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ORIGINAL RESEARCH

Pharmacogene Variation in Thai *Plasmodium vivax* Relapse Patients Treated with a Combination of Primaquine and Chloroquine

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Purpose: Pharmacogenes have an influence on biotransformation pathway and clinical outcome of primaquine and chloroquine which are often prescribed to treat *Plasmodium vivax* infection. Genetic variation may impact enzyme activity and/or transporter function and thereby contribute to relapse. The aim of the study was to assess allele, genotype frequencies and the association between pharmacogenes variation and primaquine response in Thai patients infected with *Plasmodium vivax*.

Patients and Methods: Fifty-one patients were genotyped for 74 variants in 18 genes by Sequenom MassARRAY® and Taqman® SNP Real-Time PCR.

Results: SNP frequencies were not significantly different between relapse (n=4) and non-relapse (n=47) patients. However, the *CYP2C19* c.681G>A, the frequency of the A-allele that defines the non-functional *CYP2C19*2* haplotype was significantly higher compared to the G-allele (OR=5.14, p=0.021). Patients heterozygous for *ABCG2* c.421C>A had a higher odds ratio (OR=8.75, p=0.071) and the frequency of the G-allele of *UGT2B7 c*.372G>A was higher compared to the A-allele (OR=3.75, p=0.081). *CYP2C19, ABCG2* and *UGT2B7* emerged as potential high priority genes.

Conclusion: Decreased activity of CYP2C19, ABCG2 and UGT2B7 in combination with CYP2D6 intermediate or poor metabolizer status may expose patients to a higher risk of *Plasmodium vivax* relapse. Further investigations are warranted to substantiate these findings.

Keywords: primaquine, chloroquine, *Plasmodium vivax*, relapse, pharmacogenes

Introduction

Primaquine (PQ), an 8-amino-6-methoxyquinoline (8AQ) derivative is the only drug approved by the United States Federal Drug Administration to treat acute illness and relapse of *Plasmodium vivax* (*P. vivax*) and *P. ovale* infection caused by hypnozoites that persist in the hepatocytes of infected patients.[1,2] Recently, a number of studies reported on the metabolism of PQ showing that biotransformation occurs through three main pathways which are Cytochrome P450 (CYP450) enzymes (CYP2D6, CYP2C19, CYP3A4, CYP1A2), monoamine oxidases (MAO-A and B) and flavin-containing monooxygenase-3 (FMO-3).[3–7] PQ is a pro-drug primarily metabolized by MAO-A to PQ aldehyde, which is further oxidized by aldehyde dehydrogenase (ADH) to carboxyprimaquine, the major PQ metabolite found in plasma.[4,8] Carboxyprimaquine is further oxidized by FMO to the N-hydroxylated PQ metabolite which can cause hemotoxicity.[9] Finally, PQ is metabolized via CYP2D6 to 5-hydroxyprimaquine, 5, 6-orthoquinone, and other phenolic metabolites; other P450 enzymes are also believed to contribute to PQ

metabolism.[4,5,10] Chloroquine (CQ) is metabolized into N-desethylchloroquine by CYP2C8, CYP3A4, and CYP2D6 by in vitro study.[11]

The major challenge of elimination of malaria caused by *P. vivax* and *P. ovale* in endemic areas is relapse of dormant hypnozoites that survive in the liver of the patient after primary infection. These hypnozoites can persistent in the liver for weeks, months or even years following a primary attack.[12,13] Although PQ has been used to treat *P. vivax* and *P. ovale* infections for several decades, the exact mechanisms of PQ efficacy and toxicity are still not well understood, neither have the metabolic pathways been fully elucidated. It has been postulated that human host genetics may, at least in part, contribute to the failure of PQ treatment.[7] Bennett et al[7] first reported a significant association between CYP2D6 metabolizer phenotype and relapsing *P. vivax* infection. Relapsing CYP2D6 poor (PM) and intermediate metabolizer (IM) patients had a significant higher plasma concentration of the parent drug after 24 hrs compared to non-relapsing patients. These data supported the hypothesis that the CYP2D6-dependent pathway is crucial for the bioactivation of PQ to its phenolic metabolites which are the active metabolites responsible for the elimination of dormant hypnozoites in the liver. Furthermore, these data suggested that patients with impaired CYP2D6 activity caused by genetic variation in the *CYP2D6* gene may be at a higher risk of relapse of *P. vivax*. However, a previous study by our group has also found relapsing infections in patients with normal CYP2D6 metabolism.[14] It needs to be noted that our patients have been treated with a combination of PQ and CQ (per standard Thai guidelines). Thus, we are speculating that genetic variation in other genes that contribute to PQ and CQ metabolism may also impact a patient's response to *P. vivax* treatment in Thai patients. Moreover, in addition to drug-metabolizing enzymes, transporters have been shown to affect PQ efficacy.[15,16] Sortica et al found that SLCO2B1, SLCO1A2 and SLCO1B1 were associated with the clearance of *P. vivax* in patients treated with PQ and CQ.[16] The MRP transporter, for example, can be inhibited by quinoline derivatives,[17] and Hayeshi et al demonstrated inhibitory effects of several antimalarial drugs to P-glycoprotein (P-gp) mediated transport and reported that both, PQ and chloroquine, inhibit P-gp.[18]

This study aimed to investigate genetic variation in drug-metabolizing enzymes and drug transporters and their association with relapse in Thai patients treated with a PQ/CQ combination regimen.

Materials and Methods

This exploratory investigation included 51 Thai patients from a previous study.[14] The study was approved by the Internal Ethics Review Committee on Human Research of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand (MURA 2016/657) and conducted in accordance with the Declaration of Helsinki. Briefly, symptomatic *P. vivax* patients from the Tha Song Yang malaria clinic, Tak province, Thailand were recruited from April 2014 to September 2015; all patients gave written informed consent. Patients were diagnosed with *P. vivax* infection and treated with 25 mg base/kg body weight CQ over 3 days and 0.3 mg/kg PQ daily for 14 days. Finger-prick blood samples were collected before treatment and at 1 and 2 weeks after enrollment, then every 2 weeks for 6 months, then every 4 weeks until 9 months.[14] Patients for the current study were selected based on the availability of genomic DNA and clinical data including recurrent status and date of follow up/survival data.

Genes Analyzed with MassARRAY and Real-Time PCR

DNA samples diluted to 10 ng/μL were genotyped using the Sequenom MassARRAY® System (Agena Bioscience™, San Diego, CA, USA). The panel consisted of pre-designed SNPs and indels (referred to SNVs therein), and CNV assays that target the most relevant variants in 11 important pharmacogenes. A total of 53 SNVs were interrogated and five assays were utilized to determine *CYP2D6* gene copy number variation (CNV) (Table 1). The iPLEX® PGx 68 Panel (Agena, San Diego, CA) included drug transporters (*ABCB1, SLCO1B1, SULT4A1*), Phase I enzymes (*COMT, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5*). The workflow consisted of five steps: PCR amplification, primer extension or fragmentation, dispensed extension product onto a SpectroCHIP® Array and MassARRAY MALDI-TOF mass spectrometry. Automated software provided diplotype, haplotype, and CNV calls in a single combined report. The overall process was completed in less than 10 hrs.

An additional 21 SNVs of 10 genes including *CYP2B6, CYP3A4, ABCA1, ABCB1, ABCC2, ABCC4, ABCG2, SLC25A40, SULT1A1*, and *UGT2B7* were genotyped with commercially available TaqMan® Genotyping Assays

Table 1 List of Genes and SNPs Detected by MassArray® and Taqman® RT-PCR

No.	Genes	Nucleotide Change	SNP ID	Alleles Detected
1	*CYP1A2*	g.-3860G>A, g.-163C>A, g.-729C>T, g.3533G>A, g.558C>A	**rs2069514, rs762551, rs12720461, rs56107638, rs72547513**	**1A,*1C,*1F,*1K,*7,*11*
2	*CYP2B6*	c.983T>C, c.64C>T, c.516G>T, c.785A>G	**rs28399499**, rs8192709, rs3745274, rs2279343	**2, *4, *6, *16, *18*
3	*CYP2C9*	c.430C>T, c.1075A>C, c.1076T>C, c.1080C>G, c.818delA, c.449G>A, c.1003C>T, c.1465C>T, c.269T>C, c.485C>A	**rs1799853, rs1057910, rs56165452, rs28371686, rs9332131, rs7900194, rs28371685, rs9332239, rs72558187, rs72558190**	**2, *3, *4, *5, *6, *8, *11, *12,*13, *15, *18, *25*
4	*CYP2C19*	c.681G>A, c.636G>A, c.1A>G, c.1297C>T, c.395G>A, g.19294T>A, c.358T>C, g.-806C>T	**rs4244285, rs4986893, rs28399504, rs56337013, rs72552267, rs72558186, rs41291556, rs12248560**	**2,*3, *4, *5, *6, *7, *8, *17*
5	*CYP2D6*	g.2850C>T, g.4180G>C, g.2549delA, g.1846G>A, g.1707delT, g.2935A>C, g.1758G>T, g.2615_2617delAAG, g.100C>T, g.883G>C, g.124G>A, g.137_138insT, g.1023C>T, g.4125_4133dupGTGCCCACT, g.2539_2542delAACT, g.1973_1974insG, g.3183G>A, g.2988G>A, g.4155C>T	**rs16947, rs1135840, rs35742686, rs3892097, rs5030655, rs5030867, rs5030865, rs5030656, rs1065852, rs201377835, rs5030862, rs774671100, rs28371706, hCV32407220, rs72549353, rs72549354, rs59421388, rs28371725, rs28371735**	**1,*2,*3,*4,*6,*7,*8,*9,*10, *11,*12,*14A,*14B,*15,*17, *18,*19,*20,*29,*36,*41,*69* and **5 del*
6	*CYP3A4*	c.664T>C, c.566 T>C, g.15389C>T, c.-392A>G, c.878T>C	**rs55785340, rs4987161, rs35599367**, rs2740574, rs28371759	**1B, *2, *17, *18, *22*
7	*CYP3A5*	g.27289C>A, g.6986A>G, g.27131_27132insT	**rs28365083, rs776746, rs41303343**	**1A,*2,*3,*7, *10*
8	*ABCB1*	c.1236C>T, c.2677G>T/A, c.3435C>T	rs1128503, rs2032582, **rs1045642**	**1, *2, *6*
9	*ABCA1*	c.2649A>G, c.4760A>G	rs2066714, rs2230808	n/a
10	*ABCC2*	c.-24C>T, c.3972C>T, g.68231A>G	rs717620, rs3740066, rs3740065	**1A, *1C*
11	*ABCC4*	c.912C>A, c.2269G>A	rs2274407, rs3765534	n/a
12	*ABCG2*	c.421C>A	rs2231142	n/a
13	*SLCO1B1*	g.37041T>C	**rs4149056**	**5*
14	*SULT1A1*	c.638G>A	rs9282861	n/a
15	*SULT4A1*	*1113A>G	**rs763120**	n/a
16	*SLC25A40*	g.87868008C>G	rs10239908	n/a
17	*COMT*	c.322G>A	**rs4680**	n/a
18	*UGT2B7*	c.-161C>T, c.211G>T, c.372A>G, c.802C>T	rs7668258, rs12233719, rs28365063, hCV32449742	**1d, *2a, *3*

Notes: Allele definitions are according to the Pharmacogene Variation Consortium at www.PharmVar.org. SNV coordinates are provided either on the gDNA or cDNA level based on which numbering is more commonly used in the literature. n/a, not available, ie SNP is not part of a star (*) allele definition. The reference SNP ID in bold are in the MassArray iPLEX PGx68 panel; the *CYP2D6* variants were defined as M33388 reference sequence.

(Applied Biosystems™, Carlsbad, CA, USA) following manufacturer's instructions. Details are provided in Table 1. Allelic variants were designated according to the Pharmacogene Variation Consortium (PharmVar) at www.PharmVar.org.[19,20]

Activity scores (AS) were assigned as previously described[21] and applied in CPIC guidelines.[22–25] To assess the combined impact of CYP2D6 and CYP2C19 activity, we assigned a composite AS, which is the sum of the AS assigned to each gene.

Statistical Analysis

Deviation from Hardy-Weinberg expectations was assessed using an exact and chi-square test. SNV and genotype frequencies were determined by direct counting. Linkage disequilibrium measures (r^2) and haplotype analysis were performed using Haploview version 4.2.[26] Comparisons of SNV and genotype frequencies between relapsing and non-relapsing patients were performed using the $\chi 2$ test, odds ratio and 95% confidence intervals were calculated as measurements of the strength of association. The probability of significant associations was set at $p<0.05$. Relapse-free survival (RFS) was estimated using the log-rank test and Kaplan–Meier curves. The Cox proportional hazards model was conducted to assess the impact of candidate SNPs on relapse of *P. vivax*. All statistical analyses were performed using STATA software version 14 (StataCorp LP, TX, USA).

Results

Demographic Data

The average age of the 51 patients was 26.37 (min–max; 7–71) years with 32 (63%) males and 19 (37%) females. There were four recurrent *P. vivax* infections, which occurred between 8 and 32-weeks after the initial treatment, the average time of follow-up was 7.7±1.7 months. The clinical status of 45 patients was recorded for at least 6 months.

Association of Pharmacogene Variation and Relapse

The call rate for SNV genotyping was 100% for both testing platforms. Table 1 summarizes the genes and SNVs tested by MassArray® and Taqman RT-PCR®. SNV and genotype frequencies in the relapsing and non-relapsing patients are provided in Tables 2 and 3, respectively. All SNVs were in Hardy-Weinberg equilibrium except *CYP2D6* g.4180G>C and

Table 2 SNP Frequencies of Drug Metabolizing Genes Between Relapse and Non-Relapse Groups

Gene	Genotype	N (%) (N=51)	Association Test		OR (95% CI)	*p* value
			Relapse	Non-Relapse		
			(N=4)	(N=47)		
CYP2C19						
c.681G>A	G/G	28 (54.9)	0	28	–	0.052*
rs4244285	G/A	18 (35.3)	3	15	6.40 (0.61–66.76)	0.120
	A/A	5 (9.8)	1	4	3.58 (0.30–42.97)	0.347
	G/A+A/A	23 (45.1)	4	19	7.25 (0.79–66.84)	0.082
	G	74 (72.6)	3	71	–	
	A	28 (27.5)	5	23	5.14 (0.90–35.01)	0.021**
UGT2B7						
c.372A>G	A/A	30 (58.8)	1	29	–	0.177*
rs28365063	A/G	17 (33.3)	2	15	2.13 (0.27–16.63)	0.593
	G/G	4 (7.8)	1	3	4.89 (0.38–62.46)	0.286
	A/G+G/G	21 (41.2)	3	18	4.83 (0.47–50.09)	0.293
	A	77 (75.5)	4	73	–	
	G	25 (24.5)	4	21	3.48 (0.59–20.08)	0.081
ABCG2						
c.421C>A	C/C	34 (66.7)	1	33	–	0.152*
rs2231142	C/A	15 (29.4)	3	12	8.75 (0.83–92.32)	0.071
	A/A	2 (3.9)	0	2	3.07 (0.27–35.33)	0.379
	C/A+A/A	18 (33.3)	3	14	7.07 (0.68–73.99)	0.102
	C	83 (81.4)	5	78	–	
	A	19 (18.6)	3	16	2.93 (0.41–16.65)	0.153

Notes: *Overall *p* value; **significance ($p<0.05$); ref, reference was compared to others in the sub-analysis.
Abbreviations: N, number; OR, odds ratio; 95% CI, 95% confidence interval.

Table 3 Genotype Frequencies of Drug Metabolizing Genes Between Relapse and Non-Relapse Groups

Gene	Genotype	N (%)	Association Test		*p* value
			Relapse	Non-Relapse	
		(N=51)	(N=4)	(N=47)	
CYP1A2	*1A/*1A	2 (3.9)	0	2	0.608*
	*1A/*1F	16 (31.4)	1	15	
	*1A/*1L or *1C/*1F	6 (11.8)	1	5	
	*1F/*1F	8 (15.7)	0	8	
	*1F/*1L	14 (27.5)	1	13	
	*1L/*1L	5 (9.8)	1	4	
	*1A/*1A vs others		4	45	0.379
CYP2B6	*1/*1	15 (29.4)	1	14	0.180*
	*1/*2	1 (2.0)	0	1	
	*1/*4	2 (3.9)	1	1	
	*1/*6	21 (41.2)	1	20	
	*2/*6	1 (2.0)	0	1	
	*4/*6	3 (5.9)	1	2	
	*6/*6	8 (15.7)	0	8	
CYP2C9	*1/*1	47 (92.2)	4	43	1.000*
	*1/*3	3 (5.9)	0	3	
	*3/*3	1 (2.0)	0	1	
CYP2C19	*1/*1	23 (45.1)	0	23	0.211*
	*1/*2	17 (33.3)	3	14	
	*1/*3	4 (7.8)	0	4	
	*1/*5	1 (2.0)	0	1	
	*2/*2	5 (9.8)	1	4	
	*2/*3	1 (2.0)	0	1	
	*1/*2+*2/*2 vs others	22 (43.1)	4	18	0.075*
CYP2D6	*1/*1	5 (9.8)	0	5	0.937*
	*1/*2	4 (7.8)	0	4	
	*1/*5	4 (7.8)	1	3	
	*1/*10	4 (7.8)	0	4	
	*1/*41	1 (2.0)	0	1	
	*2/*2	6 (11.8)	0	6	
	*2/*10	11 (21.6)	2	9	
	*2/*41	3 (5.9)	0	3	
	*4/*10	1 (2.0)	0	1	
	*5/*5	1 (2.0)	0	1	
	*5/*10	1 (2.0)	0	1	
	*10/*10	8 (15.7)	1	7	
	*10/*41	2 (3.9)	0	2	
CYP3A4	*1/*1	49 (96.1)	4	45	0.379*
	*1/*1B	2 (3.9)	0	2	
CYP3A5	*1A/*1A	4 (7.8)	1	3	0.492*
	*1A/*3	19 (37.3)	1	18	
	*3/*3	28 (54.9)	2	26	
	*1A/*3+*3/*3	47 (92.2)	3	44	0.286
SLCO1B1	*1/*1	47 (92.2)	3	44	0.286*
	*1/*5	4 (7.8)	1	3	

Note: *Overall *p*-value.
Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.

ABCA1 c.4760A>G ($p<0.05$) which were excluded from subsequent analyses. Nine SNVs in nine genes had minor allele frequency (MAF) of less than 5% (Table S1). The frequencies of the most common genotypes are shown in Table 3 which were *CYP1A2*1A/*1F* (31.4%), *CYP2B6*1/*6* (41.2%), *CYP2C9*1/*3* (5.9%), *CYP2C19*1/*2* (33.3%), *CYP2D6*2/*10* (21.6%), *CYP3A4*1/*1B* (3.9%), *CYP3A5*3/*3* (54.9%), and *SLCO1B1*1/*5* (7.8%). In addition, frequencies for 0, 1, 2, 3 and 4 *CYP2D6* gene copies were 1.96% (n=1), 9.80% (n=5), 45.10% (n=23), 41.18% (n=21), and 1.96% (n=1), respectively. The *CYP2D6* CNV status and genotype of the four relapsed patients were three copies (two patients with a *CYP2D6*2/*10* and 1 patient with a *CYP2D6*10/*10* genotype) and one copy (one patient genotyped as *CYP2D6*1/*5*). None of these genotypes, or any other genotypes, revealed statistically significant association between relapse and non-relapse (Tables 3 and S2).

The genotype frequencies of the majority of drug-metabolizing enzyme and transporter genes of the relapsing patients were similar with those previously described for Thai. Frequencies for the candidate genes including *CYP2D6, CYP2C9, CYP2C19, CYP3A4, CYP1A2* were not significantly different among non-relapsing and relapsing patients ($p>0.05$). As shown in Table 3 there was a trend, however, for a significant association between *CYP2C19* c.681G>A, and relapse (G/A+A/A genotypes vs G/G genotype; OR=7.25, 95% CI; 0.79–66.84, $p=0.082$). Furthermore, the c.681A SNV defining the non-functional *CYP2C19*2* allele was significantly more common than the c.681G allele in relapse versus non-relapse patients

(OR=5.14, 95% CI; 0.90–35.01, p=0.021). Moreover, the *ABCG2* c.421C>A transporter SNV had a higher odds ratio, although this did not reach statistical significance (OR=8.75, 95% CI; 0.83–92.32, p=0.071). Lastly, we also explored the relationship between a composite AS for CYP2D6 + CYP2C19 and PQ response. A composite AS, however, was not significantly different (p=0.09) (Table S3).

Survival Analysis

RFS was calculated from enrollment date to the time of *P. vivax* relapse. The effect of genetic variation in pharmacogenes on RFS was investigated by grouping patients by SNVs and genotype. Kaplan–Meier analysis revealed no statistically significant differences in RFS rates for most of the variable factors. However, a trend toward statistical significance of RFS rates was observed for patients carrying the *CYP2C19*2* allele (c.681G>A) (Log-rank test; p=0.077, Figure 1A), and *ABCG2* c.421C>A (Log-rank test; p=0.099, Figure 1B). Also, as shown in Figure 1C and D, a sub-analysis of *CYP2D6* copy number variation revealed significantly different RFS rates between 1 and 2 gene copies (p=0.007) and between 2 and 3 gene copies (p=0.065), respectively. There was no statistical difference in RFS rates among groups for any of the other variable factors (Table S4).

Relapse-Free Survival Rate by Univariate and Multivariate Analysis

Cox proportional hazards analysis was used for the determination of univariate and multivariate association analysis of the SNVs with RFS. In multivariate analyses, there was no significant difference in RFS rates between SNVs, genotype or other variables (Table 4). However, a sub-analysis showed that *CYP1A2* g.-163C>A, *CYP2C19*2* (c.681G>A), *CYP2D6* copy number, *ABCA1* c.2649A>G, *ABCB1* c.1236C>T, *ABCC2* g.68231A>G, *ABCG2* c.42 1C>A, and *SLCO1B1* g.37041T>C had statistically

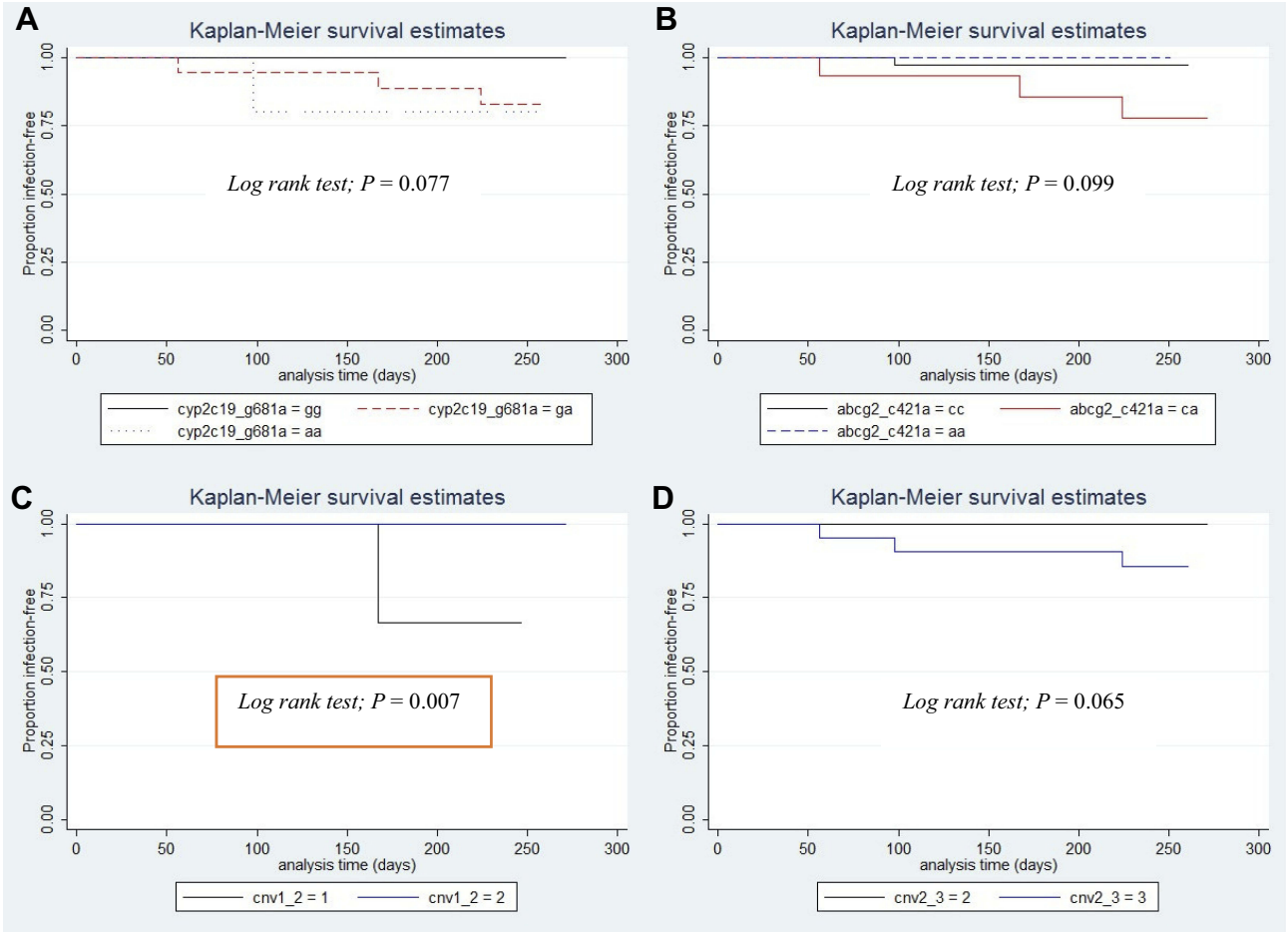

Figure 1 Kaplan–Meier estimates of relapse-free survival according to (**A**) *CYP2C19* c.681G>A genotype, (**B**) *ABCG2* c.421C>A genotype, (**C**) *CYP2D6* copy number variation (CNV) 1 vs 2 gene copies, (**D**) *CYP2D6* CNV 2 vs 3 gene copies.

Table 4 Univariate and Multivariate Analysis of the Effects of Different Variables on *P. vivax* Relapse Outcome

Variable Factors	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	***p* value**	**HR (95% CI)**	***p* value**
CYP1A2 genotype				
**1A/*1A*	Ref.		–	–
**1A/*1F*	1.66e+09	–	–	–
**1A/*1L or *1C/*1F*	4.15e+09 (2.60e+08 – 6.64e+10)	<0.001	–	–
**1F/*1F*	–	1.000	–	–
**1F/*1L*	1.72+09 (1.08 e+08 – 2.75e+10)	<0.001	–	–
**1L/*1L*	4.54+09 (2.84e+08 – 7.28e+10)	<0.001	–	–
CYP1A2 g.-163C>A			1.46 (0.08–26.25)	0.799*
C/C	Ref.			
C/A	2.30e+08	-	–	–
A/A	1.68e+08 (2.38e+07-1.19e+09)	<0.001	13.85	1.000
CYP2C19 genotype				
**1/*1*	ref.		–	–
**1/*2*	1.69e+10 (1.76e+09-1.63e+11)	<0.001	–	–
**1/*3*	1.12e-07	1.000	–	–
**1/*5*	1.12e-07	1.000	–	–
**2/*2*	1.95e+10	-	–	–
**2/*3*	1.12e-07	1.000	–	–
CYP2C19 c.681G>A			18.64 (0.79–439.28)	0.070*
G/G	Ref.			
G/A	1.52e+10 (1.58e+09-1.46e+11)	<0.001	4.24e+37	0.998
A/A	-	-	–	–
G/G vs A/A	8.08e+17	1.000	–	–
CYP2D6 genotype			1.31 (0.60–2.89)	0.505*
**1/*1*	Ref.		–	–
**1/*10*	8.32e-07	1.000	–	–
**1/*2*	8.32e-07	1.000	–	–
**1/*41*	8.32e-07	1.000	–	–
**1/*5*	4.78e+10	-	–	–
**10/*10*	1.26e+10 (6.95e+08-2.29e+11)	<0.001	1.94e-19	–
**10/*4*	8.32e-07	1.000	–	–
**10/*41*	8.32e-07	1.000	–	–
**10/*5*	8.32e-07	1.000	–	–
**2/*10*	1.74e+10 (1.37e+09-2.22e+11)	<0.001	8.97e-38	–
**2/*2*	7.85e-07	1.000	–	–
**2/*41*	8.32e-07	1.000	–	–
**5/*5*	8.32e-07	1.000	–	–
CYP2D6 CNV	1.36 (0.32–5.705)	0.678	–	
CNV: 2 copy vs 1 copy	3.55e-18	-		
CNV: 2 copy vs 3 copy	2.99e+15	-		
ABCB1 c.1236C>T			0.150 (0.01–3.233)	0.225*
C/C	Ref.		–	
C/T	5.10e+08 (7.16e+07-3.64e+09)	<0.001	0.002	1.000
T/T	1.60e+08		–	-

(*Continued*)

Table 4 (Continued).

Variable Factors	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	***p* value**	**HR (95% CI)**	***p* value**
ABCA1 c.2649A>G			4.30 (0.18–101.12)	0.365*
A/A	Ref.		–	
A/G	1.64e+09 (1.71e+08-1.58e+10)	<0.001	1.28e+08	0.999
G/G	1.46e+09	-	–	-
ABCC2 g.68231A>G			30.90 (0.13–7451.06)	0.220*
A/A	Ref.		–	
A/G	4.61 (0.48–44.30)	0.186	1.07e+32	0.997
ABCG2 c.421C>A			9.28 (0.15–557.93)	0.287*
C/C	Ref.		–	
C/A	7.57 (0.79–72.86)	0.080	1.08e+15	–
A/A	8.69e-16	1.000	-	–
C/C vs A/A	2.40e-08	1.000	–	
SLCO1B1 g.37041T>C			120.68 (0.14–102070.4)	0.163*
T/T	ref.		–	–
T/C	5.03 (0.52–48.80)	0.163	2.94e+46	–

Notes: Reference was compared to others in the sub-analysis; *overall *p*-value; vs, versus.
Abbreviations: HR, hazards ratio; 95% CI, 95% confidence interval; ref, reference.

different RFS rates ($p<0.05$). However, there were no differences in RFS rates in the multivariate analysis.

Discussion

This study investigated the frequencies of sequence variations in eleven drug-metabolizing enzymes contributing to phase I (*CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4* and *CYP3A5*) and Phase II (*COMT, SULT1A1, SULT1A4* and *UGT2B7*) metabolism of PQ and CQ as well as seven drug transporter genes (*ABCB1, ABCA1, ABCC2, ABCC4, ABCG2, SLCO1B1* and *SLC25A40*) in 51 Thai patients infected with *P. vivax*. Although the influence of drug-metabolizing enzymes and drug transporters on the efficacy of PQ treatment on *P. vivax* infection has been studied,[7,14,16,27] their contribution to relapse is still unclear. In vitro studies have shown that several phase I and II enzymes strongly relate with PQ metabolisms including CYP450 1A2, 2C19, 2C9, 2D6, 3A4, FMO-1, 3, 5 and MAO-A and B[4–6] and CYP2C8, CYP3A4, and CYP2D6 with CQ metabolism.[11] Therefore, genetic variation in these enzymes likely impacts the pharmacokinetics of both drugs to various degrees. However, to the best of our knowledge, no studies have assessed the association of pharmacogene variation and relapse other than CYP2D6 after treatment with PQ or a combination of PQ and CQ. It has been reported that CYP2D6 metabolizer status influences PQ efficacy.[7,14] Bennette et al[7] first described that two study participants (6%), one PM with a *CYP2D6*5/*6* genotype and one IM with a *CYP2D6*4/*41* genotype signifying considerable decreased activity, had multiple relapses of *P. vivax* while participants with genotypes predicting normal metabolizer (NM) status were not found to relapse. Furthermore, relapse patients had significantly higher amounts of the parent drug PQ AUC_{inf} ($p<0.001$) compared with non-relapse patients. These data support their hypothesis that the highly polymorphic *CYP2D6* gene contributed to the failure to bioactivate PQ to its active phenol metabolites that are responsible for killing hypnozoites. The clinical study by Nelwan et al[28] demonstrated high efficacy of PQ against *P. vivax* relapse in Indonesia although relatively high relapse rates of 14% were observed among treatment groups while 75% of the patients in the control arm relapsed. Unfortunately, *CYP2D6* genotyping was not performed in this study. Subsequent studies by Baird et al[29] reported that decreased CYP2D6 activity was associated with increased risk of therapeutic failure. These authors reported that patients with CYP2D6 activity scores of 1.0 or less had a higher risk of relapse compared to patients with scores >1.0 (OR=9.4, $P=0.001$). Another study conducted by Spring et al[30] confirmed that subjects with CYP2D6 IM or PM phenotypes have reduced PQ metabolism compared to those with an NM phenotype. In contrast, Chen et al[31] found that CYP2D6 phenotype or activity scores

were not significantly different between their relapse and non-relapse groups.

One explanation of why the present study did not find a relationship between *CYP2D6* genotype or metabolizer status and PQ response may be due to the co-administration of CQ. Based on in-vitro studies, CQ is metabolized into N-desethylchloroquine by CYP2C8, CYP3A4, and CYP2D6. CYP2C8 and CYP3A4 constitute low-affinity, high-capacity systems while CYP2D6 may play a more important role at low CQ concentrations contributing to low-affinity, high-capacity systems[11] of metabolism explaining why CQ may cause modest inhibition of CYP2D6 activity in humans when co-administered with debrisoquine.[32] Thus, it cannot be excluded that CQ inhibits CYP2D6 activity to a certain extent and thereby reduce the enzyme's capacity to efficiently bioactivate PQ. Furthermore, our study is limited by the small number of relapsed patients decreasing statistical power; small number size may also be a source of misclassification bias. Therefore, the findings of this study need to be viewed as preliminary. Although the patients were extensively genotyped for *CYP2D6* and CNVs, we cannot exclude the possibility that the relapsing patients possess additional rare, or novel sequence variants and/or structural variants that decrease or obliterate CYP2D6 activity and drug response.[33] We also like to stress that a lack of hypnozoites in the controls at the time of treatment initiation with PQ may have misclassified these patients as treatment successes, in fact over 22% of the relapse controls in clinical trial treated without PQ did not relapse.[28,34] Thus, the recruitment of controls lacking hypnozoites would bias the statistical analysis for patients with decreased or no CYP2D6 activity. In addition, yet another explanation needs to be entertained, ie hypnozoite resistance to PQ,[35,36] which may occur especially in some relapsing patients with normal and ultrarapid CYP2D6 activity.

Finally, there is sparse information, regarding the level of activity towards PQ that is conferred by alleles classified as decreased function alleles. For example, the *CYP2D6*10* allele, which is the most common decreased function allele in Asians and has a frequency of 34.31% in our population, appears to have considerably less activity towards tamoxifen compared to the probe drug dextromethorphan. This observation triggered specific recommendations in the tamoxifen/*CYP2D6* drug/gene pair guideline recently published by the Clinical Pharmacogenetic Implementation Consortium (CPIC) for patients carrying the *CYP2D6*10* allele.[37] It is not inconceivable that *CYP2D6*10* and other decreased function alleles metabolize PQ and/or CQ at rates that are lower than expected from their current function classifications (see variation and functionality tables available at https://www.pharmgkb.org/page/pgxGeneRef) and put carriers at risk of relapse due to the failure of producing sufficiently high levels of exposure to the metabolites that are responsible for eradicating hypnozoites.

Regarding CYP2C19, the non-functional *CYP2C19*2* allele (c.681G>A; rs4244285) was found at a statistically significantly higher allele frequency among the relapse patients (62.5%, 5/8) compared with 24.5% (23/94) among the non-relapse patients. The minor allele frequency was 26% in our study cohort making this a rather common non-functional allele in the Thai population.[38–40] Although Pybus et al[4] describe CYP2C19 as a minor contributing pathway for PQ metabolisms into its active metabolites, its contribution may assume a more prominent role in patients with compromised CYP2D6 activity. This is exemplified by one of our relapsing patients who was genotyped as *CYP2D6*2/*10* and *CYP2C19*2/*2*. A composite activity score taking CYP2D6 and CYP2C19 into account may therefore be more informative to predict the risk of *P. vivax* relapse than either of these genes alone as suggested by our data (Table S3). The use of a combined metabolism index has been proposed previously by Villagra et al[41] concluding that a

> combinatory approach represents an improvement over the current gene-by-gene reporting by providing greater scope while still allowing for the resolution of a single-gene index when needed, especially when drugs are metabolized or activated by multiple pathways

which is clearly the case for the treatment with a combined PQ/CQ regimen.

This study is the first to examine the impact of variants in the *ABCG2* efflux drug transporter on PQ response. This gene, also known as the Breast Cancer Resistance Protein (BRCP) is expressed in intestine, liver, kidney, placenta, and brain capillaries and plays an important role in the absorption, distribution, and removal of drugs across the cell membrane.[42] This transporter is believed to play a protective role by blocking drug absorption at the apical membrane of the intestine and the blood–brain barrier among other sites. At the apical membranes of the liver and kidney, it facilitates efflux of xenobiotics lowering intracellular drug levels.[43,44] Interestingly, although not significant, more subjects were heterozygous for *ABCG2* c.421C/A (rs2231142) than homozygous for the reference

allele (C/C; p=0.071). The mechanism of this variant, however, is unknown. ABCB1 is a member of the superfamily of ATP-binding cassette (ABC) transporters like ABCG2. Sortica et al[16] reported that *ABCB1* (MDR1, P-gp) T/nonG/T haplotype carriers (3435C>T, 2677G>A/T and 1236C>T) were associated with lower parasitemia clearance rates over treatment time in a model adjusted for other clinical factors; however, there was no longer statistically significant difference after false discovery rate analysis. The absence of an association between relapse and *ABCG2* in this study may be due to the small number of relapse patients and/or inhibitory effects of CQ that seems to be an inhibitor to some ABC transporters.[18] Two previous studies suggested that MAO-A/B enzyme activity relates with PQ metabolism,[4,6] however, we did not test the relationships between *MAO* variants and RFS rates.

In addition to the study limitations mentioned above, we acknowledge that adherence was assessed retrospectively by reviewing a data registry. Although a risk of *P. vivax* reinfection could not be ruled out, the risk in our region is less than 5% during the 42-day period, assuming that most *P. vivax* infection recurrences were indeed relapses,[45,46] and parasite genotyping for two polymorphic markers suggested a high probability of late relapsing infections in these volunteers.[14] Nonetheless, further work is warranted to assess the impact of *CYP2D6, CYP2C19, ABCG2* and potentially other drug-metabolizing enzymes and transporters on relapse in larger patient populations.

Conclusion

Although we did not find an association between *CYP2D6* genotype and relapse, sequence variations in other pharmacogenes emerged as additional candidate genes that may contribute to variability in PQ/CQ drug response. These findings warrant further investigation, however, in larger study populations.

Abbreviations

PQ, Primaquine; CQ, Chloroquine; CYP, Cytochrome P450; ABCB1, ATP-Binding Cassette Subfamily B Member 1; ABCC2, ATP-Binding Cassette Subfamily C Member 2; MAO, Monoamine oxidase; ABCG2, ATP-Binding Cassette Subfamily G Member 2; COMT, catechol-O-methyltransferase; SLCO1B1, solute carrier organic anion transporter family member 1B1; SULT4A1, Sulfotransferase Family 4A Member 1; SLC25A40, Solute Carrier Family 25 Member 40, UGT2B7, UDP Glucuronosyltransferase Family 2 Member B7; RFS, Relapse-free survival; CPQ, Carboxy primaquine; OR, odds ratio; HR, hazards ratio; MALDI-TOF, matrix-assisted laser desorption ionization- time-of-flight.

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Disclosure

Rhea J Longley reports a patent PCT/US17/67926 pending. The authors declare that they have no other competing interests.

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