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The analysis of *Plasmodium vivax* Duffy receptor binding domain gene sequence from resurgent Korea isolates

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Abstract The Duffy binding domain gene structures of *Plasmodium vivax* facilitate the invasion of erythrocytes. Human erythrocytes that lack Duffy blood group antigens are resistant to invasion by *P. vivax*. We have sequenced the Duffy binding domain gene from eight *P. vivax* isolates collected from malaria cases in South Korea. When compared to isolates from other regions in the world, the amino acid sequences of the Korean isolates showed unique variations in region II. From 606 sequenced amino acids, 32 variations were found. Of these, three variations were regularly found in positions 424, 437 and 503 of the Sal-1 amino acid sequence. In region III, six isolates had a loss of the 30 bp (FAESTKSAE) insert. However, six isolates had 6 bp (SD) inserts at the end of region III. Two cases had a reverse pattern. Our results suggest that the *P. vivax* currently found in South Korea are unique when compared to other isolates and can be divided, by the analysis of their molecular structure, into two strains.

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Introduction

The manifestation of malaria in Korea has unusual clinical characteristics when compared to the expected disease process. Typically, the parasite infecting a person in Korea has a very long incubation period of between 230 and 300 days. This strain has been called the North Korean strain (Shute et al. 1977). South Korea (the Republic of Korea) became free of this strain of malaria after 1979 as a result of a national malaria eradication program with WHO assistance (Paik and van der Gugten 1966; Paik et al. 1988). Since the 1980s, only imported cases of malaria have occurred. South Korea has been believed to be malaria-free since 1979. In 1993, a case of indigenous *vivax* malaria was reported in South Korean soldiers (Chai et al. 1994). Since this event, the number of reported tertian malaria cases, with characteristics typical of the Korean strain of the past, have increased exponentially for each of the past 5 years. Tertian malaria has again become endemic in Korea (Lim et al. 1999).

The Duffy binding domain gene structures of *Plasmodium vivax* help bind the parasite to an erythrocyte. Human erythrocytes that lack Duffy blood group antigens are resistant to invasion by *P. vivax*. However, almost 100% of Koreans have either Duffy a or b antigen-positive blood (Choi et al. 1984). The Duffy binding domain gene is cystein rich within region II and demonstrates natural variation which is significant in polymorphism studies for both host susceptibility and for potential vaccine development (Tsuboi et al. 1994). The main goal of this study is to determine the molecular characteristics of specific polymorphic regions, specifically the Duffy binding protein domain, of South Korean isolates by using a molecular epidemiological method and comparing the results with isolates from other regions. Characterizing the genetic sequence of the Korean strain of *P. vivax* within region II and III would be useful in both understanding the mechanism of infection within the Korean population and in serving as a

foundation for the development of preventive medications and vaccines.

Materials and methods

Blood samples were collected from eight patients. These patients, all of whom gave informed consent, were diagnosed with *P. vivax* by microscopy during the years of 1996, 1997. They were inhabitants of the Kimpo, Pajoo and Yonchon regions of Korea and their clinical characteristics are summarized in Table 1. DNA was extracted from whole blood collected in EDTA tubes using InstaGene matrix kits (Biorad, Calif.). *P. vivax* genes were amplified by PCR using the following oligonucleotide primers. These amplified the polymorphic region of the Duffy binding protein (DBP) gene(739–2557, M61095,PFADUFR):

DBPf1: 5′-TGGGACTGTAACACTAAGAAGG-3′(1043, M61095, PFADUFR);

DBPf2: 5′-TGGGGAGGAAAAAGATG-3′(739, M61095, PFADUFR);

DBPr1: 5′-TTCATTCTCAAAAGCCACCTCG-3′(1840, M61095,PFADUFR);

DBPr2: 5′-GGAAAGGGGCAAGATAATGA-3′(2475, M61095, PFADUFR); and

DBPr3: 5′-AAGCTTCTAATCAACTGCATCAGTAG-TAGC-3′ (2557, M61095,PFADUFR).

Primers were used at a final concentration of 0.1 μM in a 100 μl reaction mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP) containing 10 μl of DNA and 2.5 units of AmpliTaq polymerase (Perkin Elmer, Norwalk, Conn.). Reaction mixtures were cycled 30 times: denaturation for 1 min at 95°C, annealing for 1 min at 53°C, and extension for 2 min at 72°C in a DNA thermal cycler (Perkin-Elmer model 9600). Various size PCR products of the DBP gene were purified by Wizard PCR Preps DNA Purification System (Promega, Madison, Wis.).

All DNA sequencing was performed in both directions for each PCR product using a dye termination cycle sequencing ready reaction kit (Applied Biosystems, Foster City, Calif.) on an automated sequencer (model 377, Perkin Elmer,). The GenBank accession numbers of the Korea isolates are AF220657–AF220668.

Alignment and comparison of the 245 amino acid region were facilitated by using various programs of the Lagergene (DNASTAR, Madison, Wis.). For phylogenetic analysis, a maximum parsimony algorithm, Phylogenetic Analysis Using Parsimony, version 3.1.1 (PAUP; Swofford) was used.

Results

The size of the sequenced nucleotide was approximately 1,818 bp within region II, a 5′ cystein rich region. Korean isolates had over 99.5% nucleotide sequence similarity which distinguished them from the strains from other regions. The greatest similarity to these strains was with Belem (97%), Sal-1(96%), several Columbia isolates (96%) and Papua New Guinea isolates (95%).

Specific amino acid sequence differences were found in the Korean isolates. From a total 606 amino acids, 32 variations were found when compared to other strains. Of these, three position variations in 424, 437, 503 of the Sal-1 amino acid sequences were unique. Specifically, the 424(leucine) position, 437(tryptophan) position, and the 503(isoleucine) position of the Sal-1 strains were replaced with isoleucine, arginine, and lysine respectively in the Korean strains (Fig. 1).

In the central third of the amino acid cysteine-rich region, frequent amino-acid substitutions were found. This is consistent with previous studies (Tsuboi et al. 1994).

Six isolates demonstrated a loss of the 30 bp (FAESTKSAE) insert. However, all isolates had the 6 bp (SD) insert at the end of region III. Regional differences were not found among Korean isolates. Two cases showed a reverse pattern. These had a 30 bp (FAESTKSAE) insert, but showed a loss of the 6 bp (SD) insert at the end of region III.

Phylogenetic analysis demonstrated that the eight Korean isolates scattered irregularly from other known isolates. Phylogenetically, the Korean isolates showed no distinctive features as the sequence data base worldwide was not large enough for effective comparisons to be made (figure not shown).

Discussion

Malaria occurs in many parts of the tropical world and in some of the subtropics. It can abruptly return to areas from which it had been eradicated, as has happened in Korea, as well as spread to new areas such as America and Eastern Europe(Brachman 1998; Aramburu et al. 1999; Velibekov 2000).

All malaria parasites undergo repeated cycles of asexual development in the blood phase of the life cycle. During the obligate process of invasion into a new erythrocyte, the merozoite must interact with a specific erythrocyte receptor. For *P.vivax* DBP is essential for

Table 1 Clinical and epidemiological characteristics of collected samples. *ND* No data

Patient	Date of		Incubation time (months)	Exposure area	Response to chloroquine therapy	Mean parasitemia/ul
	Onset	Exposure				
96–07	4 June 1996	April 1995	10	Yonchon	Good	320
96–08	11 June 1996	June 1995	12	Yonchon	Good	70
96–50	28 July 1996	May 1995	14	Pajoo	Good	1,680
96–74	25 September 1996	May 1995	16	Kimpo	Good	1,040
97–11	7 July 1997	February 1996	17	Pajoo	Good	522
97–13	7 July 1997	November 1995	20	Pajoo	Good	1,767
97–14	7 July 1997	January 1996	17	Pajoo	Good	ND
97–83	21 August 1997	August 1996	12	Pajoo	Good	1,200

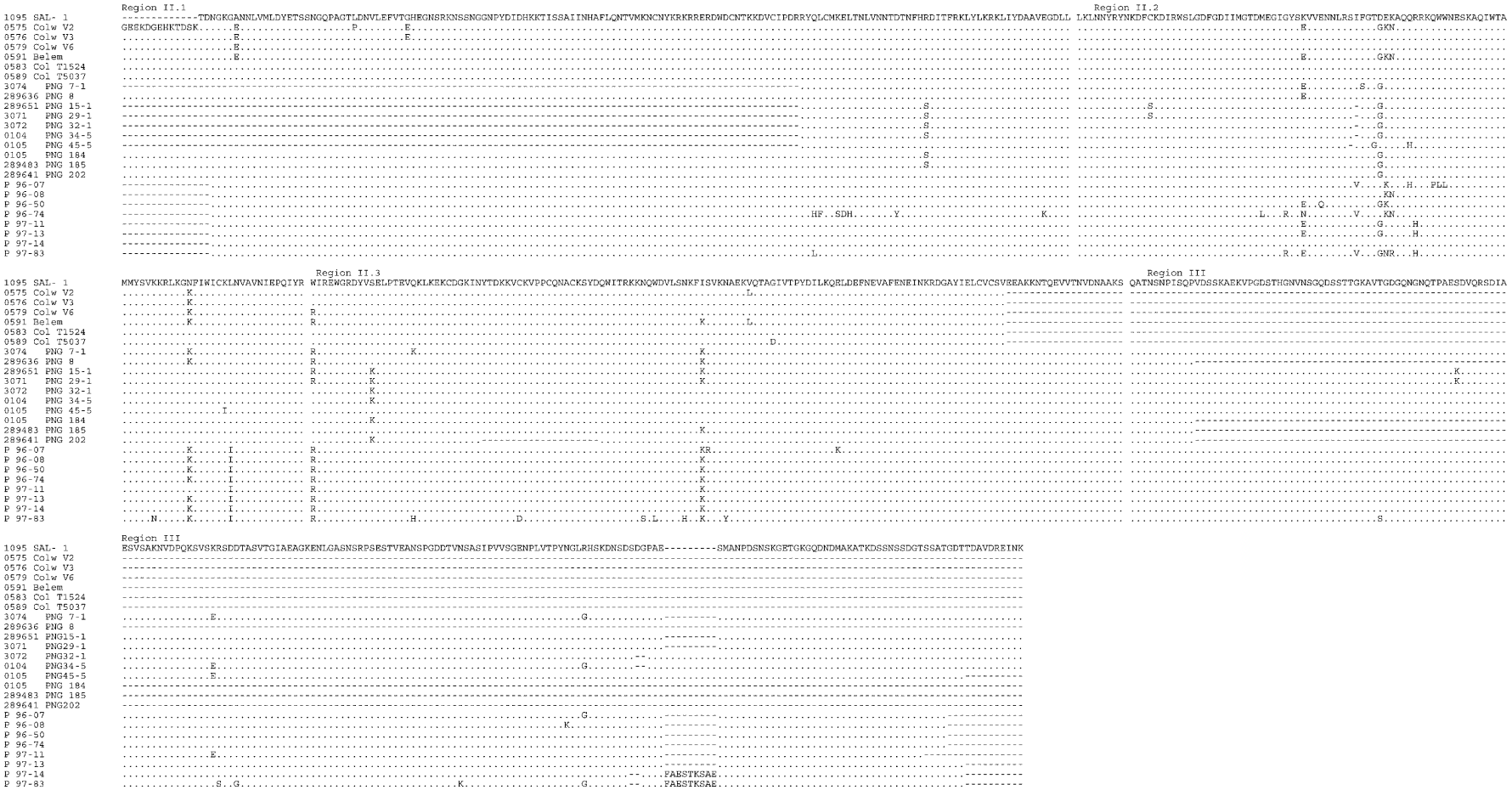

Fig. 1 Comparison of the amino acid sequence of the Duffy binding protein from Korean isolates with strains from various parts of the world. (*1* Sal-1; *2–4* Columbia wild V2,V3,V6; *5* Belem; *6*, *7* Columbia T1524, T5037; *8–19* PNG 7–1, 8, 15–1, 29–1, 32–1, 34–1, 45–1, 184, 185, 202; *20–27* DBP 96–7, 96–8, 96–50, 96–74, 97–11, 97–13, 97–14, 97–83,). *Dots* in amino acid sequences are identical to those of consensus sequences and deleted structures are shown by (-)

successful invasion. Because of this absolute requirement, DBP can potentially be used in the development of an asexual stage malaria vaccine. DBP is a type I membrane protein belonging to a family of erythrocyte binding proteins that has a stable molecular structure characterized by two cysteine-rich domains. One cysteine-rich domain, region II, was directly identified as the domain binding to the Duffy blood group antigen present on human erythrocytes (Chitnis and Miller 1994).

Amino acids of residues surrounding the receptor binding site are very variable. Changes in these residues alter the antibody epitope by immune selection (Tsuboi et al. 1994) with the central segment of the *P. vivax* region II being especially important for receptor recognition (Ranjan and Chintis 1999). Prior analysis has revealed that the polymorphism in *P.vivax* occurs at a regional level. Essentially all *P. vivax* DBP polymorphism occurs in region II. In Columbian isolates, 19 amino acid variations were found from a total 303 amino acid in the same region (Ampudia et al. 1996).

Korean strains also show frequent variation patterns in the same areas. Both the Villavicencio and Tumaco strains of the Columbia isolates show regular amino acid variations at positions 191, 192 and 417 of the Sal-1 strain. Most of the Korean isolates do not show amino acid variation at these positions, but do show regular amino acid variation at positions 424, 437 and 503 when compared to Sal-1 Papua New Guinea and Columbian isolates. These differences are unique within region II, and are not normally present in other strains. In region III, classified by the presence of 30 bp (FAESTKSAE) and 6 bp inserts, the Papua New Guinea strain has three variations (Fig. 1). However, only two types were found in Korean isolates.

After studying the regional variation found in Korean isolates, there is one unique molecular feature. The regular amino acid sequence pattern in Korean isolates suggests that South Korean *P. vivax* parasites are unique in origin and different from other *P. vivax* strains.

We carried out a phylogenetic analysis, but found that the eight Korean isolates scattered irregularly from other known isolates because fully matched, comparable sequence data are restricted to specific geographic areas (Papua New Guinea, Columbia, Brazil, El Salvador). We cannot obtain specific features of the Korean isolate compared to the other isolates using phylogenetic data,.

Understanding the genetic variation between different *Plasmodium* strains has helped us to pinpoint the strain of *P.vivax* responsible for the current malaria outbreak in Korea. The uniqueness of this strain justifies the development of preventive medications or vaccines that target specific, characteristic sites in region II as part of an ongoing malaria eradication effort in Korea.

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