ALLELIC DIMORPHISM IN THE MEROZOITE SURFACE PROTEIN-3α IN KOREAN ISOLATES OF PLASMODIUM VIVAX

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Abstract. To study the genetic diversity of re-emerging Plasmodium vivax in the Republic of Korea, nucleotide sequence variations at the merozoite surface protein-3α (PvMSP-3α) locus were analyzed using 24 re-emerging isolates and 4 isolates from imported cases. Compared with the well known Belem strain (Brazil), a large number of amino acid substitutions, deletions, and insertions were found at the locus of the isolates examined. The Korean isolates were divided into two allelic types; type I (15 isolates), similar to the Belem strain, and type II (9), similar to the Chess strain (New Guinea). Isolates from imported cases were classified into three types; type III (1 from Malaysia), similar to type B from western Thailand, type IV (1 each from Indonesia and India), and type V (1 from Pakistan), both being new types. Our results have shown that the MSP-3α locus of re-emerging Korean P. vivax is dimorphic with two allelic types coexisting in the endemic area.

INTRODUCTION

Malaria is endemic in nearly 100 countries, with an affected population of approximately two billion people, and 500 million clinical cases occur every year. Among the four species of malaria parasite, the most prevalent and extensively studied is Plasmodium falciparum, followed by P. vivax. With regard to P. vivax, the estimated number of clinical cases is 70–80 million per year; however, the epidemiologic and public health importance has been underestimated, so research is required on this species.

In the Republic of Korea, P. vivax malaria was endemic until the 1970s; this indigenous malaria had not been reported since 1984. However, indigenous malaria reappeared in a soldier in 1993, and has subsequently become a serious public health threat in the Republic of Korea. The number of reported cases has increased from 107 in 1995 to 3,930 in 1998, and then slightly decreased to 1,140 in 2003, totaling 19,164 cases by the end of 2003.

Among the proteins of the erythrocytic stages in the life cycle of Plasmodium, the merozoite surface proteins (MSP), including MSP-1, MSP-2, and MSP-3, have been shown to be involved in protective mechanisms of the host and studied as potential targets for vaccine development. The MSP-3 of P. vivax (PvMSP-3) is a protein with a molecular weight ranging from 148 to 150 kD, an alanine-rich central domain, and a series of heptad repeats that were predicted to form a coiled-coil tertiary peptide structure. The PvMSP-3 gene family consists of three intra-specific genes, PvMSP-3α, PvMSP-3β, and PvMSP-3γ; these exhibited diversity when P. vivax isolates from diverse geographic localities and origins were compared. The deduced peptide sequences and structures, corresponding to the PvMSP-3α, PvMSP-3β and PvMSP-3γ, have similarities with the MSP-3 of P. falciparum ( PfMSP-3). PfMSP-3 is a target antigen in the mediation of antibody-dependent cellular immunity, which partially protects monkeys against a lethal P. falciparum infection, via immunization. Moreover, antibodies to PfMSP-3 were found to decrease parasitemia in immunocompromised mice infected with P. falciparum. Therefore, PfMSP-3 is considered as a P. falciparum vaccine candidate. Similarly, PvMSP-3, particularly PvMSP-3α, is regarded as a candidate for inclusion in a P. vivax vaccine. PvMSP-3α was also used as a polymorphic marker in epidemiologic studies of worldwide geographic strains and isolates using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. The length and sequence polymorphisms of PfMSP-3α were analyzed, and the basis for its use in the RFLP protocol has been reported. However, in the case of the re-emerging Korean P. vivax, no studies have been performed on the genetic diversity of PvMSP-3α. Therefore, the present study was performed to provide information on the polymorphism of the PvMSP-3α locus among the re-emerging Korean isolates.

MATERIALS AND METHODS

Source of P. vivax isolates. Blood samples were collected from patients with 28 parasite-proven infections. They included 24 cases infected with re-emerging Korean P. vivax diagnosed in 1996 (n = 1; patient code SKOR-95), 1998 (6; SKOR-54, 57, 61, 63, 65, 66), 1999 (10; SKOR-67, 68, 69, 72, 75, 76, 77, 78, 79, 80), and 2000 (7; SKOR-81, 82, 83, 86, 87, 89, 90), and 4 cases of imported P. vivax malaria, 1 each from India (INDI-96), Indonesia (INDO-94), Pakistan (PAKI-93), and Malaysia (MALA-84). The travel histories of the patients were obtained by a chart review and history taking, and were used to deduce the likely origins of the infections. All the patients had a febrile illness when their blood samples were collected. Informed consent of the patients was obtained prior to blood sampling. The study was reviewed and approved by the Ministry of Health and Welfare of the Republic of Korea.

Extraction of the genomic DNA. The whole blood of 28 patients was either directly frozen at −80°C or separated into packed cells and plasma, and then frozen at −80°C. The parasite DNA was extracted using the proteinase K digestion technique, followed by extraction with phenol-chloroform, or using the QIAamp DNA Mini Kit® (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. After precipitation with ethanol, the DNA was re-dissolved in TE buffer (10 mM EDTA, 10 mM Tris-HCl, pH 8.0) and stored at −20°C until used.
Sequencing of the PvMSP-3α gene. The primer sequences were derived from the MSP-3α gene of the Belem strain (Brazil) of *P. vivax* (GenBank accession number: AF093584). The DNA from each of the 28 patients was amplified by a PCR using oligonucleotide primers that allow amplification of the complete coding region: L1 (5′-CTATTGCACCCGACAGTCA-3′) and L2 (5′-CATCACCACCCATTTGTGCTA-3′). These primers bind at positions −72/+86 (L1/L2) of the Belem MSP-3α coding sequence. The primers were used at a final concentration of 0.1 μM in 100 μL of the reaction mixture (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP) containing 10 μL of DNA and 2.5 units of the *Taq* polymerase (Roche, Mannheim, Germany). The following thermal cycling conditions were used: L1/L2: 95°C for five minutes, then 32 cycles at 94°C for 30 seconds, 59°C for 40 seconds, and 72°C for 60 seconds, followed by a final five-minute extension step at 72°C in a DNA thermal cycler (GeneAmp PCR System 9600; PE Applied Biosystems, Foster City, CA). After gel purification of the amplified fragment using the QIAEX II gel extraction kit (Qiagen), part of the gene was re-amplified by repeating the primary PCR.

The DNA cycle sequencing was performed on 28 PCR-amplified DNA samples using the Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturer’s instructions. Each reaction contained 4 μL of the Terminator Ready Reaction Mixture, 2 μL of the sequencing primer (1 μM), and 4 μL of the ampiclon. The sense-stranded sequencing primers were F1, 5′-GTCAAAAGCAGTAGTG CCAA-3′; F2, 5′-CAACAGGG-TAAAAGCAGAAAT-3′; F3, 5′-AAGGCCGAAGTCTGAAC-3′; F4, 5′-CTCATCAAAAGTGTTTAAA-3′; F5, 5′-ATCGCAGCGAGGAAGGCG-3′; and F6, 5′-GACACTTACGATGATATTAA-3′. The anti-sense-stranded primers were R1, 5′-AATGTTAGCTGCTGGTGTTG-3′; R2, 5′-TGCCCTATTTTCCGTCCTT-3′; R3, 5′-TGCCCTCCG-CACCTCGGCG-3′; R4, 5′-ATCTCTACGCTATTTCCGCG-3′; R5, 5′-GTTTAATGTTCACTACGTTG-3′; and R6, 5′-TTTCTTCTTCGTTCTACGTG-3′. The samples were run in an automated sequencer (Models 373A and 377; PE Applied Biosystems) and then analyzed using the ABI PRISM™ software (PE Applied Biosystems).

Analysis of DNA and deduced amino acid sequences. The whole gene sequences of the PvMSP-3α of the re-emerging Korean isolates and the isolates from the imported cases were aligned and compared with the previously published gene sequences from various geographic localities. The amino acid and nucleotide sequence alignments were analyzed using Clustal X version 1.81. New nucleotide sequence data obtained in this study have been deposited in GenBank with the accession numbers AY266087 (SKOR-67), AY266088 (SKOR-69), AY266089 (MALA-84), AY266090 (PAKI-93), and AY266091 (INDI-94).

RESULTS

The deduced peptide sequences of the PvMSP-3α showed a large number of amino acid substitutions, insertions, and deletions compared with the well-known Belem strain. Sequence variation was pronounced at the coiled-coil heptad repeat region of the central alanine-rich domain (Figure 1). The extreme N-terminus was relatively well conserved, whereas the central C-terminus displayed some variability. In contrast, the N-terminal half of the alanine-rich domain was diverse, and the C-terminal half of the alanine-rich domain well conserved (Figure 2).

Five allelic types, i.e., types I, II, III, IV, and V, were identified in the PvMSP-3α of the 28 isolates examined (Table 1). The 24 Korean isolates were divided into two types, type I (15 isolates showing the identical sequences) represented by SKOR-67, and type II (9 isolates showing the identical sequences) represented by SKOR-69. The two types showed striking size variations, 2.5 kb and 1.7 kb, respectively, which depended on the deletions in the N-terminus of the central alanine-rich core region (Figure 1). The deduced amino acid sequences of the allelic type I (e.g., SKOR-67) were generally similar to that of the Belem strain, despite the presence of 39 substitutions and 8 insertions (K108→E115) (Figure 1). Allelic type II (e.g., SKOR-69) showed 32 substitutions and 257 deletions (G77→A100 and K278→K439, excluding common deletions at amino acid positions 183–185 and 244–257) when compared with the Belem strain; it was similar to the Chess strain (New Guinea) (Figure 1).

The sequences of the MSP-3α locus of the four isolates from the imported cases were distinguished from those of the Belem strain and the two Korean types. They could be designated as the allelic type III (MALA-84), IV (INDI-94 and INDI-96 showing the identical sequences), and V (PAKI-93) (Figure 1 and Table 1). In the alanine-rich domain, the MALA-84 showed 49 amino acid substitutions, 153 deletions (L515→A182, E186→Q247, and C258→A320), and 4 insertions (K1086→E111). The INDI-94 and INDI-96 showed 35 substitutions and 42 deletions (K263→A269 and K578→L512). The PAKI-93 showed 86 substitutions, 4 deletions (G97→A100), and 14 insertions (A244→K257) in the alanine-rich domain (Figure 1).

Based on the sequence data, diagrammatic representations of the full PvMSP-3α gene have been constructed for the five major allelic types (Figure 2). Allelic type I (~2.53 kb) included the Belem strain and 15 Korean isolates including SKOR-67, and allelic type II (~1.78 kb) included the Chess strain and 9 Korean isolates including SKOR-69. Allelic type III (~2.09 kb) was found in the MALA-84 isolate, allelic type IV (~2.42 kb) in the INDI-94 and INDI-96 isolates, and allelic type V (~2.56 kb) in the PAKI-93 isolate (Figure 2).

DISCUSSION

Re-emerging *P. vivax* malaria in the Republic of Korea showed characteristic epidemiologic and clinical features, e.g., a summer peak, a predominance of patients with long incubation periods up to 5–12 months, atypical fever intervals, a low incidence of anemia, a high incidence of thrombocytopenia, and frequent relapses. To understand the reasons responsible for these features and to provide control strategies including vaccine development, studies on the genetic characteristics of the re-emerging malaria were prerequisite.

Genetic studies of re-emerging *P. vivax* malaria have been performed on several parasitic proteins, including the circumsporozoite surface protein (CSP), apical membrane antigen-1 (AMA-1), MSP-1 and 18S ribosomal RNA. The CSP gene of the Korean isolates showed two haplotypes, i.e., SK-A and SK-B, which were similar to the Chinese isolate CH-5 and the North
Korean (NK) strain, respectively; both haplotypes could be classified as the East Asian group. The AMA-1 gene of the re-emerging Korean \textit{P. vivax} could also be classified into two allelic types, similar to the Chinese CH-5 and CH-10 isolates, respectively. With regard to the DBP locus, two types were also shown to coexist among the Korean isolates. However, these allelic types appeared as different combinations in each isolate, and as a whole, the isolates could not be divided into two or three big genotype groups.

MSP-3 is known to be another useful marker for genetic polymorphism of malaria parasites in endemic areas; thus, studies have been performed on this genetic locus. In addition to its epidemiologic significance, this locus is also known to be a potential candidate for vaccine development; the potential vaccines should concentrate on the C-terminus (the nucleotide sequence positions 1,300–2,058) of the alanine-rich domain and the acidic C-terminal region because this region is highly conserved across a range of geographically distinct \textit{P. vivax} isolates. No information was available on the genetic characteristics of \textit{PvMSP-3}/H9251 of the Korean isolates; thus, studies at this antigenic site were required.

In the present study, \textit{PvMSP-3}/H9251 of the re-emerging Korean isolates was classified into two distinct allelic types, I and II. The gene size of \textit{PvMSP-3}/H9251 of allelic type I was similar to that of the Belem strain, although at positions 97–116, their amino acid sequences were different: AAKPEAALEEQKEELQKEL in type I (SKOR-67) and GPNAEPNAEQI in the Belem strain (Figure 1). The gene size of \textit{PvMSP-3}/H9251 of type II was similar to those of the Chess isolate and Br69-1.7 (Brazil), although at positions 558–563 and 605–612, their amino acid sequences were different: MSELEK and TAANVVKD in type II (SKOR-69), LSKLEE and KEATAAKL in the Chess isolate, and LSKLEE and TAANVVKD in the Br69-1.7 isolate (Figure 1). The sequence homology between type I and Belem (93.0%), type I and Br69-2.4 (94.0%), type II and Chess (99.2%), and type II and Br69-1.7 (97.8%) were high.
allelic types IV and V are reported for the first time in the Plasmodium vivax.

Frequency of the Plasmodium vivax, which shows the conserved (white), polymorphic (black), truncated (wavy pattern), and insertion (shaded) regions. Five allelic types were discriminated on the bases of the size and portion of the truncated and insertion regions. The allelic types I and II of the Korean isolates are similar to the Belem and Chess strains, respectively. kb = kilobases. Numbers represent amino acid positions, which are based on the Belem strain sequence.

relatively high. However, both the allelic types I (SKOR-67) and II (SKOR-69) appeared to be different from the North Korean strain (the NK strain), particularly in the sequence of the coiled-coil heptad repeat region of the alanine-rich domain of the PvMSP-3α (Figure 1). The sequence similarity between the allelic type I and NK was 87.1%, and that between the allelic type II and NK was 90.7%. The allelic types I, II, and III in this study were similar to the allelic types A, C, and B, respectively, reported from western Thailand. The allelic types IV and V are reported for the first time in the present study.

The PvMSP-3α allelic types of the Korean isolates and the isolates from the imported cases were compared with the allelic types of the PvMSP-1 gene (Han ET and others, unpublished data); close correlations were shown between the two genetic loci (Table 1). All 15 Korean isolates having allelic type I for PvMSP-3α showed allelic type A for PvMSP-1 (Table 1). Seven of the nine isolates with allelic type II for PvMSP-3α were matched with allelic type B for PvMSP-1; the remaining two isolates showed different combinations of allelic type II for the PvMSP-3α and allelic type A for PvMSP-1, respectively (Table 1). In the isolates from the imported cases, allelic type III of the PvMSP-3α correlated with allelic type C of PvMSP-1, allelic type IV with allelic types D and E, and allelic type V with allelic type F (Table 1).

Nevertheless, it was difficult to determine the epidemiologic and clinical significance of each allelic type of the PvMSP-3α locus. The types did not clearly correlate with the two different incubation periods of the patients (short and long), which is well known in re-emerging Korean P. vivax malaria. Similarly, no direct relationships were shown between the allelic types of the CSP, DBP, AMA-1, and MSP-1 of the Korean isolates and the regularity of the fever intervals, the incidence of anemia or thrombocytopenia, or the frequency of relapses.

Without firm evidence, North Korea was suggested to be the origin of the reemerging P. vivax malaria in the Republic of Korea. Previous studies on the genetic characteristics of CSP and AMA-1 support this suggestion. In the present study, neither allelic types I and II of the re-emerging Korean isolates corresponded with the NK strain in terms of the sequence of the polymorphic region of the PvMSP-3α locus. However, it can be speculated that more than three allelic types coexist in North Korea. Conversely, the di-allelic nature of PvMSP-3α in the Korean isolates may suggest a likely origin of the re-emerging malaria confined to a small number of introductions from a limited geographic area of North Korea.

The polymorphic nature of the PvMSP-3α gene can be used as a marker in the discrimination of multiple infections in epidemiologic studies. In previous studies, the percentage of multiple infections was 23% in Papua New Guinea and 35.6% in Thailand. In our study, the PvMSP-3α gene showed the presence of five allelic types among the 24 Korean isolates and the 4 isolates from imported cases. However, by direct sequencing, no cases of mixed infections, with more than two allelic types, were observed. To detect any co-existing minor sequence variants, which may not be detected by direct sequencing, PCR fragments from five isolates each of allelic types I and II and one each of types III, IV, and V were cloned and sequenced, but no isolates showed such minor variants.

To conclusively determine the genetic diversity of re-emerging Korean P. vivax, the nucleotide sequences of the PvMSP-3α full-length gene were studied. The results showed that two allelic types, each having distinct sequences, coexist among the reemerging Korean isolates.

**Table 1**

<table>
<thead>
<tr>
<th>Allelic types in the antigen</th>
<th>No. of isolates (%)</th>
<th>Example of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MSP-3α</strong></td>
<td><strong>MSP-1</strong></td>
<td></td>
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<tr>
<td>Korean isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>A</td>
<td>15 (62.5)</td>
</tr>
<tr>
<td>II</td>
<td>A</td>
<td>2 (8.3)</td>
</tr>
<tr>
<td>II</td>
<td>B</td>
<td>7 (29.2)</td>
</tr>
<tr>
<td>No. of cases</td>
<td>24 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Isolates from imported cases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>C</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>IV</td>
<td>D</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>IV</td>
<td>E</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>V</td>
<td>F</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>No. of cases</td>
<td>4 (100.0)</td>
<td></td>
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</tbody>
</table>

* MSP = merozoite surface protein.
† Data from this study.
‡ Data from Han ET and others, unpublished data.

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