Doctor of Medicine

Factors Affecting the Chloroquine and Primaquine Treatment Responses in Patients with Vivax Malaria in Korea

국내 삼일열 말라리아 환자에서 클로로퀸 및 프리마퀸 치료 반응에 영향을 미치는 요인 분석

The Graduate School
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Factors Affecting the Chloroquine and Primaquine Treatment Responses in Patients with Vivax Malaria in Korea

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ABSTRACT IN KOREAN

목적 : 삼일열 말라리아는 삼일열 원충(*P. vivax*)에 의해 발생하는 원충 감염 질환으로, 국내에서는 1993년 재출현이 확인된 후 최근 연간 500 – 600명 전후의 환자가 발생하고 있다. 이에 대한 표준 치료제는 적혈구 내에 기생하는 원충에 작용하는 클로로퀸(chloroquine)과 간 내 잠복하는 수면소체(hypnozoites)에 작용하는 프리마퀸(primaquine)이다. 하지만 최근 국내 연구들에서 2010년 이후부터 원충혈증이 소실되는 시간(parasite clearance time; PCT)이 점점 길어지고 있다는 결과들이 보고되었으며, 화학적 예방요법으로 인해 국내 삼일열 원충이 반복적으로 클로로퀸에 노출됨에 따라 클로로퀸에 대한 내성 발현에 대한 우려가 커지고 있다. 또한 간 내 수면소체의 활성화로 인한 재발(relapse)을 막기 위해 투여하는 프리마퀸은 인체 내에 흡수된 이후 간에서 Cytochrome P450 Isoenzyme 2D6 (CYP2D6) 효소의 대사를 거쳐 약제의 효과를 나타내는 대사체(active metabolite)로 변하게 되는데, 최근 CYP2D6 효소의 유전자형에 따라 프리마퀸의 치료 효과가 달라진다는 연구결과들이 보고되었다. 즉, 약물 대사가 원활하게 일어나지 않는 'poor metabolizer (PM)' 나 'intermediate metabolizer (IM)' 의 경우 적절한 용량의 프리마퀸을 투여하여도 활성화된 대사체가 충분히 생성되지 않아 약물의 효과가 크지 않을 수 있다. 이번 연구에서는 국내 환자들을 대상으로 클로로퀸에 대한 치료 반응에 영향을 미칠 수 있는 환자 요인과 함께 국내 삼일열 원충의 클로로퀸 내성 유전자를 조사하였고, CYP2D6 효소 활성화에 따른 말라리아 재발의 위험도를 확인하였다.

재료와 방법 : 클로로퀸에 대한 치료 반응과 관련이 있는 요인을 분석하기 위해 서울 및 인천, 경기도 지역의 총 9개 병원에서 전향적으로 말라리아 환자를 등록하였다. 72시간 이상 원충혈증이 지속된 경우 클로로퀸에 대한 치료 반응이 감소한 것으로 간주하여, 72시간 이내 원충혈증이 소실된 환자와 72시간 이후에도 원충혈증이 지속된 환자들 사이의 임상적 특징을 비교하였다. 또한 *P. vivax* 의 클로로퀸 내성에 의한 치료 효과의 차이를 판단하기 위하여, 수집한 환자 검체에서 *P. vivax* 의 DNA를 추출, 클로로퀸 감수성과 관련이 있는 것으로 알려져 있는 *pvmdr1*와 *pvcrt-o* 유전자의 돌연변이를 확인하였다. CYP2D6 유전자형과 말라리아 재발 사이의 관련성을 확인하기 위해 질병관리청에서 말라리아 재발 환자들의 혈액 검체를 추가로 공급받았다. 이후 전향적으로 등록된 환자들과 질병관리청에 등록된 재발 환자들의 CYP2D6 유전자형 및

표현형을 확인하였고, CYP2D6 유전자형 및 표현형에 따른 말라리아 재발의 위험도를 평가하였다.

결과 : 연구에 참여한 환자는 총 102명 이었으며, 질병관리청으로부터 38명의 말라리아 재발 환자의 혈액 검체를 공급받았다. 72시간 이상 원충혈증이 지속된 군과 그렇지 않은 군을 비교하였을 때, 단변량 로지스틱 회귀분석에서 여성이 장기간의 원충혈증과 유의한 연관성을 보였으나(남성 대 여성, 교차비 0.14, 95 % CI; 0.03−0.66; p = 0.01), 다변량 분석에서는 유의한 연관성을 보이지 않았다. 성별 요인을 제거하여 남성만을 분석하였을 때, 체중 당 클로로퀸 총 용량과 25mg/kg 미만의 클로로퀸을 투여한 비율이 두 군 사이에 유의하게 차이를 보였다. 단변량 및 다변량 로지스틱 회귀분석에서 클로로퀸 용량과 장기간의 원충혈증 사이에 유의한 연관성이 나타나지는 않았지만, 체중당 클로로퀸 총 용량이 증가함에 따라 장기간의 원충혈증의 위험이 감소하는 경향을 보였다(교차비 0.57, 95 % CI; 0.27−1.16, p = 0.12). *Pvmdr1* 유전자 분석 결과 Y976F 돌연변이는 발견되지 않았지만 모든 원충에서 F1076L 돌연변이가 확인되었으며, *pvcrt−o* 유전자 분석 결과 K10 삽입이 확인된 원충은 없었다. 재발군과 비재발군 사이에 CYP2D6 표현형을 비교하였을 때, IM 표현형에서 normal metabolizer (NM) 또는 ultrarapid metabolizer (UM) 표현형에 비해 재발이 더 많았으며(교차비 = 2.33, 95 % CI; 1.14 – 4.77, p = 0.02), activity score 가 1.5 미만인 군에서 1.5 이상인 군에 비해 재발이 더 많았다(교차비 = 2.65, 95 % CI; 1.004 – 7.00, p = 0.04).

결론 : 이번 연구에서 체중당 클로로퀸 투약 용량이 적을수록 클로로퀸에 대한 치료 반응이 감소하는 경향성을 확인하였으며, 클로로퀸 약물 내성과 연관이 있는 것으로 알려진 *P. vivax* 유전자의 변이와 클로로퀸 치료 효과 지연 사이의 연관성은 보이지 않았다. 이러한 결과는 실제 임상에서 환자의 체중에 기반한 적절한 용량의 클로로퀸을 투여하는 것이 중요할 수 있고, 클로로퀸 치료 효과에 대한 감시 강화와 *P. vivax* 말라리아의 약물 내성과 관련된 상세한 분자 메커니즘 평가가 필요하다는 점을 시사한다. 또한 낮은 CYP2D6 활성도로 인한 프리마퀸 대사체 형성의 감소가 프리마퀸 투여 후 *P. vivax* 재발률에 영향을 미칠 수 있음을 확인하였다.

중심단어 : 말라리아, 클로로퀸, 프리마퀸, 약제 내성, 재발, CYP2D6

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ABBREVIATION

CDC Centers for Disease Control

CI confidence interval

CYP2D6 cytochrome P450 isoenzyme 2D6

DMZ demilitarized zone

EM extensive metabolizer

FCT fever clearance time

G6PD Glucose-6-phosphate dehydrogenase

IC_{50} half maximal inhibitory concentration

IM intermittent metabolizer

KDCA Korea Diseases Control and Prevention Agency

MIC minimal inhibitory concentration

OR odds ratio

PCR polymerase chain reaction

PM poor metabolizer

Pfcrt P. falciparum chloroquine resistant transporter

Pfmdr1 P. falciparum multidrug resistance 1

PRR parasite reduction ratio

Pvcrt-o P. vivax putative transporter protein

Pvmdr1 P. vivax multidrug resistance 1

SNP single nucleotide polymorphism

SSRI selective serotonin reuptake inhibitors

WHO World Health Organization

UM ultrarapid-metabolizer

INTRODUCTION

Malaria, febrile infectious diseases caused by protozoa of the Genus *Plasmodium*, occurs broadly in tropical and subtropical regions as well as in temperate climates. Currently more than 40% of the world's population lives in malaria endemic areas.[1)] Vivax malaria (benign tertian malaria) caused by *Plasmodium vivax* is the second most prevalent disease following *P. falciparum* malaria. Vivax malaria is widely distributed geographically in the most densely populated regions including South Korea.[2)]

Vivax malaria has existed as an indigenous disease on the Korean Peninsula for many centuries.[3, 4)] Once the malaria was designated as a legal communicable disease in 1963 and malaria control efforts were conducted with the World Health Organization (WHO), the number of patients gradually decreased. South Korea was declared malaria eradication by WHO in 1979 and indigenous malaria was not reported after 1984 for several years.[4)] However, the vivax malaria has reemerged in 1993. After the first patient has been reported in Paju-gun, Gyeonggi-do Province, near the Demilitarized Zone (DMZ) in 1993,[5, 6)] the number of annual cases have been rapidly increased with up to 4,142 cases in 2000.[7)] Since then, around 500 to 600 patients each year have been occurring mostly in northwestern area including Gyeonggi-do Province and the Incheon (Figure 1), posing a major public health problem in South Korea.

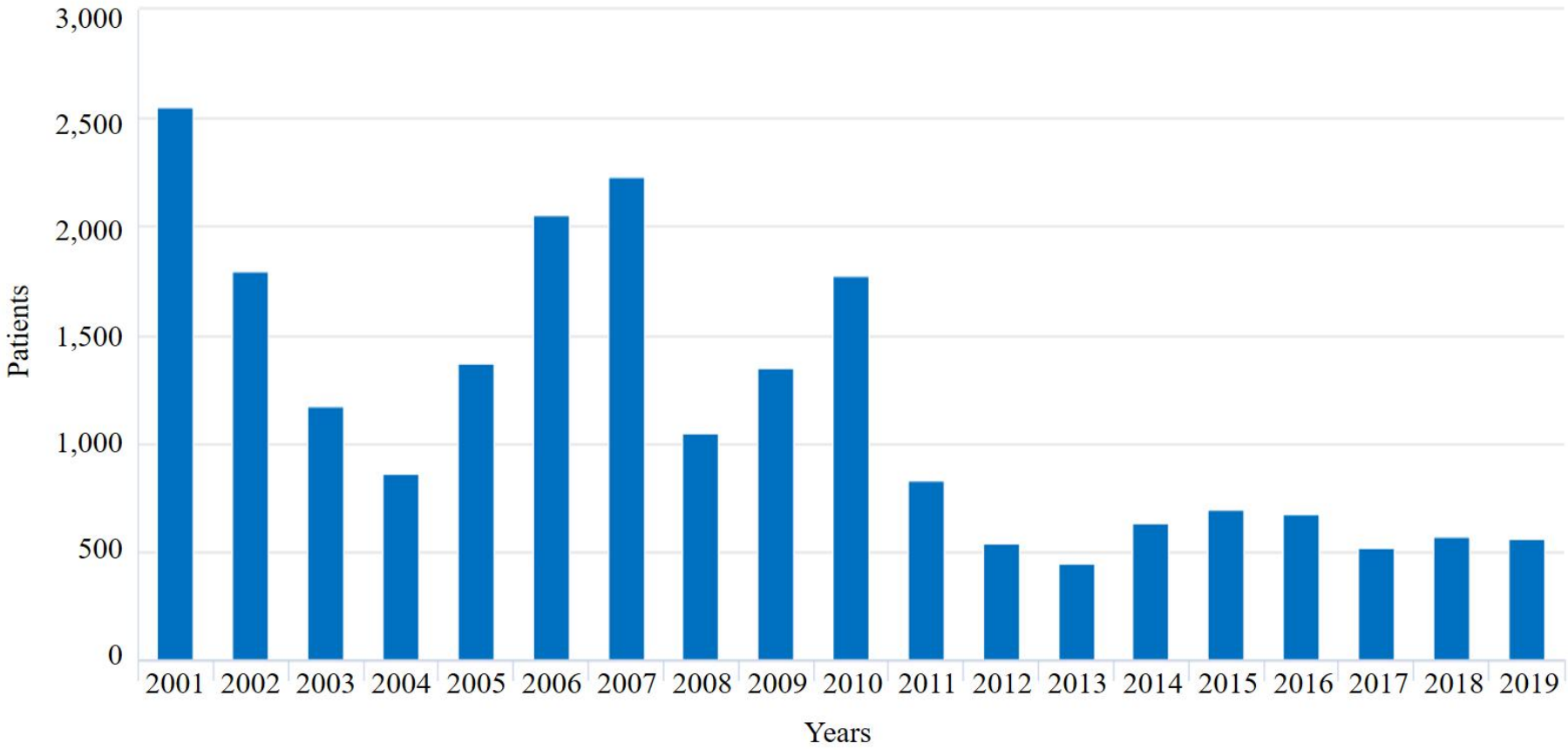

Figure 1. Number of malaria cases, reported at 1 year intervals, from 2001 through 2019. Retrieved from http://www.cdc.go.kr/npt/biz/npp/nppMain.do

In the life cycle of vivax malaria, sporozoites are injected to the host by infected female *Anopheles* mosquito during the blood meal. *P. vivax* sporozoites first infect hepatocytes, and take two distinct pathways. Some develop directly into liver-schizonts, which release merozoites to initiate the asexual cycle in the blood, and the others remain as hypnozoites (dormant stages) that do not divide and stay dormant.[8] If not treated properly, this hypnozoites can cause new blood-stage infections weeks to months after the initial attack and is conventionally considered to be the source of relapse in *P. vivax* malaria. Therefore, treatment of vivax malaria requires the clearing of blood schizonts and killing hypnozoites termed 'radical cure'.[9]

The standard blood schizonticide for vivax malaria in South Korea is hydroxychloroquine or chloroquine, which became the first-line antimalarial drug for *P. vivax* infection from the early 1950s.[10] In recent studies, however, it has been reported that the parasite clearance time (PCT) is being extended since when vivax malaria reemerged in South Korea,[11, 12] and *P. vivax* malaria indigenous to South Korea tend to have longer PCT than in tropical countries of Southeast Asia (Table 1). Delaying the effect of chloroquine treatment maybe dependent on various factors, such as sub-therapeutic weight-adjusted chloroquine dose, chloroquine resistance of *P. vivax* strains, initial high-parasitemia level, non-adherence to the prescribed regimen, and cytochrome P450 enzyme genotype of host related to chloroquine metabolism.

Table 1. Parasite clearance times in patients treated with chloroquine for *P. vivax,* East Asian countries other than South Korea

Study (years)	Country (Location)	Population	Chloroquine treatment regimen	Sample size	Parasite clearance time (hours)
Pukrittayakamee S, et al. (2000)[13]	Thailand	Adults (> 15 y)	25 mg of base/kg for 36 hours	30	65 (mean)
Krudsood S, et al. (2007)[14]	Thailand	Adults (> 15 y)	25 mg base/kg for three days	51	55.8 (mean; range 23-106)
Poravuth Y, et al. (2011)[15]	Cambodia, Thailand, India, and Indonesia	Aged between 3-60 years	Adults: 620 mg on Days 0 and 1 and 310 mg on Day 2 Children: 10 mg/kg on Days 0 and 1 and 5 mg/kg on Day 2	209	32 (median)
Muhamad, et al. (2011)[16]	Thailand (Tak province; Thai□Myanmar border)	Aged between 15-60 years	2,000 mg chloroquine phosphate for three days	130	30 (median, 95% CI 18-36)
Höglund, et al. (2016)[17]	Thailand (Tak province; Thai□Myanmar border)	Aged between 17-52 years	25 mg base/kg for three days	75	30 (median, 95% CI 18-36)
Phung Duc Thuan, et al. (2016)[18]	Vietnam (Binh Phuoc Province)	Aged ≥ 3 years	25 mg base/kg for three days	65	36 (median, IQR 30-48)

WHO recommends chloroquine at a total dose of 25 mg base/kg in areas with chloroquine-sensitive *P. vivax*. Lower total doses are not recommended since they accelerate the emergence of resistance.[9] However, the United States Centers for Disease Control (CDC)[19] and malaria treatment guideline from the United Kingdom[20] recommend a fixed dose regardless of body weight. A previous study in Korea showed that the dose of chloroquine did not reach adequately according to the patient's gradually increasing body weight, and in many cases, a suboptimal dose was administered.[11]

The resistance to chloroquine by the asexual blood stages of *P. vivax* had emerged within a decade and evidences of reduced chloroquine efficacy is accumulating in many *P. vivax* endemic areas.[21, 22] The mutant alleles of the *P. vivax* multidrug resistance 1 (p*vmdr1*) and *P. vivax* putative transporter protein (*pvcrt-o*), which are orthologous to *P. falciparum* multidrug resistance 1 (*pfmdr1*) and *P. falciparum* chloroquine resistant transporter (*pfcrt*) genes, respectively, have been identified as associated with chloroquine resistances in *P. vivax* in Southeast Asia both *in vivo* and *in vitro*.[23-25] In South Korea, chemoprophylaxis with chloroquine was initiated among military personnel in 1997, with the cumulative number reaching more than 1.4 million by the end of 2007.[26] Therefore, there is growing concern that mass chemoprophylaxis with poor compliance could have increase the possibility of chloroquine-resistant *P. vivax* strain emerging. Two cases of chloroquine-resistance *P. vivax* were reported in patients in Korea[27] and the F1076L variant of the *pvmdr1* gene has been reported.[28]

Clinically significant recurrence is known to occur in about less than 5% of vivax malaria patients in South Korea.[29, 30] Recurrences of *P. vivax* parasites in peripheral blood may derive from different sources: from sporozoites of a new infection (re-infection), from sub-patent asexual parasitemia due to resistance to the treatment (recrudescence) or from hepatic hypnozoites (relapse) (Figure 2).[31] Considering the low prevalence of malaria in Korea and the pharmacokinetics of chloroquine with a long half-life, most of the recurrences are considered to be relapse due to the activation of the hypnozoites in the liver.

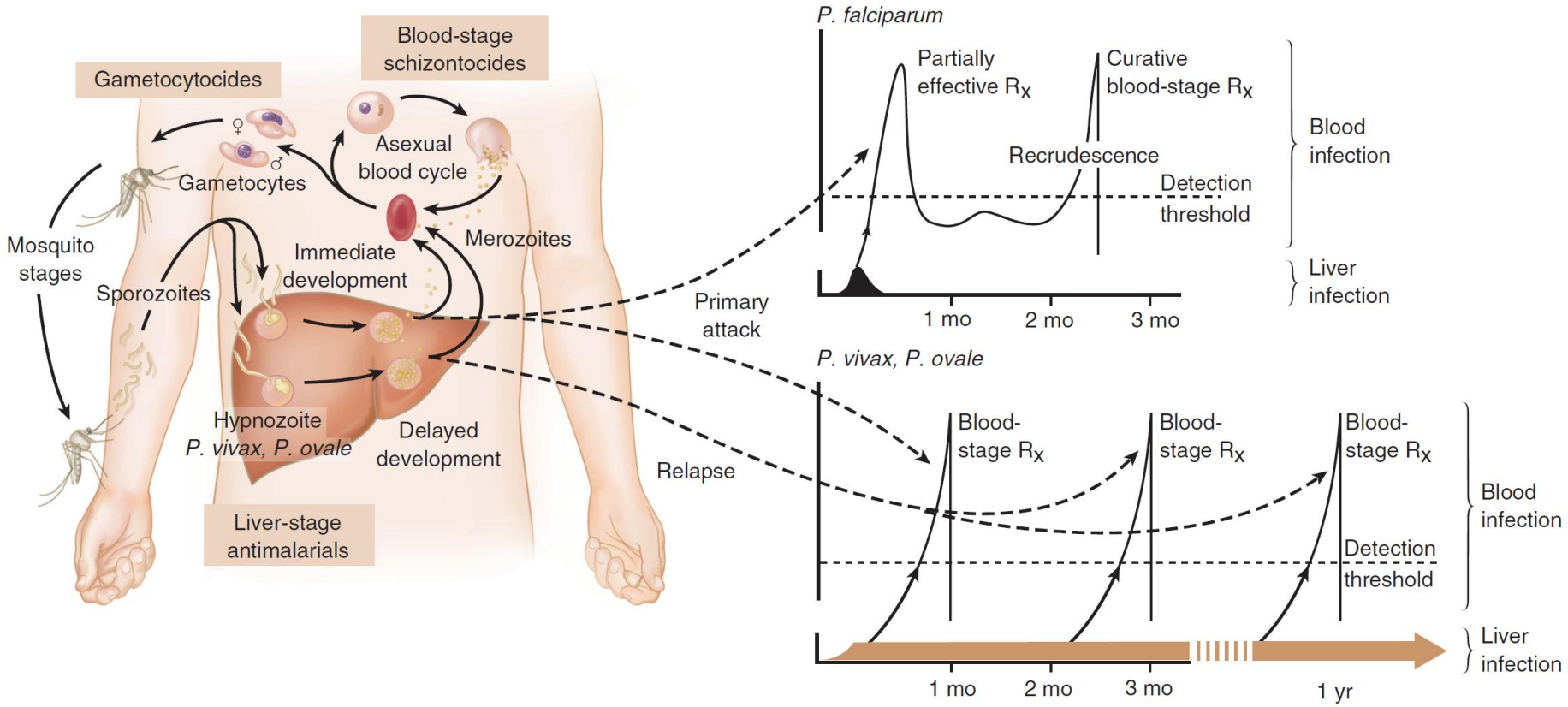

Figure 2. The *Plasmodium* life cycle and disease patterns of recrudescence and relapse. Adapted from Mandell, Douglas and Bennett's Principles and Practices of Infectious Diseases 8th Ed, 2014, Elsevier

The only currently available approved drug for eliminating hypnozoites is primaquine, and the antimalarial properties of primaquine are known to be dependent on the production of oxidized metabolites from metabolization by the cytochrome P450 isoenzyme 2D6 (CYP2D6) (Figure 3).[32] CYP2D6 is highly polymorphic and there are more than 46 known major alleles which can be determined and predict a CYP2D6 phenotype (poor, intermediate, normal and ultrarapid)[33] that have different levels of activity for drug metabolization. Patients with 'poor metabolizer (PM)' or 'intermediate metabolizer (IM)' show an impaired function in CYP2D6 enzyme, so even if an appropriate dose of primaquine is administered, the active metabolite is not sufficiently produced and recurrence may occur.[34, 35]

To identify various factors associated with vivax malaria treatment in South Korea, we identified host factors related to decrease chloroquine therapeutic efficacy and the prevalence of mutation in chloroquine resistance genes in the clinical isolated *P. vivax* from patients. In addition, we analyzed the CYP2D6 profiles of patients to identify the association between primaquine metabolization and relapse of malaria.

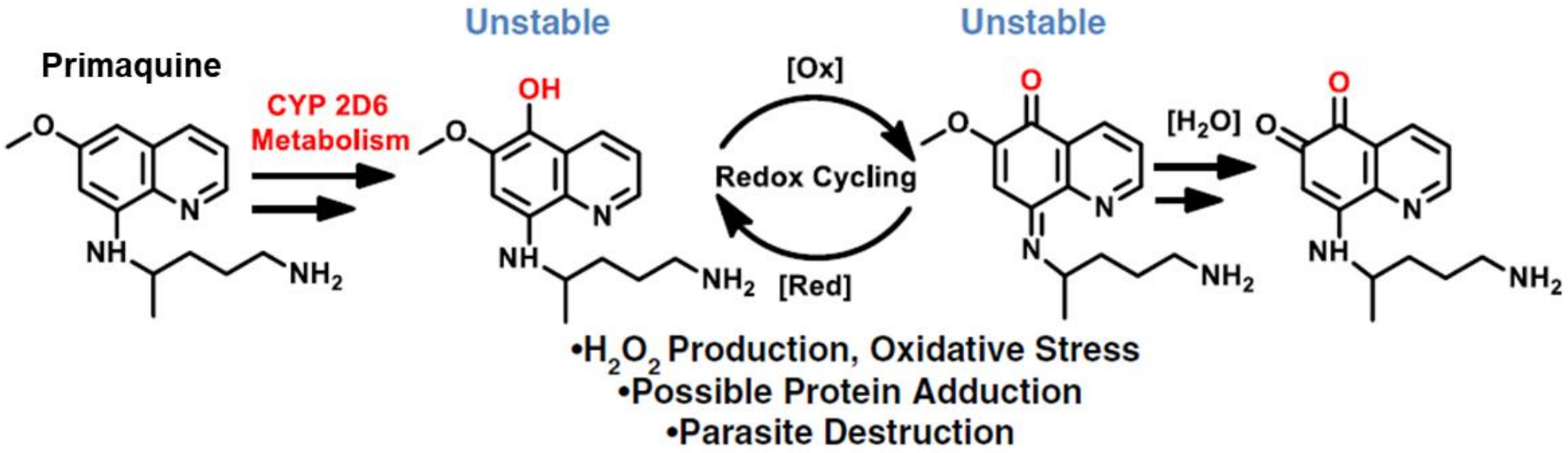

Figure 3. Proposed mechanism of primaquine CYP2D6 metabolic activation and liver stage antimalarial activity. Shown is the proposed mechanism of activation by CYP2D6. Unmodified primaquine has little to no liver stage antimalarial activity. CYP2D6 hydroxylates primaquine on multiple locations of the quinoline core, however, hydroxylation at the 5-position produces and unstable intermediate. The 5-hydroxyprimaquine is capable of redox cycling back and forth to the corresponding quinone-imine. This redox cycle has been shown to produce H2O2, ROS, and subsequent oxidative damage. The 5-hydroxyprimaquine and corresponding quinone-imine species are inherently unstable and will react with water to form the stable 5,6-ortho-quinone. Adapted from Marcsisin, *et al.* Pharmacol Ther 2016;161:1-10

MATERIALS AND METHODS

1. Study sites and population

A. Prospectively registered patients

This study was performed at 9 hospitals (National Health Insurance Service Ilsan Hospital, Inje University Ilsan Paik Hospital, Dongguk University Ilsan Hospital, Severance Hospital, Catholic Kwandong University International St. Mary's Hospital, Asan Medical Center, Gimpo Woori Hospital, Ajou University Medical Center, and Armed Forces Capital Hospital) in and around malaria endemic areas (Gyeonggi-do Province, Incheon and Seoul), South Korea (Figure 4). We prospectively enrolled the patients with > 16 years of age in whom *P. vivax* malaria had been diagnosed and who had been prescribed chloroquine in combination with primaquine from 2018 to 2020. This study was approved by the institutional review board of each hospitals.

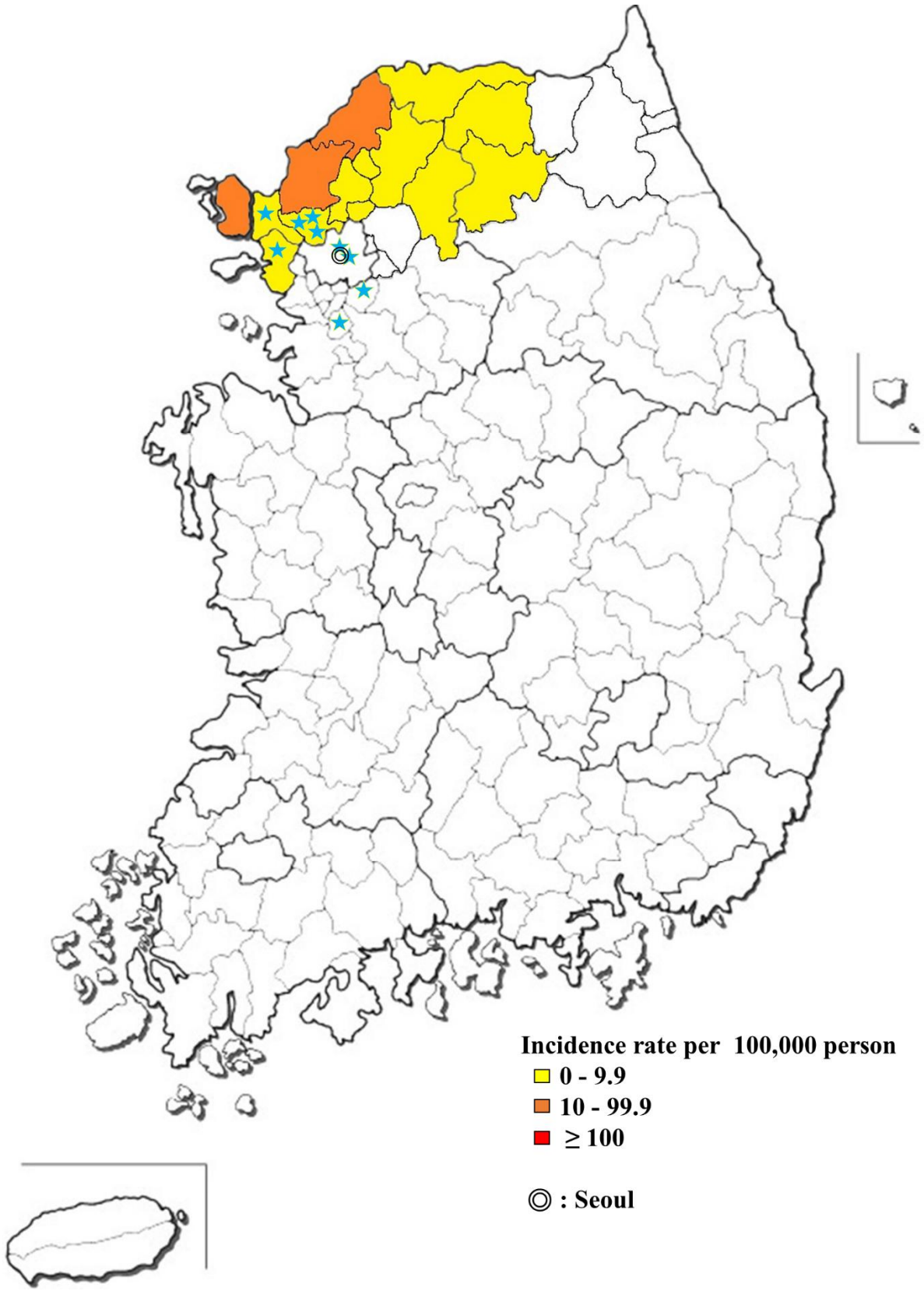

Figure 4. Major risk areas of *P. vivax* malaria in South Korea, 2020, and location of hospitals that performed this study

B. Retrospectively enrolled recurred patients

Considering the low recurrence rate which is around 5% of vivax malaria patients in Korea, we have made the effort to collect more blood samples of patients who have recurred vivax malaria. Malaria, indigenous or imported, is a mandatory notified disease in South Korea and all patients are reported to Korea Diseases Control and Prevention Agency (KDCA) with infectious disease reporting systems. In addition, blood samples of most malaria patients are sent to KDCA for confirmation of malaria and further analysis. The DNA from the blood samples of the 38 patients who have recurred malaria between 2015 and 2018 were provided by the KDCA and included in the analysis.

2. Clinical characteristics and parameters

A. Baseline demographics

We collected patient's age, sex, and body weight for baseline characteristics. Since very low prevalence of Glucose-6-phosphate dehydrogenase (G6PD) deficiency in Koreans,[36] G6PD deficiency test was not performed.

B. Diagnosis for *P. vivax* malaria

P. vivax malaria was diagnosed by peripheral blood smears (thick and thin smears stained by Giemsa) and/or polymerase chain reaction (PCR). Parasite density was determined by Giemsa-stained blood slides at a magnification of 1000× using WHO-recommended methods.[37]

C. Chloroquine and primaquine dosage

Patients were treated with chloroquine for three days as soon as were diagnosed. Primaquine was administered from the next day after three days of chloroquine treatment and usually for 14 days. The dosages of drugs administered to each patient was determined at the physician's discretion. We checked both the total dosage and the dose per patient's body weight administered during the treatment period.

D. Clearance of parasitemia

We estimated patients' initial parasitemia at the time of diagnosis and parasite counts were measured every day in the case of hospitalized patients until the parasitemia became undetectable. In patients who had undergone outpatient treatment, parasitemia was measured every day of visit. The

results were reported as negative only after at least 200 fields of the thin film had been examined without encountering a parasite. PCT was calculated, defined as the time in hours from chloroquine administration until the first blood smear negative for parasites after which the patient's follow-up smears were also negative.

E. Fever clearance time (FCT)

FCT was defined as the time in hours from chloroquine administration until the patient's body temperature decreased to < 37.5°C for 48 consecutive hours.[38] We checked for fever every 4–6 hours or whenever the patient felt febrile.

F. Recurrence of vivax malaria

We identified recurrence patients based on the medical records of each medical institution and the data reported by KCDA. Recurrent vivax malaria was defined as having reported two or more attacks of *P. vivax* malaria within two years.

3. Molecular analysis

A. Identification of *pvmdr1* and *pvcrt-o* polymorphism

Parasite genomic DNA was extracted from the whole-blood samples collected from prospectively enrolled patients using a commercially available QIAamp DNA blood kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. The *pvmdr1* and *pvcrt-o* genes were amplified by nested or semi-nested PCR performed with the minor modifications of the previous description[25] using gene-specific primers (Table 2 and 3). The PCR of *pvmdr1* and *pvcrt-o* genes was performed in the following reaction mixture: 2 μl of 10× buffer II, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.25 μM of each primer and 1 U TaKaRa LA Taq DNA polymerase (TaKaRa BIO, Otsu, Shiga, Japan), and 1 μl of genomic DNA. The primary reaction of the *pvmdr1* gene was performed under the following conditions: initial denaturation at 94 ◦C for 10 min, followed by 35 cycles of 94 ◦C for 30 s, 61 ◦C for 30 s, and 72 ◦C for 4min, and a final extension period at 72 ◦C for 10 min. The second reaction of the *pvmdr1* gene was performed under the following conditions: initial denaturation at 94 ◦C for 10 min, followed by 35 cycles of 94 ◦C for 40 s, 58 - 62 ◦C for 30 - 60 s, and 72 ◦C for 50–60 s, and a final extension period at 72 ◦C for 10 min. And the second reaction was conducted in the following reaction mixture: 2 μl of 10× buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer, 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ) and 1 μl of amplicon. The

primary reaction of the *pvcrt-o* gene was performed under the following conditions: initial denaturation at 94 ◦C for 10 min, followed by 30 cycles of 94 ◦C for 30 s, 58 ◦C for 30 s, and 72 ◦C for 3.5 min, and a final extension period at 72 ◦C for 10 min. The second reaction cycling conditions for *pvcrt-o* were as follows: initial denaturation at 94 ◦C for 10 min, followed by 35 cycles of 94 ◦C for 30 s, 59 ◦C for 1 min, and 72 ◦C for 1min, and a final extension period at 72 ◦C for 10 min. The second PCR to amplify these fragments was conducted the same as *pvmdr1*. The second PCR products of *pvmdr1* and *pvcrt-o* genes were sequenced directly to identify the single nucleotide polymorphism (SNP). Amino acid sequences were compared with sequences of Sal I (Salvador I): GenBank accession nos. AY571984 for *pvmdr1*[39] and AF314649 for *pvcrt-o*.[40] The deduced amino acid sequences were aligned and analyzed using Lasergene software (DNASTAR, Madison, WI).

Table 2. List of PCR primers designed for amplification of *pvmdr1* genes

Genes	Primers	Product size
pvmdr1,	F: 5′-TTGAACAAGAAGGGGACGTT-3′	4290bp
primary reaction	R: 5′-CTTATATACGCCGTCCTGCAC-3′	(82-4371 nt)
pvmdr1,	F: 5′-CAGCCTGAAAGATTTAGAAGCCTT-3′	539bp
secondary reaction 1	R: 5′-CATCCACGTCCACAGTGGAAC-3′	(1455-1993 nt)
pvmdr1,	F: 5′-GGATAGTCATGCCCCAGGATTG-3′	604bp
secondary reaction 2	R: 5′-CATCAACTTCCCGGCGTAGC-3′	(2751-3354 nt)
pvmdr1,	F: 5′-GATGAGCCTGCTGATGCGATTCTAC-3′	745bp
secondary reaction 3	R: 5′-ATATACGCCGTCCTGCACCGAG-3′	(3624-4368 nt)

Reference sequence: AY571984

Table 3. List of PCR primers designed for amplification of *pvcrt-o* genes

Genes	Primers	Product size
pvcrt-o,	F: 5′-ATCCCGTCATCCGCCTCA-3′	3780bp
primary reaction	R: 5′-CGCACAGCGTAATCAAAGG-3′	(158-3937 nt)
pvcrt-o,	F: 5′-ATCCCGTCATCCGCCTCACT-3′	1137bp
secondary reaction 1	R: 5′-AGTTTCCCTCTACACCCG-3′	(158-1294 nt)
pvcrt-o,	F: 5′-GATTGCCTCGCTGATTGTA-3′	836bp
secondary reaction 2	R: 5′-AATCGTCGCACATCTTGGA-3′	(1548-2383 nt)
pvcrt-o,	F: 5′-CATAGCCATCGCCTACTACTTT-3′	757bp
secondary reaction 3	R: 5′-CGCACAGCGTAATCAAAGG-3′	(3181-3937 nt)

Reference sequence: AF314649

B. CYP2D6 genotype

CYP2D6 genotyping analysis was commissioned to SPMED Co.,Ltd., Busan, South Korea. Patient DNA was isolated and purified from blood samples using SPMED™ Genotyping Kit: CYP2D6 (in vitro license No. 20-297, SPMED Co.,Ltd., Busan, South Korea) according to the manufacturer's instructions and CYP2D6 *2, *3, *4, *5 (deletion), *6, *9, *10, *14, *17, *18, *21, *29, *41, *49, *52, *60, *XN (duplicate) were analyzed. CYP2D6 metabolizer status was inferred from the genotypes using the activity score (AS). An AS of 0.0 indicates a poor metabolizer (PM), an AS of 0.25 to 1.0 indicates an intermediate metabolizer (IM), an AS of 1.25 to 2.25 indicates a normal metabolizer (NM), and an AS of > 2.25 indicates an ultrarapid metabolizer (UM) (Figure 5).[41)]

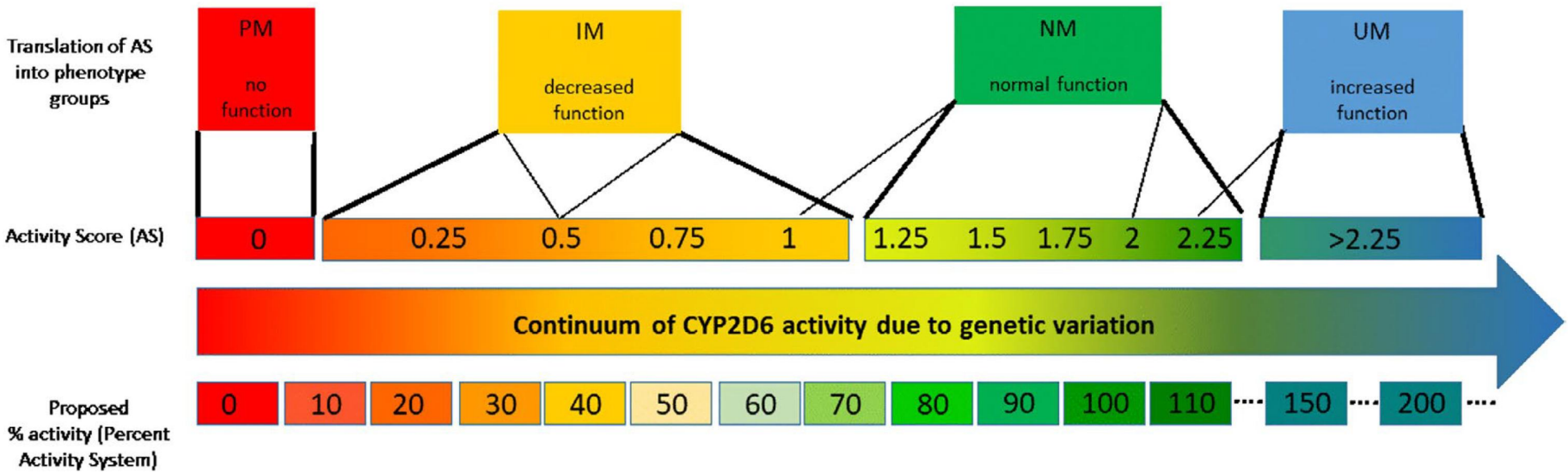

Figure 5. Comparison of the Clinical Pharmacogenetics Implementation Consortium method and percentage activity method for translating CYP2D6 genotype to phenotype. Thin lines represent different ways to translate activity score (AS) into phenotype and the bold lines represent the recommended CYP2D6 genotype to phenotype translation consensus system. IM, intermediate metabolizer; NM, normal metabolizer; PM, poor metabolizer; UM, ultrarapid metabolizer. Adapted from KE Caudle, *et al.* Clin Transl Sci 2020;13(1) 116-124

4. Statistical analysis

Descriptive statistics were expressed as means, medians, or proportions. Statistical comparison was performed using the χ-square test and Mann-Whitney test, and multiple logistic regression analysis were conducted to determine the relevance of clinical variables. All tests of significance were two-tailed, and differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed using SPSS Statistics 23 (IBM, https://www.ibm.com).

RESULTS

1. Patients characteristics

A total of 102 patients were enrolled prospectively during the study period, including one patient with four times of malaria attack. All of them were diagnosed as indigenous vivax malaria in Korea due to lack of recent overseas travel history. The mean age of the patients were 43.4 years old and the 76% of patients were male. The mean body weight of the patients were 73.5 kg. Each medical center enrolled one to twenty one patients. Additionally, the KCDA provided blood samples of 38 suspected recurred patients (Figure 6).

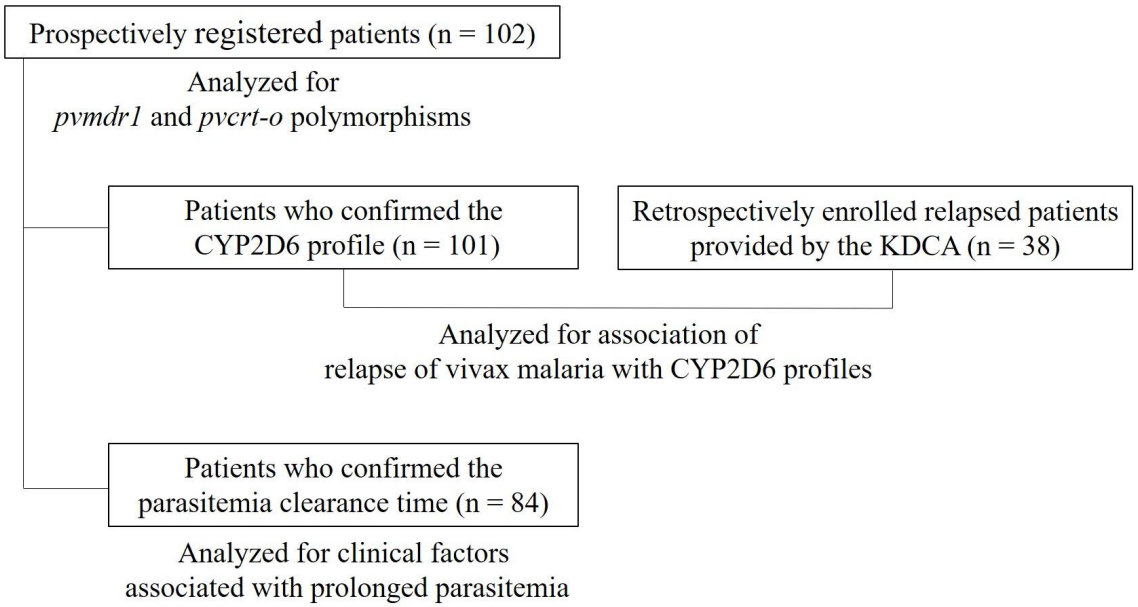

Figure 6. Schematic flow chart of patient distribution in this study

2. Clinical factors associated with prolonged parasitemia

Among the 102 patients, 84 patients have been measured parasite counts every day until the parasitemia became undetectable. Considering the usual treatment response of *P. vivax* to chloroquine, prolonged parasitemia - parasitemia presence at 72 hours after chloroquine treatment - could be considered as the reduced sensitivity to chloroquine. To identify the risk factors for prolonged parasitemia, clinical characteristics were compared between the two groups with and without *P. vivax* parasitemia at 72 hours after first dose of chloroquine.

Among the 84 patients, 55 (65.5%) showed parasitemia on blood smear at 72 hours after first dose of chloroquine administration. There were significant differences in the proportion of female, primaquine daily dose per body weight and FCT between the two groups with or without prolonged parasitemia (Table 4). To compensate for the bias due to the small portion of female in entire patients, we analyzed clinical factors again according to sex. Of a total of 63 male patients, 36 (57.1%) showed parasitemia at 72 hours after first dose of chloroquine administration. Chloroquine total dose per body weight, the percentage of administration of less than 25 mg/kg of, and FCT were additionally identified as statistically significant different factors in male (Table 5), not in female (data not presented).

Table 4. Demographic and clinical characteristics of patients with *P. vivax* according to parasitemia at 72 hours after first dose of chloroquine

Variable	Parasitemia < =72 hours (n = 29)	Parasitemia > 72 hours (n = 55)	*p* value
Age, mean years ± SD	44.5 ± 18.3	44.0 ± 17.0	0.91
Male sex (%)	27 (93.1)	36 (65.5)	0.01
Body weight, kg ± SD	73.9 ± 10.8	73.7 ± 13.4	0.88
Initial parasitemia, /µL ± SD	2559.2 ± 4355.2	6455.5 ± 8208.1	0.15
< 2,000/µL	8	8	
2,000 - 20,000 /µL	3	17	
> 20,000 /µL	0	1	
Chloroquine total dose, mean mg/kg ± SD	23.6 ± 2.9	22.6 ± 3.4	0.19
Chloroquine dose < 25 mg/kg (%)	17 (58.6)	40 (72.7)	0.19
Primaquine daily dose, mg/kg ± SD	0.33 ± 0.08	0.29 ± 0.08	0.03
Fever clearance time (FCT), hours ± SD	28.9 ± 24.3	56.3 ± 33.1	0.04

Table 5. Demographic and clinical characteristics of male patients with *P. vivax* according to parasitemia at 72 hours after first dose of chloroquine

Variable	Parasitemia < =72 hours (n = 27)	Parasitemia > 72 hours (n = 36)	*p* value
Age, mean years ± SD	43.9 ± 18.7	42.2 ± 15.1	0.68
Body weight, kg ± SD	74.9 ± 10.5	79.2 ± 11.3	0.13
Initial parasitemia, /µL ± SD	1335.5 ± 1665.3	4870.5 ± 4057.4	
< 2,000/µL	8	6	
2,000 - 20,000 /µL	2	8	
> 20,000 /µL	0	0	
Chloroquine total dose, mean mg/kg ± SD	23.5 ± 3.0	21.7 ± 3.2	0.03
Chloroquine dose < 25 mg/kg (%)	16 (59.3)	30 (83.3)	0.046
Primaquine daily dose, mg/kg ± SD	0.34 ± 0.08	0.30 ± 0.09	0.047
Fever clearance time (FCT), hours ± SD	74.9 ± 10.5	79.2 ± 11.3	0.03

Univariable and multivariable logistic regression analysis were conducted to determine the independent associated factors with prolonged parasitemia. By univariable analysis, sex were statistically independent factor associated with prolonged parasitemia. By multivariable analysis, however, no clinical characteristics remained significantly associated with prolonged parasitemia (Table 6).

Table 6. Univariable and multivariable logistic regression analysis for prolonged parasitemia of vivax malaria

Variable	Univariable analysis			Multivariable analysis		
	OR	**95% CI**	***p* value**	**OR**	**95% CI**	***p* value**
Age	0.99	0.97- 1.03	0.91	0.99	0.94 - 1.04	0.73
Male sex	0.14	0.03 - 0.66	0.01	0.16	0.02 - 1.67	0.13
Initial parasitemia, /µL ± SD	1.00	1.00 - 1.00	0.12	1.00	1.00 - 1.00	0.19
Chloroquine total dose, mean mg/kg	0.91	0.78 - 1.05	0.19	0.57	0.27 - 1.16	0.12
Chloroquine dose < 25 mg/kg (%)	1.88	0.73 - 4.86	0.19	6.57	0.57 - 75.23	0.13

3. *Pvmdr1* and *pvcrt-o* polymorphisms in relation to chloroquine sensitivity

The prevalence of mutations in *pvmdr1* and *pvcrt-o* genes of *P. vivax* were analyzed with extracted genomic DNA from the blood samples of prospectively collected 102 patients. The SNPs of the *pvmdr1* gene was the same in all clinical specimens, and the K10 insertion status of the *pvcrt-o* gene was also the same in all clinical specimens. Therefore, these SNPs could not explain in the difference of the clinical characteristics and duration of parasitemia (Table 7).

Table 7. *Pvmdr1* and *pcrt-o* polymorphism frequency

***pvmdr1* allele**		***pvcrt-o***	**Frequency (%)**
Y976F	F1076L	K10 insertion	
Y	F	-	0/102 (0.0)
Y	F	K	0/102 (0.0)
Y	L	K	0/102 (0.0)
Y	L	-	102/102 (100.0)
F	L	-	0/102 (0.0)
F	L	K	0/102 (0.0)

4. Association with recurrence of vivax malaria with impaired CYP2D6 genotype and phenotype

Among the 102 prospectively enrolled patients, CYP2D6 genotype analysis were possible in 101 patients. Among these 101 patients, 10 patients were identified as recurred patients. CYP2D6 genotyping was performed additionally with already stored 38 DNA samples from the recurred patients, provided by the KDCA.

A total of 16 CYP2D6 alleles were observed among the 139 samples examined. Table 8 summarizes the CYP2D6 genetic profiles, activity score and phenotypes for the 38 samples provided by KDCA. Table 9 summarizes the CYP2D6 genetic profiles, activity score, phenotypes and recurrence in prospectively collected 101 patients treated for *P. vivax*. For the 139 patients included in this analysis, the most common genotype was *10B/*10B which was identified in 36 samples, and no patient had PM phenotype. Sixty five, seventy three and one patient were identified to have IM, NM and UM phenotypes respectively. In line with the previous studies, CYP2D6 phenotypes were classified and analyzed with two categories: PM and IM vs NM and UM. And genotype determined activity scores were classified and analyzed with two group, under 1.5 (< 1.5) and more than 1.5 (≥ 1.5) activity score.

Table 8. Cytochrome P450 2D6 genetic profiles, activity score and phenotypes in retrospectively collected 38 patients with recurred *P. vivax* malaria

Years	Genotype	Activity score	Phenotype	Years	Genotype	Activity score	Phenotype
2015	*2/*41	1.5	NM	2017	*2/*10B	1.25	NM
2015	*10B /*21B	0.25	IM	2017	*10B/*10B	0.5	IM
2015	*1/*10B	1.25	NM	2017	*2/*10B	1.25	NM
2015	*10B/*10B	0.5	IM	2017	*10B/*10B	0.5	IM
2015	*1/*1	2	NM	2017	*1/*10B	1.25	NM
2015	*2/*10B	1.25	NM	2017	*10B/*10B	0.5	IM
2015	*1/*10B	1.25	NM	2017	*10B/*10B	0.5	IM
2016	*1/*5	1	IM	2017	*10B/*41	0.75	IM
2016	*1/*5	1	IM	2017	*2/*10B	1.25	NM
2016	*1/*1	2	NM	2018	*10B/*52	0.5	IM
2016	*5/*41	0.5	IM	2018	*1/*10B	1.25	NM
2016	*5/*10B	0.25	IM	2018	*10B/*10B	0.5	IM
2016	*5/*10B	0.25	IM	2018	*2/*10B	1.25	NM
2016	*1/*10B	1.25	NM	2018	*10B/*10B	0.5	IM
2016	*1/*5	1	IM	2018	*1/*2	2	NM
2016	*10B/*10B	0.5	IM	2018	*1/*10B	1.25	NM
2016	*1/*5	1	IM	2018	*4/*10B	0.25	IM
2016	*10B/*10B	0.5	IM	2018	*1/*5	1	IM
2016	*1/*10B	1.25	NM	2018	*10B/*10B	0.5	IM

Table 9. Cytochrome P450 2D6 genetic profiles, activity score, phenotypes and recur in prospectively collected 101 patients treated for *P. vivax*

Years	Genotype	Activity score	Phenotype	Recur	Years	Genotype	Activity score	Phenotype	Recur
2018	*5/*10B	0.25	IM	No	2019	*1/*1	2	NM	No
2018	*10B/*10B	0.5	IM	No	2019	*1/*2	2	NM	No
2018	*1/*10B	1.25	NM	No	2019	*1/*1	2	NM	No
2018	*10B/*10B	0.5	IM	No	2019	*10B/*10B	0.5	IM	No
2018	*1/*10B	1.25	NM	No	2019	*1/*10B	1.25	NM	Yes
2018	*10B/*49	0.75	IM	No	2019	*1XN/*5	2	NM	No
2018	*10B/*10B	0.5	IM	No	2019	*10B/*10B	0.5	IM	Yes
2018	*10B/*10B	0.5	IM	No	2019	*2/*41	1.5	NM	No
2018	*5/*10B	0.25	IM	No	2019	*1/*10B	1.25	NM	No
2018	*1/*1	2	NM	No	2019	*1/*2	2	NM	No
2018	*2/*10B	1.25	NM	No	2019	*1/*10B	1.25	NM	No
2018	*10B/*10B	0.5	IM	No	2019	*1/*10B	1.25	NM	No
2018	*5/*10B	0.25	IM	Yes	2019	*1/*10B	1.25	NM	No
2018	*1/*10B	1.25	NM	No	2019	*1/*21B	1	IM	No
2018	*1/*10B	1.25	NM	No	2019	*1/*10B	1.25	NM	No
2018	*5/*10B	0.25	IM	No	2019	*10B/*10B	0.5	IM	No
2018	*1/*10B	1.25	NM	No	2019	*1/*10B	1.25	NM	No
2018	*1/*41	1.5	NM	No	2019	*1/*5	1	IM	No
2018	*1/*2	2	NM	Yes	2019	*1/*10B	1.25	NM	No
2018	*10B/*10B	0.5	IM	No	2019	*2/*10B	1.25	NM	No
2018	*2/*41	1.5	NM	No	2019	*10B/*10B	0.5	IM	No
2018	*10B/*10B	0.5	IM	No	2019	*2/*5	1	IM	No
2018	*5/*14B	0.5	IM	No	2019	*1/*10B	1.25	NM	No
2018	*1/*10B	1.25	NM	No	2019	*10B/*10B	0.5	IM	Yes
2018	*2/*14B	1.5	NM	Yes	2019	*1/*2	2	NM	No
2018	*1/*2	2	NM	No	2019	*1/*1	2	NM	No
2018	*10B/*10B	0.5	IM	No	2019	*1/*1	2	NM	No
2018	*1/*10B	1.25	NM	No	2019	*1/*10B	1.25	NM	No
2018	*4/*10B	0.25	IM	Yes	2019	*5/*10B	0.25	IM	No
2018	*10B/*10B	0.5	IM	No	2019	*1/*10B	1.25	NM	No
2018	*10B/*10B	0.5	IM	No	2019	*10B/*10B	0.5	IM	No
2018	*10B/*10B	0.5	IM	No	2019	*2/*10B	1.25	NM	No
2018	*1/*10B	1.25	NM	No	2019	*1/*1	2	NM	No
2018	*10B/*10B	0.5	IM	No	2019	*10B/*10B	0.5	IM	No
2018	*1/*2	2	NM	No	2020	*10B/*10B	0.5	IM	No
2018	*1/*10B	1.25	NM	No	2020	*10B/*10B	0.5	IM	No
2018	*1/*10B	1.25	NM	No	2020	*1/*1	2	NM	No
2018	*2/*5	1	IM	No	2020	*1/*10B	1.25	NM	No
2018	*2/*10B	1.25	NM	No	2020	*10B/*41	0.75	IM	No
2018	*10B/*52	0.5	IM	Yes	2020	*10B/*10B	0.5	IM	No
2018	*1/*1	2	NM	No	2020	*5/*10B	0.25	IM	No
2018	*1/*10B	1.25	NM	No	2020	*2/*10B	1.25	NM	No
2018	*1/*14B	1.5	NM	No	2020	*2/*2	2	NM	No
2018	*1/*41	1.5	NM	No	2020	*1/*1	2	NM	No
2018	*10B/*10B	0.5	IM	No	2020	*10B/*10B	0.5	IM	Yes
2018	*10B/*10B	0.5	IM	No	2020	*1/*10B	1.25	NM	No
2018	*1/*2	2	NM	No	2020	*1/*2XN	3	UM	No
2018	*5/*10B	0.25	IM	No	2020	*1/*2	2	NM	No
2018	*1/*10B	1.25	NM	No	2020	*1/*21B	1	IM	No
2019	*10B/*10B	0.5	IM	Yes	2020	*1/*41	1.5	NM	No
2019	*1/*10B	1.25	NM	No					

IM, intermediate metabolizer; NM, normal metabolizer; PM, poor metabolizer; UM, ultrarapid metabolizer.

Clinical characteristics were compared between 92 patients with single attack and 10 patients with multiple attacks, who were considered to have recurred vivax malaria. Furthermore, the CYP2D6 genotypes were compared the 91 non-recurred patients and the 48 recurred patients. When clinical characteristics were compared between the non-recurred and recurred groups with prospectively collected patients, only FCT had significant difference between two groups (Table 9). When CYP2D6 phenotypes were compared between the non-recurred and recurred groups combined with prospectively collected and retrospectively enrolled patients, the recurrences were more common in the IM phenotype compared to NM or UM phenotype, odds ratio (OR) = 2.33 (95% CI; 1.14 - 4.77, p = 0.02). Furthermore, the recurrences were more common in the lower activity score group (< 1.5) when compared to the higher activity score groups (≥ 1.5), OR = 2.65 (95% CI; 1.004 - 7.00, p = 0.04) (Table 10).

Table 10. Demographic and clinical characteristics of prospectively collected 102 patients according to recurrences of *P. vivax* malaria

Variable	No- recurred patients (n = 92)	Recurred patients (n = 10)	*p* value
Age, mean years ± SD	43.1 ± 17.5	46.0 ± 12.1	0.55
Male sex (%)	72 (78.3)	4 (50.0)	0.09
Body weight, kg ± SD	73.4 ± 12.3	73.8 ± 16.1	0.97
Initial parasitemia, /µL ± SD	5499.6 ± 7403.1	1794.7 ± 1383.6	0.51
< 2000/µL	17	2	
2,000 - 20,000 /µL	21	1	
> 20,000 /µL	1	0	
Chloroquine total dose, mean mg/kg ± SD	23.1 ± 3.2	22.5 ± 3.9	0.74
Chloroquine dose < 25 mg/kg (%)	62 (68.1)	5 (62.5)	0.71
Primaquine daily dose, mg/kg ± SD	0.3 ± 0.1	0.3 ± 0.1	0.41
Persistent parasitemia (> 72 hours) (%)	50 (65.8)	5 (62.5)	0.99
Fever clearance time, hours ± SD	40.3 ± 28.5	94.5 ± 27.6	0.04

Table 11. Unadjusted odds ratio for recurred *P. vivax* malaria associated with cytochrome P450 2D6 genotype and activity score

Variable	No-recurred patients (n = 91)	Recurred patients (n = 48)	Odds ratio (95% CI)	*P* value
Phenotype				
Poor or intermediated metabolizer	36	29	2.33 (1.14 – 4.77)	0.02
Normal or ultrarapid metabolizer	55	19		
Genotype-determined activity score				
< 1.5	66	42	2.65 (1.004 – 7.00)	0.04
≥ 1.5	25	6		

DISCUSSION

Although the mortality of vivax malaria is lower than that of falciparum malaria, *P. vivax* continues to inflict a huge burden on public health and greatly affecting the quality of life for many people in tropical, subtropical, and temperate countries. With efforts such as increases in access to health-care services, rapid diagnosis and treatment with highly effective antimalarial drug regimens, most countries in Southeast Asia are making steady progress in reducing the burden of malaria. In South Korea, however, the endemicity of vivax malaria is becoming a growing concern after 1993's reemergence despite the ongoing national eradication program.[12] Therefore, the aim of this study was to determine the clinical and microbial information of patients to identify various factors associated with vivax malaria treatment to expedite the appropriate treatment strategy.

PCT is a parameter not affected by relapse or re-infection, and can suggest evidence of reduced chloroquine efficacy and the development of chloroquine resistance. Therefore we evaluated the risk factors associated with prolonged parasitemia and further the chloroquine resistant gene profiles of *P. vivax* isolates. When comparing the two groups with or without prolonged parasitemia, the female sex was significantly associated with the prolonged parasitemia in univariable logistic regression analysis, however, not in multivariable analysis. There was a significant difference in primaquine daily dose per body weight between the two groups in both the entire patients and the male patients. Primaquine has activity against both blood and liver stages, including against chloroquine-resistant strains.[42, 43] One study identified concurrent use of chloroquine and primaquine could result in a synergistic effect against asexual forms of *P. vivax*, as has been shown for *P. falciparum* parasites.[44] In this study, whereas, primaquine was administered from the next day after three days of chloroquine treatment. Patients with clearance of parasitemia within 72 hours were not exposed to primaquine. The difference in PCT cannot be explained by the difference in primaquine daily dose per body weight. Therefore we excluded the primaquine daily dose from the logistic regression analysis. Nevertheless, in following studies on the efficacy of chloroquine, the likelihood that such possible synergism results in the underestimating of resistance for chloroquine of *P. vivax* should be considered. High initial parasitemia before treatment is a major determinant of therapeutic efficacy, and itself may indicate resistance to chloroquine.[45] However, in this study, there was no statistical significant difference between two groups and association with prolonged parasitemia. The main reason is maybe due to that initial parasitemia was measured in only about half of enrolled patients (37 among 84 patients). Moreover, since the hemoglobin level was not calculated together, the possibility that the parasite count was not checked correctly cannot be ruled out.

The cure of blood-stage *P. vivax* with chloroquine depends on the exposure of parasites to adequate blood concentrations.[46] Increasing rates of overweight and obesity may promote a significant impact on drug exposure[47] and suboptimal doses of chloroquine can prolong the parasite clearance and increase the rate of early recurrence and with emergence of drug resistance. Recent pooled meta-analysis study[48] identified that one third of study patients with vivax malaria received less than the WHO recommended target dose of chloroquine of 25 mg/kg and increasing the total mg/kg chloroquine dose reduced early recurrences. In particular, even in regions with long relapse periodicity, such as in Korea,[49] 5 mg/kg increase in chloroquine dose reduced the recurrence rate between day 7 and day 42.[48] Previous study in Korea also showed that chloroquine is mostly under-dosed in the treatment of vivax malaria,[11] and 57 out of 84 patients (67.8%) received a lower dose of chloroquine than the dosing recommended by WHO in this study. When analyzed only male patients to avoid bias due to the small portion of female and the difference of drug metabolism between sex, chloroquine total dose per body weight and the percentage of administration of less than 25 mg/kg of chloroquine were significantly different between the two groups. Univariable and multivariable logistic regression analysis did not show a significant association between chloroquine dose and prolonged parasitemia, however, the risk of prolonged parasitemia tended to decrease as the chloroquine total dose per body weight increased (hazard ration 0.57, 95% CI; 0.27 - 1.16; $p = 0.12$). This results might be affected with other patient-related confounding factors such as drug absorption, metabolism, excretion, or fat distribution which affect the plasma concentrations of the drug and its major metabolite. Moreover, the prolonged half-life of chloroquine could make the lack of a significant association between the administered dose and the concentrations of chloroquine metabolite. Nevertheless, these results suggest a probable influence of chloroquine dose per body weight on PCT so that clinicians should consider following weight-based treatment guidelines for chloroquine. The reason that some malaria treatment guidelines limit the maximum dose of chloroquine per day is thought to be probably because the possibility of retinopathy, an important side effect, increases as the dose of chloroquine increases. It is known that the risk of retinopathy caused by chloroquine is most accurately assessed on the basis of daily dose per body weight, chloroquine > 2.3 mg/kg (hydroxychloroquine > 5.0 mg/kg), with duration more than 5 years without other risk factors.[50] Therefore, it is considered that the possibility of retinopathy is unlikely in the case of short-term administration such as malaria treatment. No cases of retinopathy that occurred after malaria treatment were found in the review of literatures.

Increasing chloroquine resistance enables parasite growth in high drug concentrations, which slows

parasite clearance. A previous meta-analysis showed clearance of parasitemia assessed by microscopy in day 3 was 100% predictive of chloroquine sensitivity.[22] Chloroquine resistance of *P. vivax* has been first reported in Papua New Guinea in 1989,[51] and sporadic cases have been observed in other Asian countries subsequently as well.[22] The mechanism of chloroquine resistance is probably similar in both *P. vivax* and *P. falciparum*, however, the molecular mechanisms in *P. vivax* are less studied and there is still a lack of a confirmed marker for chloroquine resistance unlike the *P. falciparum*. Therefore, preliminary studies on chloroquine resistance in *P. vivax* have focused on the orthologues of the genes *pfcrt* and *pfmdr1*, known to be genetic determinants in *P. falciparum* resistance.[52] In recent studies in South Korea, chloroquine resistance genes have been reported[27, 28] although a clinically significant level of chloroquine resistant malaria has not yet been identified. Therefore, we performed DNA sequencing of *P. vivax* isolates collected from enrolled patients to determine any mutations or SNPs in the chloroquine-resistant molecular markers.

The substitution Y976F in *pvmdr1* gene has been associated with a decreased chloroquine sensitivity in several studies in Southeast Asia, especially in Thailand, Myanmar, and Indonesia.[53] One study identified that *P. vivax* isolates with Y976F mutation showed significantly increased the half maximal inhibitory concentration (IC_{50}) values for chloroquine *in vitro*, although the cut-off IC_{50} value for chloroquine resistance is uncertain.[23] In other studies form Madagascar[54], Brazil[55] and Honduras[56], however, Y976F mutation alone was not sufficient to cause the failure of chloroquine treatment and can only affect treatment outcome when associated with the F1076L mutation. The abundance of *pvmdr1* F1076L in isolates from Mangaluru has been considered an indication of emerging chloroquine resistance,[57, 58] but isolates with only F1076L mutations were not associated with a chloroquine resistance in other study.[25] These observation may supports that chloroquine resistance of *P. vivax* requires the presence of both mutations. In this study, the *pvmdr1* analysis showed that mutations at amino acid position Y976F were not found but mutation at position F1076L was identified in all isolates. These results may suggest that endemicity of F1076L mutation indicate the progress stage for chloroquine resistance *P. vivax* in Korea. The insertion of lysine (K) in the first exon (K10) in *pvcrt-o* gene was also identified as a possible marker for *P. vivax* resistance to chloroquine.[25, 55] Several studies have found a negative association between K10 insertion and reduced chloroquine IC_{50}, while others have shown that *pvcrt-o* expression decreased susceptibility to chloroquine by 2.2 fold.[23, 59] The overall frequency of K10 insertion in isolated from this study isolates was 0%. Although K10 insertion in *pvcrt-o* gene is not detected, this needs to be monitored continuously under regular drug resistance epidemiological surveillance. In conclusion, the coidentity

of SNPs in the chloroquine-resistant gene in all of isolates shows that it is difficult to explain the difference in PCT due to SNPs in Korea.

Prospective studies are needed to more accurately assess whether PCT has increased, and the definitive diagnosis of chloroquine resistance requires documentation of adequate drug exposure, to confirm the growth of parasites in concentrations of drug above the minimal inhibitory concentration (MIC)[21] with pharmacologic research such as serum drug levels and an *ex vivo* drug assay.

Recurrent vivax malaria is a great obstacle to human and economic development in affected population, and there are studies showing that recurrent patients could become difficult to treat and sometimes critically ill.[60] Recurrence of vivax malaria could have resulted from recrudescence, relapse, or re-infection. Considering the low prevalence of malaria in Korea, we have assumed that re-infection contributed very little to recurrence. In addition, previous studies have identified that the delayed elimination of chloroquine allows sufficient blood concentrations that prevent recurrence of chloroquine-sensitive *P. vivax* for about 35 days.[22] Hence, no recurrent parasitemia should be noted within 28 days of treatment in patients taking a complete treatment course with adequate absorption.[21] Failures beyond 28 days are indicative of the failure of primaquine to kill the hypnozoites or re-infection. No patients has been identified to recur within 28 days from the start of chloroquine by the national infectious diseases reporting systems in South Korea, and even community health centers usually confirms the clearance of parasitemia after 28 days of diagnosis. Thus we have presumed that the recrudescence has little impact on malaria recurrence, and most of the recurrences have been assumed to be relapse in Korea.

The use of primaquine to prevent relapse of *P. vivax* may support malaria elimination activities, so optimizing primaquine dosing regimen and pharmacodynamical properties are important to clearing hypnozoites. The factor most strongly associated with relapse in primaquine therapy is the total amount of primaquine administered to the patient.[61] Traditionally, the recommended dose of primaquine has been 15 mg/day for 2 weeks. However, it is recommended to increase the daily dose of primaquine to 30 mg/day for vivax malaria which is prevalent in tropical regions[62] and the latest WHO guideline recommend as 0.25 - 0.5 mg per kg of body weight to prevent relapse.[9] In previous study in Korea, vivax malaria is known to have a relapse rate of about 1% when primaquine was administrated for 14 days with 15 mg/day. As the overall body weight of patients increased recently, relapse occasionally occurred when primaquine was administered at the above dose, and KCDA recommends that 0.25 mg per kg of body weight be administered orally once a day for 14 days.[63] In this study, the average primaquine dose of patients was 0.31 ± 0.09 mg/kg and the recommended

dosage were sufficiently administered. When clinical characteristics were compared between the non-recurred and recurred groups, there was no difference between the two groups for primaquine daily dose.

CYP2D6 is an enzyme involved in the metabolism of 20 - 25% of the commonly used drugs such as codeine, selective serotonin reuptake inhibitors (SSRIs), many antihypertensive agents[64, 65], and primaquine.[32] Polymorphism in the CYP2D6 gene greatly impact the enzymatic activity of CYP2D6, thus has been implicated in the formation of active metabolites that are responsible for the pharmacological effect of primaquine. A previous report showed that primaquine treatment failures were directly attributable to an intermediate or null phenotype of CYP2D6,[66] and other study using a mouse model demonstrated the dependence of primaquine treatment efficacy on CYP2D6 metabolism.[67] Several cases of vivax malaria recurrence in patients with intermediate or poor metabolizer CYP2D6 genotypes have also been reported.[68-70] Other study on CYP2D6 have found that approximately 50% of Southeast Asians possess the homozygous CYP2D6*10 allele, which is associated to decreased CYP2D6 function,[71] and more than half of the global burden of *P. vivax* occurs in this region. In the current study, the most common genotype was also *10B/*10B (36 of 139, 25.9%) and almost half of the patients (65 of 139, 46.8%) had an IM phenotype. In the poor or intermediated metabolizer, recurrence risk was statistically higher than that of normal or ultrarapid metabolizer (OR = 2.33, $p = 0.02$), and in case with genotype-determined activity score less than 1.5, recurrence risk was statistically higher than in case with 1.5 or higher (OR = 2.65, $p = 0.04$). These findings show that primaquine treatment with appropriated dose to achieve radical cure may be hampered by the low CYP2D6 activity due to low exposure to the active metabolite. Moreover, some commonly used drugs inhibiting CYP2D6 can render the NM phenotype as a functional PM phenotype. Routine screening of CYP2D6 activity in patients with vivax malaria is not currently practical because of the high cost and technical expertise required. However, papulation in the region with high frequencies of impaired CYP2D6 alleles would likely benefit from increased or prolonged primaquine treatment as standard of regimen. Further study should be conducted to other possible non-hypnozoite plasmodial sources of recurrence, such as erythrocytic parasites in bone marrow, spleen and the skin.[72]

A number of limitations should be accounted for when interpreting our findings. First, the number of enrolled patients in this study is limited. Thus our study population would not represent the actual situation in South Korea. Second, administration of chloroquine and primaquine were not directly observed and the drug compliance could not be confirmed. Third, the proportion of patients who

confirmed that the parasitemia clearance in blood smear until discharge was low. In other words, patients who were discharged from the hospital while parasitemia did not disappear until the time of discharge, or those who were not hospitalized with mild diseases, did not perform a peripheral blood smear until they visited the outpatient clinic, so an accurate PCT could not be calculated. In addition, a significant number of patients with parasitemia over 72 hours had to be excluded from the PCT calculation. Fourth, it is not clear whether the samples received from the KCDA were relapse or re-infection because most of the patients were still living in the endemic area. Molecular genotyping methods can discriminate between genetically homologous and heterologous infections. However, genotyping cannot discriminate between a recrudescence of the blood-stage infection or a relapse with a homologous strain. Furthermore, as relapses are often heterologous activation of hypnozoites,[73, 74] reliable distinction of the causes of recurrent infection within an endemic area is not possible at present.[73, 75] Selective whole-genome amplification might be helpful for future characterization of *P. vivax* recurrences,[76] determination of the origin of which is particularly problematic in endemic areas.[77] Last, KCDA provided blood samples of recurred patients without clinical information of each patients, so there was a limitation to the analysis of clinical parameters associated with relapse of vivax malaria.

CONCLUSION

In this study, we determined that decreased treatment response to chloroquine of vivax malaria in South Korea would be associated with insufficient dosage of chloroquine, suggesting that it is important to administer an appropriate dosage of chloroquine based on the patient's body weight in clinical practice. Genetic polymorphism of the chloroquine resistance gene in *P. vivax* were not identified in this study, however, enhanced monitoring of decreasing chloroquine efficacy and the evaluation of detailed molecular mechanisms involved in drug resistance of *P. vivax* malaria is essential. Moreover, CYP2D6-dependent metabolism of primaquine may be a key determinant of anti-hypnozoite activity of primaquine that prevent *P. vivax* malaria relapse.

This study is important that informs what should be considered for malaria treatment strategy in South Korea, which aims to re-eradication of malaria with the goal of 2024.

REFERENCES

1. Organization WH. A framework for malaria elimination: World Health Organization; 2017.
2. Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of *Plasmodium vivax* malaria. Am J Trop Med Hyg. 2001;64(1-2 Suppl):97-106.
3. Feighner BH, Pak SI, Novakoski WL, Kelsey LL, Strickman D. Reemergence of *Plasmodium vivax* malaria in the republic of Korea. Emerg Infect Dis. 1998;4(2):295-7.
4. Ree HI. Unstable vivax malaria in Korea. Korean J Parasitol. 2000;38(3):119-38.
5. Lee JS, Lee WJ, Cho SH, Ree H-I. Outbreak of vivax malaria in areas adjacent to the demilitarized zone, South Korea, 1998. Am J Trop Med Hyg. 2002;66(1):13-7.
6. Cho SY, Kong Y, Park SM, Lee JS, Lim YA, Chae SL, et al. Two vivax malaria cases detected in Korea. Korean J Parasitol. 1994;32(4):281-4.
7. Park JW, Klein TA, Lee HC, Pacha LA, Ryu SH, Yeom JS, et al. Vivax malaria: a continuing health threat to the Republic of Korea. Am J Trop Med Hyg. 2003;69(2):159-67.
8. Adams JH, Mueller I. The Biology of *Plasmodium vivax*. Cold Spring Harb Perspect Med. 2017;7(9).
9. Organization WH. Guidelines for the treatment of malaria: World Health Organization; 2015.
10. WHO. The Use of Antimalarial Drugs–Report of a WHO Informal Consultation. 2001.
11. Park SY, Park YS, Park Y, Kwak YG, Song JE, Lee KS, et al. Increasing Malaria Parasite Clearance Time after Chloroquine Therapy, South Korea, 2000-2016. Emerg Infect Dis. 2020;26(8):1852-5.
12. Yeom JS, Jun G, Kim JY, Lee WJ, Shin EH, Chang KS, et al. Status of *Plasmodium vivax* malaria in the Republic of Korea, 2008-2009: decrease followed by resurgence. Trans R Soc Trop Med Hyg. 2012;106(7):429-36.
13. Pukrittayakamee S, Chantra A, Simpson JA, Vanijanonta S, Clemens R, Looareesuwan S, et al. Therapeutic responses to different antimalarial drugs in vivax malaria. Antimicrob Agents Chemother. 2000;44(6):1680-5.
14. Krudsood S, Tangpukdee N, Muangnoicharoen S, Thanachartwet V, Luplertlop N, Srivilairit S, et al. Clinical efficacy of chloroquine versus artemether-lumefantrine for *Plasmodium vivax* treatment in Thailand. Korean J Parasitol. 2007;45(2):111-4.
15. Poravuth Y, Socheat D, Rueangweerayut R, Uthaisin C, Pyae Phyo A, Valecha N, et al. Pyronaridine-artesunate versus chloroquine in patients with acute *Plasmodium vivax* malaria: a randomized, double-blind, non-inferiority trial. PLoS One. 2011;6(1):e14501.

16. Muhamad P, Ruengweerayut R, Chacharoenkul W, Rungsihirunrat K, Na-Bangchang K. Monitoring of clinical efficacy and *in vitro* sensitivity of *Plasmodium vivax* to chloroquine in area along Thai Myanmar border during 2009-2010. Malar J. 2011;10:44.
17. Hoglund R, Moussavi Y, Ruengweerayut R, Cheomung A, Abelo A, Na-Bangchang K. Population pharmacokinetics of a three-day chloroquine treatment in patients with *Plasmodium vivax* infection on the Thai-Myanmar border. Malar J. 2016;15:129.
18. Thuan PD, Ca NTN, Van Toi P, Nhien NTT, Thanh NV, Anh ND, et al. A Randomized Comparison of Chloroquine Versus Dihydroartemisinin-Piperaquine for the Treatment of *Plasmodium vivax* Infection in Vietnam. Am J Trop Med Hyg. 2016;94(4):879-85.
19. Guidelines for treatment of malaria in the United States. CDC 2019.
20. Lalloo DG, Shingadia D, Bell DJ, Beeching NJ, Whitty CJ, Chiodini PLJJoI. UK malaria treatment guidelines 2016. J Infect. 2016;72(6):635-49.
21. Baird JK, Leksana B, Masbar S, Fryauff DJ, Sutanihardja MA, Suradi, et al. Diagnosis of resistance to chloroquine by *Plasmodium vivax*: timing of recurrence and whole blood chloroquine levels. Am J Trop Med Hyg. 1997;56(6):621-6.
22. Price RN, von Seidlein L, Valecha N, Nosten F, Baird JK, White NJ. Global extent of chloroquine-resistant *Plasmodium vivax*: a systematic review and meta-analysis. Lancet Infect Dis. 2014;14(10):982-91.
23. Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, et al. Chloroquine resistant *Plasmodium vivax*: *in vitro* characterisation and association with molecular polymorphisms. PLoS One. 2007;2(10):e1089.
24. Brega S, Meslin B, de Monbrison F, Severini C, Gradoni L, Udomsangpetch R, et al. Identification of the *Plasmodium vivax* mdr-like gene (*pvmdr1*) and analysis of single-nucleotide polymorphisms among isolates from different areas of endemicity. J Infect Dis. 2005;191(2):272-7.
25. Lu F, Lim CS, Nam DH, Kim K, Lin K, Kim TS, et al. Genetic polymorphism in *pvmdr1* and *pvcrt-o* genes in relation to *in vitro* drug susceptibility of *Plasmodium vivax* isolates from malaria-endemic countries. Acta Trop. 2011;117(2):69-75.
26. Yeom JS, Ryu SH, Oh S, Choi DH, Song KJ, Oh YH, et al. Evaluation of anti-malarial effects of mass chemoprophylaxis in the Republic of Korea army. J Korean Med Sci. 2005;20(5):707-12.
27. Lee KS, Kim TH, Kim ES, Lim HS, Yeom JS, Jun G, et al. Short report: chloroquine-resistant *Plasmodium vivax* in the Republic of Korea. Am J Trop Med Hyg. 2009;80(2):215-7.

28. Kim YK, Kim C, Park I, Kim HY, Choi JY, Kim JMJTAjotm, et al. Therapeutic efficacy of chloroquine in *Plasmodium vivax* and the pvmdr1 polymorphisms in the Republic of Korea under mass chemoprophylaxis. 2011;84(4):532-4.
29. Moon KT, Kim YK, Ko DH, Park I, Shin DC, Kim C. Recurrence rate of vivax malaria in the Republic of Korea. Trans R Soc Trop Med Hyg. 2009;103(12):1245-9.
30. Kwak YG, Lee HK, Kim M, Um TH, Cho CR. Clinical characteristics of vivax malaria and analysis of recurred patients. Infect Chemother. 2013;45(1):69-75.
31. Bennett JE, Dolin R, Blaser MJ. Mandell, douglas, and bennett's principles and practice of infectious diseases: 2-volume set: Elsevier Health Sciences; 2014.
32. Potter BM, Xie LH, Vuong C, Zhang J, Zhang P, Duan D, et al. Differential CYP 2D6 metabolism alters primaquine pharmacokinetics. Antimicrob Agents Chemother. 2015;59(4):2380-7.
33. Marcsisin SR, Reichard G, Pybus BS. Primaquine pharmacology in the context of CYP 2D6 pharmacogenomics: Current state of the art. Pharmacol Ther. 2016;161:1-10.
34. 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(14):1381-2.
35. Baird JK, Louisa M, Noviyanti R, Ekawati L, Elyazar I, Subekti D, 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(4):e181449.
36. Goo YK, Ji SY, Shin HI, Moon JH, Cho SH, Lee WJ, et al. First evaluation of glucose-6-phosphate dehydrogenase (G6PD) deficiency in vivax malaria endemic regions in the Republic of Korea. PLoS One. 2014;9(5):e97390.
37. Organization WH. Basic malaria microscopy: Part I. Learner's guide: World Health Organization; 2010.
38. Subramony H, Tangpukdee N, Krudsood S, Poovorawan K, Muangnoicharoen S, Wilairatana P. Evaluation of efficacy of chloroquine for *Plasmodium vivax* infection using parasite clearance times: a 10-year study and systematic review. Ann Acad Med Singap. 2016;45(7):303-14.
39. Sá JM, Nomura T, Neves JdA, Baird JK, Wellems TE, del Portillo HAJEp. *Plasmodium vivax*: allele variants of the mdr1 gene do not associate with chloroquine resistance among isolates from Brazil, Papua, and monkey-adapted strains. Exp Parasitol. 2005;109(4):256-9.

40. Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, et al. Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. J Infect Dis. 2001;183(11):1653-61.

41. 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. 2020;13(1):116-24.

42. Pukrittayakamee S, Imwong M, Looareesuwan S, White NJ. Therapeutic responses to antimalarial and antibacterial drugs in vivax malaria. Acta Trop. 2004;89(3):351-6.

43. Baird JK, Hoffman SL. Primaquine therapy for malaria. Clin Infect Dis. 2004;39(9):1336-45.

44. Bray PG, Martin RE, Tilley L, Ward SA, Kirk K, Fidock DA. Defining the role of *PfCRT* in *Plasmodium falciparum* chloroquine resistance. Mol Microbiol. 2005;56(2):323-33.

45. Russell B, Chalfein F, Prasetyorini B, Kenangalem E, Piera K, Suwanarusk R, et al. Determinants of *in vitro* drug susceptibility testing of *Plasmodium vivax*. Antimicrob Agents Chemother. 2008;52(3):1040-5.

46. Baird JK. Chloroquine resistance in *Plasmodium vivax*. Antimicrob Agents Chemother. 2004;48(11):4075-83.

47. White NJ. Pharmacokinetic and pharmacodynamic considerations in antimalarial dose optimization. Antimicrob Agents Chemother. 2013;57(12):5792-807.

48. Commons RJ, Simpson JA, Thriemer K, Humphreys GS, Abreha T, Alemu SG, et al. The effect of chloroquine dose and primaquine on *Plasmodium vivax* recurrence: a WorldWide Antimalarial Resistance Network systematic review and individual patient pooled meta-analysis. Lancet Infect Dis. 2018;18(9):1025-34.

49. Battle KE, Karhunen MS, Bhatt S, Gething PW, Howes RE, Golding N, et al. Geographical variation in *Plasmodium vivax* relapse. Malar J. 2014;13:144.

50. Marmor MF, Kellner U, Lai TY, Melles RB, Mieler WF, American Academy of O. Recommendations on Screening for Chloroquine and Hydroxychloroquine Retinopathy (2016 Revision). Ophthalmology. 2016;123(6):1386-94.

51. Rieckmann KH, Davis DR, Hutton DC. *Plasmodium vivax* resistance to chloroquine? Lancet. 1989;2(8673):1183-4.

52. Price RN, Douglas NM, Anstey NM. New developments in *Plasmodium vivax* malaria: severe disease and the rise of chloroquine resistance. Curr Opin Infect Dis. 2009;22(5):430-5.

53. Tacoli C, Gai PP, Siegert K, Wedam J, Kulkarni SS, Rasalkar R, et al. Characterization of

Plasmodium vivax pvmdr1 Polymorphisms in Isolates from Mangaluru, India. Am J Trop Med Hyg. 2019;101(2):416-7.

54. Barnadas C, Ratsimbasoa A, Tichit M, Bouchier C, Jahevitra M, Picot S, et al. *Plasmodium vivax* resistance to chloroquine in Madagascar: clinical efficacy and polymorphisms in *pvmdr1* and *pvcrt-o* genes. Antimicrob Agents Chemother. 2008;52(12):4233-40.

55. Orjuela-Sanchez P, de Santana Filho FS, Machado-Lima A, Chehuan YF, Costa MR, Alecrim M, et al. Analysis of single-nucleotide polymorphisms in the *crt-o* and *mdr1* genes of *Plasmodium vivax* among chloroquine-resistant isolates from the Brazilian Amazon region. Antimicrob Agents Chemother. 2009;53(8):3561-4.

56. Jovel IT, Mejia RE, Banegas E, Piedade R, Alger J, Fontecha G, et al. Drug resistance associated genetic polymorphisms in *Plasmodium falciparum* and *Plasmodium vivax* collected in Honduras, Central America. Malar J. 2011;10:376.

57. Joy S, Mukhi B, Ghosh SK, Achur RN, Gowda DC, Surolia N. Drug resistance genes: *pvcrt-o* and *pvmdr-1* polymorphism in patients from malaria endemic South Western Coastal Region of India. Malar J. 2018;17(1):40.

58. Anantabotla VM, Antony HA, Parija SC, Rajkumari N, Kini JR, Manipura R, et al. Polymorphisms in genes associated with drug resistance of *Plasmodium vivax* in India. Parasitol Int. 2019;70:92-7.

59. Sa JM, Yamamoto MM, Fernandez-Becerra C, de Azevedo MF, Papakrivos J, Naude B, et al. Expression and function of *pvcrt-o*, a *Plasmodium vivax* ortholog of *pfcrt*, in *Plasmodium falciparum* and Dictyostelium discoideum. Mol Biochem Parasitol. 2006;150(2):219-28.

60. Kevin Baird J. Malaria caused by *Plasmodium vivax*: recurrent, difficult to treat, disabling, and threatening to life--the infectious bite preempts these hazards. Pathog Glob Health. 2013;107(8):475-9.

61. Schmidt LH, Fradkin R, Vaughan D, Rasco J. Radical cure of infections with *Plasmodium* cynomolgi: a function of total 8-aminoquinoline dose. Am J Trop Med Hyg. 1977;26(6 Pt 1):1116-28.

62. Hill DR, Baird JK, Parise ME, Lewis LS, Ryan ET, Magill AJ, et al. Primaquine: report from CDC expert meeting on malaria chemoprophylaxis I. Am J Trop Med Hyg. 2006;75(3):402-15.

63. Yeom J-S, Park Y-K. Treatment of Korean vivax malaria in Korea. J J Korean Med Assoc. 2007;50(1):88-92.

64. Preissner SC, Hoffmann MF, Preissner R, Dunkel M, Gewiess A, Preissner S. Polymorphic

cytochrome P450 enzymes (CYPs) and their role in personalized therapy. PLoS One. 2013;8(12):e82562.

65. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther. 2013;138(1):103-41.

66. Zanger UM, Raimundo S, Eichelbaum M. Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. Naunyn Schmiedebergs Arch Pharmacol. 2004;369(1):23-37.

67. Pybus BS, Marcsisin SR, Jin X, Deye G, Sousa JC, Li Q, et al. The metabolism of primaquine to its active metabolite is dependent on CYP 2D6. Malar J. 2013;12(1):1-7.

68. He X, Pan M, Zeng W, Zou C, Pi L, Qin Y, et al. Multiple relapses of *Plasmodium vivax* malaria acquired from West Africa and association with poor metabolizer CYP2D6 variant: a case report. BMC Infect Dis. 2019;19(1):704.

69. Mat Salleh NH, Rahman MFA, Samsusah S, De Silva JR, Ng DC-E, Ghozali AH, et al. Case report: recurrence of *Plasmodium vivax* malaria due to defective cytochrome P450 2D6 function in Pos Lenjang, Pahang, Malaysia. Trans R Soc Trop Med Hyg. 2020;114(9):700-3.

70. Ramírez AM, González CL, Maniega TS, Liarte ÁG, García DD, Suárez ML, et al. Several *Plasmodium vivax* relapses after correct primaquine treatment in a patient with impaired cytochrome P450 2D6 function. Malar J. 2020;19(1):1-7.

71. Droll K, Bruce-Mensah K, Otton SV, Gaedigk A, Sellers EM, Tyndale RF. Comparison of three CYP2D6 probe substrates and genotype in Ghanaians, Chinese and Caucasians. Pharmacogenetics. 1998;8(4):325-33.

72. Markus MB. Biological concepts in recurrent Plasmodium vivax malaria. Parasitology. 2018;145(13):1765-71.

73. Imwong M, Snounou G, Pukrittayakamee S, Tanomsing N, Kim JR, Nandy A, et al. Relapses of *Plasmodium vivax* infection usually result from activation of heterologous hypnozoites. J Infect Dis. 2007;195(7):927-33.

74. de Araujo FCF, De Rezende AnM, Fontes CJF, Carvalho LH, de Brito C. Multiple-clone activation of hypnozoites is the leading cause of relapse in *Plasmodium vivax* infection. PLoS One. 2012;7(11):e49871.

75. Chen N, Auliff A, Rieckmann K, Gatton M, Cheng Q. Relapses of *Plasmodium vivax* infection result from clonal hypnozoites activated at predetermined intervals. J Infect Dis. 2007;195(7):934-41.

76. Cowell AN, Loy DE, Sundararaman SA, Valdivia H, Fisch K, Lescano AG, et al. Selective whole-genome amplification is a robust method that enables scalable whole-genome sequencing of *Plasmodium vivax* from unprocessed clinical samples. mBio. 2017;8(1).

77. Popovici J, Friedrich LR, Kim S, Bin S, Run V, Lek D, et al. Genomic Analyses Reveal the Common Occurrence and Complexity of P*lasmodium vivax* Relapses in Cambodia. mBio. 2018;9(1).

ABSTRACT IN ENGLISH

Background: Vivax malaria is a protozoal infection caused by *P. vivax*. After re-emergence in 1993, about 500 - 600 patients have been occurring recently In South Korea. Chloroquine which is the blood schizonticide and primaquine which eliminate dormant hypnozoites in the liver are the standard therapeutic agents for vivax malaria. However, recent studies reported that the parasite clearance time (PCT) is being extended since 2010 in Korea. Moreover, there is growing concern about the emerging of chloroquine resistance of *P. vivax* which are repeatedly exposed to chloroquine due to massive chemoprophylaxis. Primaquine is metabolized by the enzyme cytochrome P450 isoenzyme 2D6 (CYP2D6) to change into an active metabolite having antimalarial properties. Recently, several studies have reported that the antimalarial properties of primaquine varies depend polymorphic CYP2D6 activity. In the case of 'poor metabolizer (PM)' or 'intermediate metabolizer (IM)', the therapeutic effect of the drug may not be significant because of the low level of primaquine metabolites even if appropriate dosage is administered. In this study, we identified host factors related to decrease chloroquine therapeutic efficacy and the prevalence of mutation in chloroquine resistance genes in the clinical isolated *P. vivax* from patients in Korea. In addition, we analyzed the CYP2D6 profiles of patients to identify the association between primaquine metabolization and recurrence of malaria.

Methods: Vivax malaria patients were enrolled prospectively in 9 hospitals in Gyeonggi-do Province, Incheon and Seoul. Parasitemia more than 72 hours have been considered as decreased therapeutic efficacy to chloroquine, and clinical characteristics were compared between the two groups with and without *P. vivax* parasitemia at 72 hours after first dose of chloroquine. The prevalence of mutations in chloroquine resistance genes (*pvmdr1* and *pvcrt-o*) in the clinical isolated *P. vivax* from patients were analyzed. To identify the relationship between CYP2D6 genotype and relapse of vivax malaria, the DNA from the blood samples of patients who were suspected recurred malaria were provided by the Korea Diseases Control and Prevention Agency (KDCA). The CYP2D6 genotype and phenotype of prospectively enrolled patients and recurred patients registered with the KCDA were analyzed and we compared the difference between recurred patients and non-recurred patients.

Results: A total of 102 patients were enrolled prospectively during the study period, including one patient with four times of malaria attack. Additionally, the KCDA provided blood samples of 38 suspected recurred patients. The female sex was significantly associated with the prolonged parasitemia in univariable logistic regression analysis, not in multivariable analysis. When analyzed male patients by removing the sex factor, chloroquine total dose per body weight and the percentage

of administration of less than 25 mg/kg of chloroquine were significantly different between the two groups. Univariable and multivariable logistic regression analysis did not show a significant association between chloroquine dose and prolonged parasitemia, however, the risk of prolonged parasitemia tended to decrease as the chloroquine total dose per body weight increased (hazard ration 0.57, 95% CI; 0.27 - 1.16; p = 0.12). The *pvmdr1* analysis showed that mutations at amino acid position Y976F were not found but mutation at position *F1076L* was identified in all isolates. The overall frequency of K10 insertion in isolated from this study was 0%. When comparing the CYP2D6 phenotype and genotype between the recurred and non-recurred groups, risk of recurrence was statistically higher in the poor or intermediated metabolizer, than that of normal or ultrarapid metabolizer (OR = 2.33, p = 0.02). In case with genotype-determined activity score less than 1.5, risk of recurrence was statistically higher than in case with 1.5 or higher (OR = 2.65, p = 0.04).

Conclusion: In this study, we determined that decreased treatment efficacy to chloroquine of vivax malaria may be associated with insufficient dosage of chloroquine, suggesting that it is important to administer an appropriate dosage of chloroquine based on the patient's body weight in clinical practice. Genetic polymorphism of the chloroquine resistance gene in *P. vivax* were not identified in this study, however, enhanced monitoring of decreasing chloroquine efficacy and the evaluation of detailed molecular mechanisms involved in drug resistance of *P. vivax* malaria is essential. Moreover, CYP2D6-dependent metabolism of primaquine may be a key determinant of anti-hypnozoite activity of primaquine that prevent *P. vivax* malaria relapse.

Keywords: *P. vivax*, malaria, chloroquine, primaquine, drug resistance, CYP2D6