PCR - AMPLIFICATION, SEQUENCING, AND COMPARISON OF THE *VAR*/PFEMP-1 GENE FROM THE BLOOD OF PATIENTS WITH FALCIPARUM MALARIA IN THE PHILIPPINES

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Abstract. The adhesion of *Plasmodium falciparum*-infected erythrocytes to vascular endothelium and to uninfected red blood cells (RBCs) plays a key role in the pathology of severe malaria. Adhesion is known to be mediated in part by the antigenically-variant erythrocyte membrane protein-1 (PfEMP-1), which is encoded by the *var*-gene family of *P. falciparum*. It has recently been reported that *in vitro* a single parasite simultaneously transcribes multiple *var*-genes but that, through a developmentally regulated process, the parasite selects only one PfEMP-1 that will to reach the surface of the host RBC. Were this to be true *in vivo*, one would expect a correlation between the type of *var*/PfEMP-1 that is expressed on the parasite-infected RBC and the severity of clinical disease.

In order to test this assumption, we determined the sequence of the *var*-gene that was expressed by the parasites in patients' blood samples. Seven blood samples were collected from patients with or without severe clinical symptoms (cerebral malaria): two samples were from patients diagnosed as having imported falciparum malaria at the International Medical Center of Japan (IMCJ); the five others were from patients of the Davao Regional Hospital in Davao, The Philippines. The parasites (ring stage) in the blood samples were cultured for 24 hours; the matured trophozoites, in which the *var*-gene selection had taken place, served as material for mRNA isolation. The cDNA corresponding to the Duffy-binding-like (DBL)-1 domain of the *var*-gene was amplified by RT-PCR, using a region-specific primer set. The amplified cDNAs were cloned into the plasmid vector; the resultant clones (32) were sequenced on both strands.

The results indicated that there was considerable diversity in the sequence of the DBL-1 domain among the clones, even among those from a single patient. In conclusion, it was difficult to demonstrate the correlation between the type of *var*-gene transcripts found in the RBCs of malaria patients and the severity of their symptoms.

INTRODUCTION

The protozoan parasite *P. falciparum* causes lethal malaria (WHO, 1990). The adhesion of erythrocytes infected with *P. falciparum* to vascular endothelium and to uninfected red blood cells (rosetting) is the main reason for the pathogenesis of severe malaria (MacPherson *et al,* 1985). The binding is mediated by the antigenically - variant erythrocyte membrane protein - 1 (PfEMP-1) that is encoded by members of the *P. falciparum var*-gene family (Magowan *et al,* 1988). Recent research has revealed that a single parasite simultaneously transcribes multiple *var*-genes but, through a developmentally regulated process, the parasite selects only one PfEMP-1 to reach the surface of the host cell (RBC) (Chen *et al,* 1998b). Fig 1 shows the activation and selection of *var*-genes. Several *var*-genes at different chromosomal loci are activated early in development. An undefined selection process results in only one *var* transcript (mRNA), which is produced as the parasite progresses from the ring to the trophozoite stage. The same *var*/PfEMP-1 is expressed by subsequent generations, although individual parasites may switch the previously expressed *var*-gene at a frequency of about 10^{-2} per generation and consequently express a different PfEMP-1 variant. In-spite of the presence of such a switching mechanism, the majority of the parasites will express a single PfEMP-1 in the patient. There may, therefore, be some correlation between the type of PfEMP-1 that is expressed on the host RBC and the severity of clinical disease. In order to test this assumption, we tried to determine the correlation between the type of *var*/PfEMP-1 found on parasite-infected RBCs (iRBC) of malaria patients and the severity of their disease.

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MATERIALS AND METHODS

Collection of blood samples

Heparinized blood was extracted from 2 patients (patients A and B) who had been diagnosed as having

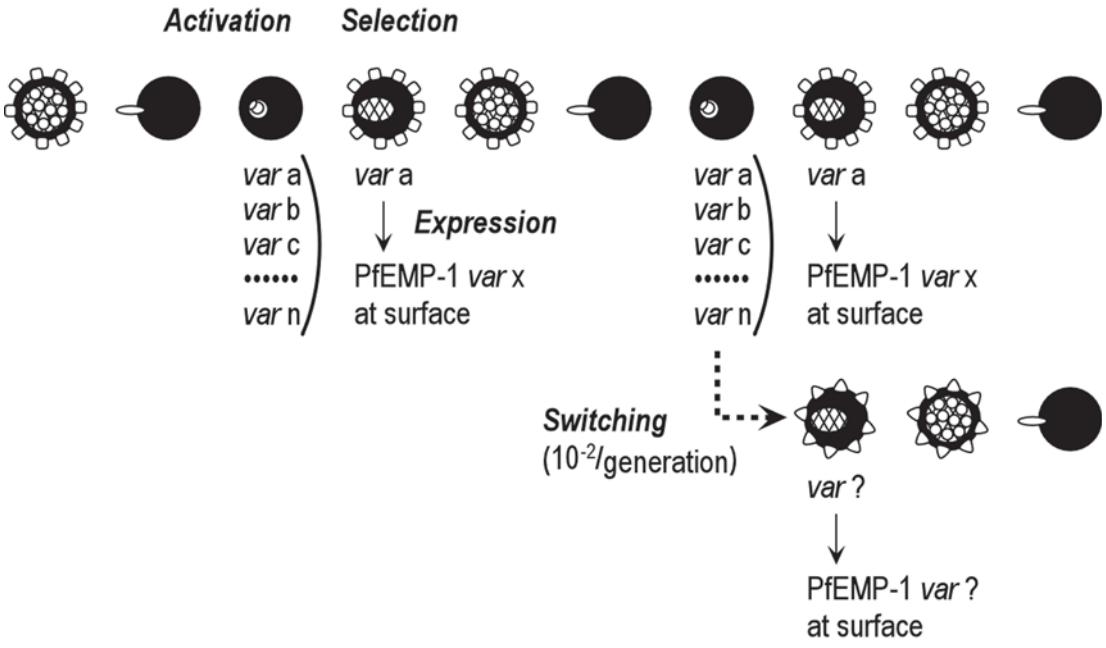

Fig 1- Activation, selection, and expression mechanisms of PfEMP-1: proposed by Chen *et al* (1988b).

imported-falciparum malaria at the International Medical Center of Japan, (IMCJ). EDTA-anticoagulated blood was extracted from 5 falciparum malaria patients (patients C to G) who had been admitted to the Davao Regional Hospital, the Philippines. Patients' details are summarized in Table 1. The aim of the study was explained to the patients by members of the medical staff; the patients gave their informed consent prior to plebotomy.

***In vitro* culture of blood samples**

The blood samples were cultured in a multi-gas incubator for 24 to 48 hours (Trager and Jensen, 1976). During this short-term culture, the ring form parasites in the blood developed into trophozoites.

Preparation of mRNA

mRNA was isolated from the blood samples that had been cultured for 24 hours with the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Japan, Tokyo). In order to eliminate the contaminated DNA, which may have served as a template for an undesirable amplification of *var*-gene from the parasite genome, the mRNA was treated once with RNase-free deoxyribonuclease (RT Grade; Nippon Gene, Tokyo) for 30 minutes at 37ºC before reverse transcription.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The *var*-gene was amplified from the mRNA sample by RT-PCR. Reverse transcription, a cDNA synthesis according to the mRNA template, was performed using the You-Prime First-Strand Beads System (Amersham Pharmacia Biotech Japan, Tokyo). The reaction continued for 1 hour at 37ºC. The synthesized cDNA then served as the template for the PCR amplification of the *var*-gene using specific oligonucleotide primers. The primers, DBL-1.1 and DBL-1.2, were designed as degenerate (mix) primers from short, conserved amino-acid sequences that flank relatively variable stretches (400-700 base pairs) in the DBL-1 domain of PfEMP-1 (MacPherson *et al*, 1985). The sequence of each oligonucleotide primer (20 mer) was as follows: DBL-1.1: 5´-GG(A/T) GC(A/T) TG(C/T) GC(A/T) CC(A/T) T(A/T)(C/T) (A/C)G-3´; DBL-1.2: 5´-A(A/G) (A/G) TA(C/T) TG(A/T) GG(A/T) AC(A/G) TA(A/G) TC-3´ (Magowan *et al*, 1988). The PCR reaction was performed in a 50µl reaction mixture containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$, 200µM each of the four dNTPs, 2µM each of the oligonucleotide primers and 4 to 8µl of the template. The template added to the reactions was either the cDNA or the mRNA before reverse transcription. The reaction mixture was subjected to

Table 1
Summary of patients details.

	History	Chief complaint	Diagnosis	Drugs administered before blood extraction
Patient A	Traveled in a malaria endemic area	Fever, behavioral change	Cerebral malaria	None
Patient B	Lived in a malaria endemic area	Fever	Uncomplicated malaria	None
Patient C	Lived in a malaria endemic area	Fever	Uncomplicated malaria	None
Patient D	Lived in a malaria endemic area	Fever, cough	Uncomplicated malaria	None
Patient E	Lived in a malaria endemic area	Fever	Uncomplicated malaria	None
Patient F	Lived in a malaria endemic area	Fever, behavioral change	Cerebral malaria	None
Patient G	Traveled in a malaria endemic area	Fever	Uncomplicated malaria	None

Two patients (patients A and B) were diagnosed as having imported falciparum malaria at the IMCJ; the other 5 were falciparum malaria patients (patients C to G) admitted to the Davao Regional Hospital.

35 cycles of amplification in a programable heating block (PE Biosystems Japan, Tokyo). The addition of *Taq* DNA polymerase (2.5 units, PE Biosystems Japan) was withheld until the reaction temperature reached 95ºC for the Hot-start protocol, in order to ensure the high specificity of the products. The program used was as follows: 95ºC, 5 minutes (Pre PCR); 95ºC, 30 seconds; 47ºC, 30 seconds and 65ºC, 2 minutes (35 cycles of PCR); 65ºC, 5 minutes (Post PCR). After amplification, the reaction mixture was electrophoresed on a 1.5% agarose gel with TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0). Gels were stained with ethidium bromide and the PCR products (amplified fragments) were visualized by UV light.

Prediction and analysis of amino acid sequences of the *var* transcripts

The PCR products of sizes 400 to 700 base pairs (bp) were excised from the gel and purified with a QIAQuick™ spin column (QIAGEN, Hilden, Germany). A part (2μl) of each purified product was subjected to cloning with the pCR®2.1 plasmid vector (Invitrogen, CA, USA). The recombinant plasmid was transformed with *Escherichia coli* INVaF' strain. The bacteria were plated on Luria-Bertani (LB) agar which contained ampicillin at 50μg/ml. Four to twelve of the colonies that emerged were randomly selected from each plate: these were grown overnight to saturate 3ml of LB liquid medium. Plasmid clones were purified from each bacterial culture with a Quantum Plasmid Miniprep Kit (Bio Rad Japan, Tokyo). The inserted DNA in the plasmid clones was sequenced on both strands using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) using M13 sequencing primers. The DNA sequence obtained was translated into an amino acid sequence using a genetic information processing program (GENETIX; Software Development Co, Ltd, Japan). Amino acid sequence data were aligned for comparison using a Clustal W multiple sequence alignment program (Thompson *et al,* 1994). Glycosaminoglycan (GAG) binding motifs (clusters of positively charged amino acid residues contained in the consensus sequences XBBXBX or XBBBXXBX) were searched for in the obtained sequences using the same software. The presence of GAG-binding motif suggests the involvement of the protein (DBL domain of the PfEMP-1) is the rosette formation of the iRBC.

RESULTS

The DNA sequences of the amplified *var* transcripts from the blood samples had a high degree of similarity (more than 50%) with those that had been

deposited as the DBL-1 domain of *var*-gene in the GenBank™ database (FCR3S1.2-var1: AF003473). The similarities were all high enough to suggest that the DNA sequences obtained in this study were certainly those of the *var* transcripts.

In order to investigate features of the PfEMP-1 protein, the DNA sequences were translated into amino acid sequences. Fig 2 shows the alignment of the predicted amino acid sequences of the DBL-1 domain of FCR3S1.2-var1 and the 32 sequences from the seven patients. Two to 12 clones of *var* transcripts were sequenced for each patient. The boxed amino acids were those identical to FCR3S1.2-var1. There was considerable diversity in the DBL-1 domain among the clones, even among those from a single patient. The greatest similarity was found between the amino acid sequence of FCR3S1.2-var1 and that of a patient sample (59.4%). Five patients (B, C, D, E and G) were classified as having uncomplicated falciparum malaria; the other two (Patients A and F) were diagnosed as having cerebral malaria (behavioral changes were observed). Although similarities in amino acid residues were found in several parts of the sequences, we could not find a distinctive sequence (motif) in the amino acid sequences for either complicated or uncomplicated group.

There were several (0 to 4) GAG-binding motifs found among the obtained DBL-1 sequences: they had

Fig 2- Predicted amino acid sequences of *var* transcripts of FCR3S1.2-var1 (GenBank™ accession # AF003473) and those from cultured patient's blood. Identical amino acid residues are boxed. GAG-binding motifs (Chen *et al*, 1998a) are indicated by underlining.

positional similarities between each sequence (Fig 2, underlined). However, the number of GAG-binding motifs in the DBL-1 domain, had no correlation to the severity of clinical illness. *Var* transcripts with four GAG-binding motifs were found in the patients with no complications, whereas *var* transcripts with no GAG-binding motif were found in the patients with cerebral malaria.

DISCUSSION

Cerebral malaria is the most serious complication of infection with *P. falciparum.* Cerebral malaria is due to the massive sequestration of infected and uninfected erythrocytes in the microvasculature of the brain. The virulence of the parasite is a function of its capacity to cause infected erythrocytes to adhere to endothelial cells (sequestration) and other erythrocytes (rosetting). The DBL-1 domain of PfEMP-1 was found to be a binding ligand to heparin sulfate on RBCs and was throught to be involved in rosetting (Chen *et al,* 1998a). The same author reported that parasites in the trophozoite stage select a *var*-gene and express only one PfEMP-1 to reach the surface of iRBCs (Chen *et al*, 1998b). Since rosetting is one of the key phenomena seen in the patients with severe malaria, there should be some correlation between the type of PfEMP-1 selected and the severity of malaria. In the present study, this hypothesis was investigated in malaria patients in the Philippines. The blood samples, that were originally ring stage parasites rich, were cultured for 24 hours in order to obtain the transcripts after the *var*-gene selection (that might directly relate their pathogenicity). The results of our DNA and amino acid sequence analyses, however, showed that there is considerable diversity in the DBL-1 domain among clones, even among those from a single patient. These patients might be simultaneously infected with parasites that express different PfEMP-1 variants (multi-clonal infection). The diversity in the DBL-1 domain in the parasite clone may be the result of the 'switching' of *var* transcripts. The selected *var*-gene type was shown to be switched at a frequency of about 10^{-2} per generation (Chen *et al,* 1998b). Vector mosquitos may also contribute to the diversity of DBL-1 by transmitting parasites with different PfEMP-1 variants from one patient to another. Consequently, parasites with different PfEMP-1 variants may be mixed within a single patient's blood. The expression of only one *var*/PfEMP-1, as shown in parasite culture *in vitro,* may be difficult to demonstrate in malaria patients.

Several (0-4) GAG-binding motifs were found in each amino acid sequence from the patients, which suggests the involvement of the protein (DBL domain of the pfEMP-1) in the rosette formation of the iRBC. Although further studies are required, our protocol could have amplified the rosette-relating *var* transcripts from the patients' samples. No correlation, however, was found between the number of GAG-binding motifs in the sequence and the patients' symptoms. Indeed, no clear differences were found in either the features (*ie*, distinctive sequence) or the structure (*ie*, number and position of GAG-binding motif) of the DBL-1 domain of the PfEMP-1 between the sequences from uncomplicated and cerebral malaria. In conclusion, this study did not demonstrate the correlation between the type of *var*-gene transcripts found in the iRBCs of malaria patients and the severity of the symptoms. This could be attributable to the small number of samples used. In order to find the pathology-associated motif of the PfEMP-1, further trials to amplify, clone and sequencing the *var* transcripts of the blood of malaria patients are needed.

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