Short Report: Emergence of New Alleles of the MSP-3 α Gene in *Plasmodium vivax* Isolates from Korea

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Abstract. Nucleotide sequence analysis of the *Plasmodium vivax* PvMSP-3 α gene was conducted on blood from 143 malaria patients admitted to Korea University Medical Center from 1996 to 2007 in the Republic of Korea (ROK). From 1996 to 2002, the PvMSP-3 α alleles were of two types, SKOR-67 (2.53 kb) and SKOR-69 (1.78 kb), which differed in length and amino acid sequence. Two new variants with similar size to SKOR-67 were first observed in 2002 and in 2006–2007 accounted for nearly 50% (25/51) of the sampled isolates. The new variants had the same amino acid sequence as SKOR-69 in the N-terminal region, but in Blocks I and II and in the C-terminal region, they were similar to previously reported isolates from Thailand, Papua New Guinea, India, Brazil, and Ecuador strains.

The *Plasmodium vivax* merozoite surface protein-3 α (PvMSP-3 α) is a vaccine candidate surface protein¹ that is geographically polymorphic.^{2,3} PvMSP-3 α has a large central domain with 20–30% alanine residues, which occupies the first and fourth positions of the typical heptad repeat unit.^{4,5} These heptad repeat units are predicted to form coiled alpha-helical structures that may facilitate anchoring the proteins to the merozoite surface.^{2,4,6} Globally, three major alleles of the PvMSP-3 α gene are known to be widely distributed; their sizes are approximately 1.5, 2.1, and 2.4 kb.³ Rayner and colleagues showed nucleotide diversity among isolates of the PvMSP-3 α gene collected over 40 years from various geographic areas. In Papua New Guinea (PNG), Bruce and others^{2,6} identified 24 alleles of the PvMSP-3 α gene based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Two major types, 1.5 and 1.9 kb, with deletions in Block I, have been observed in many Thai isolates.⁷ In western Thailand, parasites belonging to the two major deletion types in Block I accounted for more than 25% of the isolates analyzed.⁷

Since the first report of autochthonous *Plasmodium vivax* transmission in the Republic of Korea (ROK) in 1993, malaria rapidly increased to a high of 4,142 cases by 2000 (Table 1).⁸ Previous PvMSP-3 α gene sequences identified two distinct geographically coexisting Korean types, SKOR-67 and SKOR-69 that were different in length and amino acid sequence.⁹ The sequence homology of the PvMSP-3 α gene between the SKOR-67 and Belem strains, and between the SKOR-69 and Chesson strains, were 93.0% and 99.2%, respectively.⁹ The aim of this study was to identify genetic variation of the PvMSP-3 α gene from Korean isolates over time (1996–2007).

The nucleotide sequence of the PvMSP-3 α gene was analyzed from Korean isolates from 1996 through 2007. A total of 143 Korean patients (22–60 yrs of age) admitted to Korea

university medical center with confirmed *P. vivax* positive blood smears were recruited under an approved human-use protocol (Korea National Institutes of Health, Seoul, ROK). The medical history of the volunteer malaria patients indicated autochthonous transmission of malaria in the ROK. All cases received supervised treatment with 2,000 mg hydroxychloroquine sulfate given orally (800 mg initial dose, followed by 400 mg at 6, 24, and 48 h). Following hydroxychloroquine therapy, outpatients received oral primaquine (15 mg/day for 14 days). Whole blood from each patient was collected and stored at –80°C. The DNA was extracted from frozen pellets using AquaPure genomic DNA kits (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. After precipitation with 100% ethanol, DNA was dissolved in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and stored at –20°C until used.

The PvMSP-3 α gene was amplified by PCR using two primers: P1 5'-CAGCAGACACCATTTAAGG-3' and P2 5'-CCGTTTGTGATTAGTTGC-3'. The primers' final concentration was 0.1 μ M in 100 μ L of reaction mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.2 mM of each dNTP) with 10 μ L of DNA and 2.5 units of ExTaq polymerase (TaKaRa Shuzo Co., Shiga, Japan). The thermal cycler (Biosystem model 2720, Applied Biosystems, Foster City, CA) conditions were 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec/56°C for 30 sec/72°C for 2.5 min, with a final 5-min extension step at 72°C, followed by restriction digest with *EcoR* I (5 units of enzyme/reaction; TaKaRa Shuzo Co.) and *Pst* I (5 units of enzyme/reaction; TaKaRa Shuzo Co.). The PvMSP-3 α amplified products were size fractionated by electrophoresis on 1.0% agarose gels with ethidium bromide at 0.5 mg/mL. Three cases with two separate PCR amplified bands (2.2kb and 1.5kb) were interpreted as double infections (Table 1).

The amplified DNA products from only one PCR were cloned into the pCR II plasmid from a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Three white colonies selected from each of the samples were completely sequenced in both directions. Sequences from the subcloned PCR products were identical to those obtained by direct sequencing in eight samples. Sequences obtained in this study have been deposited to GenBank with accession nos. EF204144–EF204171. From

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TABLE 1
Incidence of allelic types of PvMSP-3 α in Korea, within the reported malaria cases, and estimated number of ROK soldiers on hydroxychloroquine/primaquine chemoprophylaxis from 1996 to 2007

Allelic type/year	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	Total
SKOR-67* (2,246 bp)	8 (57.1)§	5 (50.0)	7 (63.6)	10 (62.5)	8 (47.0)	1 (20.0)	5 (45.4)	3 (42.9)	0	0	8 (32.0)	11 (42.3)	66
SKOR-69* (1,463 bp)	6 (42.9)	5 (50.0)	4 (36.4)	6 (37.5)	7 (41.2)	4 (80.0)	4 (36.4)	0	0	0	2 (8.0)	4 (15.4)	42
KPVMSP3a02-24† (2,264 bp)	0	0	0	0	0	0	2 (18.2)	4 (57.1)	0	0	9 (36.0)	3 (11.5)	18
KPVMSP3a05-4† (2,243 bp)	0	0	0	0	0	0	0	0	0	1 (100.0)	6 (24.0)	7 (26.9)	14
Double infection (SKOR-67, 69)	0	0	0	0	2 (11.8)	0	0	0	0	0	0	1 (3.9)	3
Total number	14	10	11	16	17	5	11	7	826	1	25	26	143
Annual number of malaria cases in ROK‡	356	1,724	3,932	3,621	4,142	2,488	1,763	1,107	826	1,324	2,021	2,180	25,484
Estimated number of ROK soldiers on chemoprophylaxis‡	0	16,000	37,000	60,000	90,000	120,000	145,000	165,000	165,000	165,000	200,000	200,000	1,363,000

* Han and others.⁹

† Variant strains.

‡ Korea Center for Disease Control and Prevention.⁸

§ Percent (%) in parentheses.

FIGURE 1. Schematic representation of the structure of the PvMSP-3 α gene. A deletion within Block I accounts for the difference in size between SKOR-69 and SKOR-67. The approximate locations of the two primers are shown at the bottom (P1, P2). The *EcoR* I and *Pst* I restriction sites are indicated by the arrows below. The *EcoR* I restriction site is shown in Block I, 4th region. One *Pst* I restriction site is shown between Block I and Block II; the other is in Block II. Regions are graphically differentiated by patterns of shading, although identical patterns do not indicate identical sequences in different strains.

the 143 amplified PCR products, two allelic variants, with respect to size (2.2 and 1.5 kb), were observed. The 2.2 kb samples exhibited three different patterns with respect to *EcoR* I or *Pst* I digestion. The previously reported SKOR-67 type (2.2 kb) was cleaved by *EcoR* I into 989 bp and 1,300 bp, whereas SKOR-69 (1.5 kb) was not cleaved. Among the 2.2 kb products, one new variant (KPVMSP3a02-24) was first observed in 2002 and another (KPVMSP3a05-4) in 2005, which differed in sequence from the original SKOR-67 sequences of similar length. Among the new variants, two clear restriction patterns were produced by digestion with *Pst* I (Figure 1).

Two previously identified Korean alleles (SKOR-67 and SKOR-69) were identical with few frequency differences observed in 1996. The incidence of SKOR-69 rapidly decreased in more recently collected samples (Table 1), while new alleles KPVMSP3a02-24 and KPVMSP3a05-4 appeared and increased in frequency over time (Table 1).

There were four distinct PvMSP3a sequences: SKOR-67 ($N = 27$), SKOR-69 ($N = 17$), KPVMSP3a02-24 ($N = 6$), and KPVMSP3a05-4 ($N = 10$). The latter 16 new isolates were different from worldwide isolates. The nucleotide sequences of six new KPVMSP3a02-24 variants were closest to SKOR-69 in the N-terminal (100%) and in the first part of both Block I (98.7%) and Block II regions (99%) with three mutation sites; however, the second and third part of the Block I region was closest to the Ecuador and Thai-NYU isolates (99.8%) with one mutation site, whereas the fourth part of Block I was similar to IEC Brazil, India-NYU, and PNG isolates (96.6–98.9%) with one or three mutation sites (Figure 2). The 10 new KPVMSP3a05-04 sequences were identical to SKOR-69 in the N-terminal (100%) and first part of Block I (100%), whereas the second part of Block I was identical to the Ecuador and Thai NYU isolates (100%), and the third part of Block I was closest to the PNG and India-NYU isolates (98.9–99.5%). The nucleotide sequence of the fourth part of Block I in the new KPVMSP3a05-04 variant was different from sequences found elsewhere, whereas Block II was similar to SKOR-67 (98%).

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FIGURE 2. Alignment of the PvMSP-3 α amino acid sequences from Korean and other worldwide *P. vivax* isolates. The alignment of the sequences is made in reference to the Belem strain (AF093584). The amino acid positions are numbered at the top of the alignment, where the different regions of the gene are also indicated (see Figure 1). Dots and dashes represent identical residues and deletions, respectively. Block I is divided into four regions according to the sequence similarity to the variant Korean strains.

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