highest risk of relapse, and most of the non-relapsed patients had CYP2D6*10 allele. This result is not in line with that of Brasil et al [36], 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 of outcome might be justified that the prediction method of CYP2D6 enzyme activity used in this study focuses on analyzing the whole gene coding region information, thereby taking more alleles into account and leading 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 shows heterogeneous phenotypes exhibiting both normal and abnormal enzyme activity [39]. Therefore, identifying the heterogeneous CYP2D6 with similar genotypes is the key to predicate the enzyme activity of CYP2D6 [20, 28], regardless of the method used for detection. Meanwhile, the prediction method should be further optimized, because the effect of primaquine resistance in patients with relapsed vivax malaria is not eliminated.

Although this study applied first-generation sequencing method to analyze polymorphism in the coding region of CYP2D6 gene, the strategy of triplicated parallel repetition was adopted for each PCR reaction and the sequencings of its products. Such effort helped eliminate the rate of error in single PCR 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 in the endemic areas where primaquine is used to intervene vivax malaria. However, this study has some limitations. 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, all the non-relapsed patients were selected from 2018, which is not equivalent to the relapsed patients enrolled for four consecutive years. Sampling bias may affect the experimental results due to changes of various factors over the years. In addition, other confounding factors which may amplify the identification of relapsed vivax malaria patients, such as the existence of primaquine-resisted *Plasmodium*, are not ruled out, despite the identification of relapsed cases has been achieved by comparing the genetic origin of infected strains. Finally, CYP2D6 enzyme activity was analyzed by assigning the activity

score of zygotic allelic, a method recommends by Caudle et al [31], yet this study defined more CYP2D6 gene locus polymorphisms. Since there is no benchmark standard to scores the activity of these loci polymorphisms, the prediction of CYP2D6 enzyme activity in this study may not be completely homogenous with the findings of Caudle et al.

Conclusion

In conclusion, the prediction of CYP2D6 enzyme activity based on whole gene coding region sequencing preliminarily reveals CYP2D6 gene mutation polymorphisms in relapsed *P. vivax* patients who showed poor curative effect of primaquine in Yunnan. This study makes up for the analysis of genetic correlation factors in relapsed *P. vivax* patients in China, and reaches the following findings: (1) Among the multiple mutations of CYP2D6 gene, combined mutations at three loci, including c.408, c.886 and c.1457, are most closely related to decreased CYP2D6 enzyme activity. Mutations at c.408 and c.1457 induce compromised CYP2D6 enzyme activity, yet a larger sample size is need to validate whether such decreased activity is aggravated by c.886 mutation; (2) The key to predict CYP2D6 enzyme activity is to define the genotype accurately and accordingly, based on 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 a promising molecular epidemiological method for investigating CYP2D6 gene mutation polymorphism.

Abbreviations

WHO: World Health Organization; CYP2D6: Cytochrome P450, family 2, subfamily D, polypeptide 6; G6PD: Glucose-6-Phosphate Dehydrogenase; CDS: coding DNA sequence; PCR: polymerase chain reaction; 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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Figures

Figure 1

1 The map of China highlighting the geographic location of Yunnan Province. Different colors represent different administrative areas, and green labels indicate the main source of infection in areas with many imported cases. 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

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

Figure 3

The 9 exons are indicated by numbered boxes with DNA polymorphisms indicated on top. Predicted amino acid changes are indicated below; no change is synonymous mutation. R: Relapsed cases; N: Non-relapsed cases.

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