Original article

Genetic polymorphisms in the *pvdhfr*, *pvmdr1*, and *pvdhps* genes of *Plasmodium vivax* in patients at a secondary hospital in South Korea

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Abstract

Background: *Plasmodium vivax* is a major pathogen that causes malaria in Korea. Several genetic polymorphisms in dihydrofolate reductase (*pvdhfr*), *P. vivax* multidrug resistance protein 1 (*pvmdr1*), and *P. vivax* hydroxymethylpterin pyrophosphokinase-dihydropteroate synthetase (*pvdhps*) genes are known to be associated with drug resistance in *P. vivax*. The objective of this study was to profile the known polymorphisms of *P. vivax* resistance genes in patients at a secondary hospital in Korea.

Methods: A total of 12 patients with confirmed *P. vivax* infections were enrolled for this study. Sanger sequencing was performed for the *pvdhfr*, *pvmdr1*, and *pvdhps* genes to detect polymorphisms of these drug resistance genes.

Results: Each specimen had single or double polymorphism in *pvdhfr*. One specimen had a polymorphism in *pvdhps.* However, no specimen had any polymorphisms in *pvmdr1*. There was no strain with multi-polymorphisms exceeding double polymorphisms, which reported the geographic location of treatment failure.

Conclusion: No specimen showed chloroquine-resistance polymorphism in *pvmdr1.* Treatment with first-line therapy was successful. The prevalence of F57L in *pvdhfr* was higher than that reported previously. This change must be confirmed by further monitoring and surveillance of the strains with multi-polymorphisms.

Keywords: Drug resistance, Genetic polymorphism, *Plasmodium vivax*, Korea

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Introduction

Most *Plasmodium vivax* infection in Korea occurs near the demilitarized zone (DMZ). From 2014, about 500 cases of *P. vivax* infections have occurred in Korea every year. Long-term military chemoprophylaxis since 2005 has contributed to a decrease in malaria cases. However, this might have facilitated the development of chloroquine (CQ)-resistant *P. vivax* strains [1].

CQ has been used for decades as a first-line treatment for uncomplicated *P. vivax* cases. Since the first report of CQ treatment failures in *P. vivax* malaria in Papua, Indonesia in 1989 [2,3], reports of antimalarial treatment failures related to genetic polymorphisms in *P. vivax* dihydrofolate reductase (*pvdhfr*), *P. vivax* multidrug resistance protein 1 (*pvmdr*1) and *P. vivax* hydroxymethylpterin pyrophosphokinase-dihydropteroate synthetase (*pvdhps*) have increased, causing worldwide concerns about drug-resistant strain spread [4-9]. Especially, strains with multi-polymorphisms are thought to be the cause of antimalarial treatment failure. Despite the continuous occurrence of malaria infections in Korea, studies of CQ resistance polymorphisms were not reported. Here, we profiled known polymorphisms of *P. vivax* genes associated with drug resistance from clinical specimens in a secondary hospital in Korea.

Materials and methods

Patients with confirmed *P. vivax* infections in Uijeongbu St. Mary's Hospital were enrolled from July 2014 to June 2017. A total of 12 *P. vivax* infection samples were collected. *P. vivax* infections were confirmed through SD Bioline Malaria Ag Pf/Pan™ rapid diagnosis test kits (Abbott, Chicago, IL, USA) and referral *Plasmodium spp*. PCR (polymerase chain reactin; SMLab, Seoul, Korea). Electronic medical records and complete blood count (CBC) findings were reviewed for all patients. In the 2015 World Health Organization (WHO) guidelines [10], antimalarial drug resistance is defined as the survival or increased number of parasites despite the use of an antimalarial drug at an usual recommended dose. According to the definition by the WHO, treatment failure in this study was defined as the persistence or an increase in parasite count despite the first-line therapy.

DNA extraction, PCR, and Sanger sequencing of *pvdhfr*, *pvmdr1*, and *pvdhps* genes were performed following methods reported in previous studies [6,11-13]. The following primers were used for PCR and Sanger sequencing [13]: primer PV1F (sense) 5′-CAGTGAAGGGACAAAGAATGAACC-3′, primer PV1R (antisense) 5′-ACTCGGGGAAGAAGACGTCAC-3′ for *pvdhfr* (560 bps), primer PV7F (sense) 5′-GCCATGTTCATTTCTGAGACGCTG-3′, primer PV7R (antisense) 5′-TCGCTCTGATGGCAAACACTC-3′ for *pvmdr1* (337 bps), primer PV9F (sense) 5′-GCGGTTT-ATTTGTCGATCCTGTG-3′ and primer PV9R (antisense) 5′-TTTTTCCTGGCATCACTTGCTG-3′ for *pvdhps* (244 bps). Amino acid sequences were compared with reference wild-type sequences, where insertions and deletions were manually verified. Identified single-nucleotide polymorphisms (SNPs), insertions, and deletions were compared with reported SNPs known to be associated with antimalarial-resistance in previous studies [5,6,8,11,14,15].

Results

Ten of 12 patient were males. Initial hemoglobin level varied. CBCs on the first hospital visit of the twelve patients showed no specific pattern, although all cases showed cytopenia including various degrees of thrombocytopenia (Table 1). All 12 specimens had polymorphisms in *pvdhfr*. Only one out of 12 specimens

had a polymorphism in *pvdhps*. No polymorphism was present in *pvmdr1*. Four previously reported nonsynonymous polymorphisms (F57L, S58R, H99S, S117N) and one previously reported in-frame deletion (c.292_309del, = T98A) were detected in *pvdhfr*. A nonsynonymous polymorphism (M399I) was detected in *pvdhps*. F57L in *pvdhfr* was only found as double polymorphisms, with c.292_309del found in 5 out of 12 cases. Two cases had C.292_309del and three cases had H99S in *pvdhfr*. One case had both S58R and S117N in *pvdhfr*. Only one case had M399I in *pvdhps*, with C.292_309del in *pvdhfr* (Table 2). All species with H99S did not have tandem repeat insertions or deletions (e.g., C.292_309del) in *pvdhfr*.

Table 1. CBC results of patients with confirmed *Plasmodium vivax* infections

Patient No.	Sex	Age (yr)	WBC (10^3/μL)	Hb (g/dL)	PLT (10^3/μL)	Neu (%)	Lym (%)	Mono (%)	Eos (%)	Baso (%)
1	M	22	3.55	15.1	42	81.0	12.0	6.0	0.0	1.0
2	M	22	6.16	6.5	122	66.1	21.3	10.6	1.8	0.2
3	M	64	2.56	15.1	63	78.0	12.0	8.0	0	2.0
4	F	50	2.40	13.1	65	81.2	12.3	5.7	0.5	0.3
5	M	57	5.39	9.4	28	86.0	6.0	8.0	0.0	0.0
6	M	64	3.51	13.8	53	82.0	13.0	3.0	1.0	1.0
7	M	23	5.00	11.7	64	93.0	2.0	4.0	0.0	1.0
8	M	22	6.48	13.6	107	49.0	38.0	12.0	1.0	0.0
9	F	68	4.96	11.8	31	92.0	1.0	2.0	0.0	0.0
10	M	29	4.15	15.6	40	51.9	20.8	18.0	6.5	2.8
11	M	53	1.28	13.8	16	62.0	31.0	2.0	1.0	0.0
12	M	43	1.67	15.5	26	80.0	20.0	0.0	0.0	0.0

Abbreviations: CBC, complete blood count; WBC, white blood cells; Hb, hemoglobin; PLT, platelets; Neu, neutrophils; Lym, lymphocytes; Mono, monocytes; Eos, eosinophils; Baso, basophils; M, male; F,female.

Table 2. Identified polymorphisms in *pvdhfr* of *Plasmodium vivax*

Variants	No. of cases
pvdhfr F57L, C.292_309del	5
pvdhfr C.292_309del	2
pvdhfr H99S	3
pvdhfr S58R, S117N	1
pvdhfr C.292_309del & pvdhps M399I	1

Nine out of 12 cases were cured with first-line regimen of three days of hydroxycholoroquine (HCQ) followed by two weeks of primaquine (PQ). The patient No. 8 with the S58R/S117N double polymorphism in this study recovered after three days of atovaquone (AQ) followed by two weeks of PQ. Patient No. 10 who was transferred out after three days of HCQ was confirmed to be cured later (Table 3).

One of the three H99S patients was lost to follow-up after the first visit. However, the other two patients recovered after three days of HCQ followed by two weeks of PQ. All c.292_309del cases with or without F57L in *pvdhfr* recovered after three days of HCQ followed by two weeks of PQ. The patient with c.292_309del in *pvdhfr* and M399I in *pvdhps* was transferred out after three days of HCQ medication. It was confirmed that this patient recovered later.

Table 3. Clinical course of patients with confirmed *Plasmodium vivax* infections

Patient No.	Medication	Parasite count (/μL) [FU day*]					
		Initial	1st FU	2nd FU	3rd FU	4th FU	5th FU
1	3 days of HCQ followed by 2 weeks of PQ	1,704	305 [2]	298 [3]	negative [8]		
2	3 days of HCQ followed by 2 weeks of PQ	10,903	negative [4]				
3	3 days of HCQ followed by 2 weeks of PQ	6,104	518 [2]	110 [3]	26 [4]	negative [5]	
4	3 days of HCQ followed by 2 weeks of PQ	1,344	60 [2]	30 [3]	29 [4]	32 [5]	negative [9]
5	FU loss after 3 days of AQ	63,117	FU loss				
6	3 days of HCQ followed by 2 weeks of PQ	6,809	271 [4]	negative [11]			
7	3 days of HCQ followed by 2 weeks of PQ	8,400	426 [3]	negative [14]			
8	3 days of AQ followed by 2 weeks of PQ	12,247	negative [5]				
9	3 days of HCQ followed by 2 weeks of PQ	9,313	3,432 [2]	195 [3]	77 [4]	negative [5]	
10	Transferred out after 3 days of HCQ	428	transferred				
11	3 days of HCQ followed by 2 weeks of PQ	36,632	1,432 [2]	164 [3]	152 [4]	negative [6]	
12	3 days of HCQ followed by 2 weeks of PQ	17,889	11,289 [2]	1,234 [3]	326 [4]	197 [5]	negative [6]

*Days from initial medication.

Abbreviations: FU, follow-up; HCQ, hydroxycholoroquine; PQ, primaquine; AQ, atovaquone.

Discussion

Among polymorphisms detected in *pvdhfr* (F57L, S58R, H99S, S117N, and c.292_309del), the S58R/S117N double polymorphism has been previously reported in Indonesia, Thailand, Ethiopia, China, East Timor, Philippines, Vanuatu, Vietnam, Papua New Guinea, Madagascar, Iran [16], and French Guiana with *in vitro* resistance to drug combination of sulfadoxine and pyrimethamine (SP) [11]. Because the S58R/S117N double polymorphism case in this study was treated with AQ and PQ, we could not assume that this polymorphism resulted in SP or CQ resistance *in vivo*.

It has been reported that the F57L polymorphism in the *pvdhfr* gene can increase resistance of *P. vivax* to antifolate agents such as pyrimethamine [4,9]. F57L strains are known to be less prevalent in the region where CQ is used as a first-line regimen [17], which means low selection pressure of antifolate agents. In a previous study [18], *pvdhfr* F57L was found in 23% (22/97) of *P. vivax* strains in Korea. In the present study, it was found in 42% (5/12) of *P. vivax* strains (Table 2). This might be due to the difference in sample size. Actual change within the period between the two studies is also possible.

M399I in the *pvdhps* gene was reported in a previous report [19], although its association with drug resistance was unknown. This polymorphism was unlikely to cause *in vivo* CQ resistance because the patient recovered after HCQ medication (Table 3).

Unlike previous reports from other geographic locations, multi-polymorphisms exceeding double polymorphisms were not found in the present study. These reports of multi-polymorphisms are from the region in which antimalarial treatment failures and are thought to be the cause of treatment failures [11,15,16]. This justifies further monitoring of genetic profile and drug resistance of *P. vivax* in Korea.

Although every specimen had single or double polymorphisms known to cause antimalarial drug resistance, there was no reported treatment failure with first-line therapy. Because all cases except two with AQ therapy were cured with first-line HCQ therapy and none of the 12 cases had polymorphism in *pvmdr1*, the current regimen of three days of HCQ followed by two weeks of PQ seems to be proper as a first-line

therapy in Korea.

To the best of our knowledge, this is the first study analyzing polymorphisms in *pvdhfr*, *pvdhps* and *pvmdr1* genes altogether in Korea. Although all cases were susceptible to first-line therapy, *pvdhfr* F57L proportion in this study was higher than that in a previous report. Whether this change was true without a selection pressure by antifolate drugs or it was just an error due to small sample size was unclear. This is a limitation of this study. To clarify this change and to surveil emergence of multi-polymorphism strain reported abroad, further monitoring of genetic profile and drug resistance of *P. vivax* is needed.

Ethics statement

This study protocol was approved by the Institutional Review Board of the Catholic University of Korea (IRB No. UC14TISI0006). This study was performed in accordance with the Declaration of Helsinki. Written consent was obtained from all patients in accordance with local regulations.

Conflicts of interest

No potential conflicts of interest relevant to this article were reported.

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