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

Nozomu Ikenoue<sup>1</sup>, Shin-ichiro Kawazu<sup>2</sup>, Toshimitsu Hatabu<sup>2</sup>, Elena A Villacorte<sup>3</sup>, Pilarita T Rivera<sup>3</sup> and Shigeyuki Kano<sup>2</sup>

<sup>1</sup>Gunma University School of Health Sciences, Gunma, Japan; <sup>2</sup>Research Institute, International Medical Center of Japan, Tokyo, Japan; <sup>3</sup>College of Public Health, University of the Philippines, Manila, The Philippines

**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 vargenes 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<sup>-2</sup> per generation and consequently express a different PfEMP-1 variant. Inspite 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.

## MATERIALS AND METHODS

#### **Collection of blood samples**

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

Correspondence: Nozomu Ikenoue, Gunma University School of Health Sciences, Gunma, Japan.

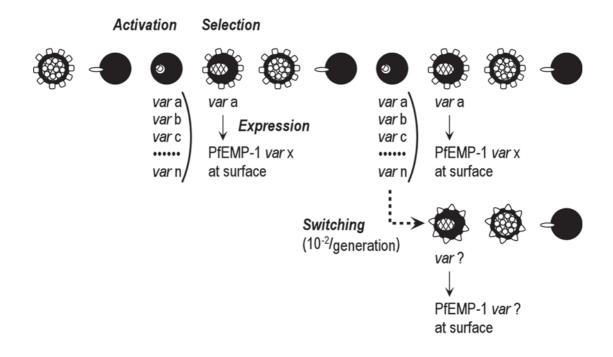


Fig 1- Activation, selection, and expression mechanisms of PfEMP-1: proposed by Chen et al (1988b).

imported-falciparum malaria at the International Medical Center of Japan, (IMCJ). EDTAanticoagulated 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 37°C 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 37°C. 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<sub>2</sub>, 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

	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	a None
Patient C	Lived in a malaria endemic area	Fever	Uncomplicated malaria	a None
Patient D	Lived in a malaria endemic area	Fever, cough	Uncomplicated malaria	a None
Patient E	Lived in a malaria endemic area	Fever	Uncomplicated malaria	a 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	a None

# Table 1 Summary of patients details.

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 95°C for the Hot-start protocol, in order to ensure the high specificity of the products. The program used was as follows: 95°C, 5 minutes (Pre PCR); 95°C, 30 seconds; 47°C, 30 seconds and 65°C, 2 minutes (35 cycles of PCR); 65°C, 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<sup>TM</sup> spin column (QIAGEN, Hilden, Germany). A part (2µl) of each purified product was subjected to cloning with the pCR<sup>®</sup>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 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<sup>™</sup> 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

each plate: these were grown overnight to saturate 3ml

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<sup>TM</sup> 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

FCR3S1, 2var-1		107
Patient A-01		107
	GÁCAP <u>PRELHL</u> CYONCEGNIDPAKITSTINLÍSUDÚLLAKKYGGIS ITADYPKYÖJAOYHADNPDFKTHICTULARSFADIGDI VRGKOLIFUGTNEEKKÁLEENIKK	
Patient A-02	BACAP <u>FRRISIO</u> TNLEOUDPDEVTTTDNLUVDVCLAAKHEGNSINTHYOKYDAOYASSASSSOICTMLARSFADIGDUTRGKDLYRONNKKDOVEKEGLETNLKT	106
Patient A-03	GACAP <u>YRRLHL</u> CHHNLEKMEANNYDSGKATHTLLAEVCMTAYHEGDSIKAHHEPHONKYGDSASOLCTVLACSFADIGDIIRGKDLYLQYDDEEKIKROQLEKNLKD	107
Patient A-04	GACAP <mark>YRRLHL</mark> CDHNLEN[ISDFDHINN  TLL ADVCLAAKFEGD SLEKHRAO YO SKNEDFKTHICTELARSFADI ADIVRGDNREKAKL <u>EKKLKE</u>	100
Patient A-05	GACAP <u>FRRUSV</u> CDYNLEKMGRTSTTKHDLLAEVCIGAYYEAESLINYRAOYDAEHHDTGFTTCTMLARSFADIGDIIRGKDLYLG- <u>DKKKKO</u> NGKKTETEREKL <u>EOKLKD</u>	109
Patient A-06	GACAPERRENE COMMERTED AND THE LADVIC A A KEEGD SEEK HAADY OSK NE DEKT HICTEL ARSEAD IAD I VRGODLYRODNEKT	100
Patient A-07	BACAP <u>YRRLHL</u> OHHNLETUETTSKTSTDTLLAEVCYAAKFEGES <u>LKKYHA</u> OHOVTYSDSOLCTELARSFADIGDIVRGKDPFYGNP-OEK <u>DORKKLEKNLKK</u>	101
Patient A-08	GACAPFRRLSVCDYNLEKMGTKKIDNTHNLULEVCLAAKHEGESLOGYYGKMDAOYHDFGSTICTVLARSFADIGDIITRGKDLYLGNPEEIKOROOLENKLKK	103
Patient A-09	GACAPERRINLETTIDTSTTHOLLLEVCMAAKYEGESLTRYHRD/QVXYRDSPSGCTVLARSFADIGDVRGRDLVRGND-KKKEKRDELDKKLKE	101
	BACAP FARLING ONLENISD FDHINNHT LAÖVCIAAK FEGÖSLEN HANOYÖSKNE DEKT HI ETELARSFADIÖD I VRGÖNEKT	100
Patient A-10	GACAP FREIT CONNECTION CONTRACTOR CONT	
Patient A-11	GACAPFRRLYLCDYNLETINKTTSTPTNKLLLEV GMAAKYEGDLUKTRHLEHOLRNNDSAYOLCTVLARSFADIODURGKOLYLGNP-EENKORDEI <u>EKNLKD</u>	103
Patient A-12	GACAP <u>ERRLHL</u> CDH <u>NLENUSDFDHINNHTLLAD</u> YCLAAKFEGDSLEKHRAOYOSKNEDFKTHICTELARSFADIADIVRGRDLYRGDNREKTKL <u>EKKLKE</u>	100
Patient B-01	GACAP <mark>YRRLHL</mark> CDYNESIDTTSTTHTLLAEVCMAAKYEGOSIKTHYTPHOOKYKDTGT-ASELCTVLARSFADIGDIVRGRDLYLGNKKKNO-AREKEKL <u>ENKLKE</u>	105
Patient B-02	GACAP <u>FRRLHL</u> COYNLESIDTTSTTHTLLAEVCMAAKYEGOSIKTHYTPHOOKYKDTGT-ASELCTVLARSFADIGDIVRGRDLYLGNKKKNO-AREKEKLENKLKE	105
Patient C-01	GACAPYRRONLCOKNLEYLDNTNTDDTDDLLGNVLVTAKYEGESIV <u>AKHPHK</u> DNSOVCTALARSFADIGDIVRGROMFLPNKDDKVOKGLOV	92
Patient C-02	GACAPFRRÜFLCDHHLSYMKDDKIDNTHNLLLEVQYÄAKYEGOSEV <u>ekhkemiten</u> pdsoictalarsfadigdi∏rgKdüfiGYnekdreekko∐odnlKd	102
Patient C-03	GACAPFRRLFLCDHHLSYMKDDKIDNTHNLLLEVCYAAKYEGOSLVEKHKEYITENPDSOICTALARSFADIGDIIRGKDLFIGYNEKDREEKKOLODNLKD	102
Patient D-01	GACAPYRRLHLOHHNLETTTOTNNYNSSNAKHNLLLEV CWAAYYEGDLTNTHYTRYOO IYGNSPYOLCTVLARSFAD I GDI VRGKDLFRGNN-EEKKORKELDKKLKD	106
Patient D-02	GACAP <mark>YRELEL</mark> CHINLETIOTNYNSSNAKHNLLLEVCMAAYYEGDLINTHYTRYOGIYGNSPYQLCTVLARSFADIGDIVRGKDLFRGNN- <u>EEKKOR</u> KEL <u>DKKLKD</u>	106
Patient D-02	GACAPTRELSTORE UND THAT THE LIGHT AND	104
Patient D-04	GACAP <u>FRRLHL</u> CHHNLETTETNNYNSSNAKHNLLYDYCMAAKYEGDSTKTPYPKHKOTNNDSASOLCTVLARSFADIGDIYRGRDLFYGNP-OEKDORKKLDDKLKE	106
Patient E-01	GACAP <u>YRRLHM</u> CDRNLEEIIYPDKIKKTENLLVDVLLAAKHEGEMIITKNLKEVDNANYESKICTALARSFADIGDITRGKDHFL <u>GHKQRK</u> INLENRLQT	98
Patient E-02	GACAP <u>FREHE</u> CDYNLENISDFNNINNDTLLVDVOLAAKYEGDSIKTHHPOYKLTYDBSOICTVLARSFADIGDIVRGKDLYRRD <u>NK-KNKL</u> EKNLKT	97
Patient E-03	GACAP <u>YRRRHI</u> CDLNLEHIDVHNVONIHDLLGNVLVTAKYEGESIVEKHPNRGSSEVCTALARSFADIGDITRGRDWFLPNKDDXVOKGLRE	92
Patient E-04	GACAP <u>YRREHE</u> CDYNLENISDFNNINNDTLLVDVCLAAKYEGDSIKTHHPOYKLTYDDSQICTVLARSFADIGDIVRGKDLYRRD <u>NK-KNKL</u> EKNLKT	97
Patient F-01	gacap <u>frrlflod</u> ohlahmeddkinntdnlllevslaakvegesitknypkdgnnkegictalarsfadigditrgkdlflqytkdekkekvoknlkr	100
Patient F-02	GACAP <u>YRRIHL</u> CHHNLESTIOTNNYNSSNAKHKLULEVÖMAAKYEGOSIKTYYTOHOLTNNDSASQLCTVLARSFADIGDIVRGKDLFYGNT-OESAORKKLEONLKE	106
Patient F-03	GACAR YRRONI CIRRIEVI INKNTENTHOU I GNUL VTAKYEGEG UVKNHPNKGGGEVCTAL ARGEAD I GD I VRGKONGKONGKON	92
Patient F-04	BACATIMATUCUNAL ETIMAT AT EN INCLUMENTAL LA CUNAL LA CUERTA LA CUERTA DE LA CUERTA DE LA CUERTA DE LA CUERTA DE BACAP <u>ERRIAL</u> ONNE ETIMAT TA TENTAL LA CUERTA LA CUERTA DE LA	103
Patient G-01	GACAP <u>FREHE</u> COOHLEHIKHOEITRHNLLADVCLAAKFEGESLKNYRAOYOKTNSDVNINICTMLARSFADIGDI <u>IKRKRS</u> LSW <u>KRKT</u>	97
Patient G-02	GACAPYRRLHLCHHNLETINNTTSTTSDTLLAEVCYAAKFEGESITGRYPOHOOTNEGSQLCTMLARSFADIGDIVRGKDPFYGNP-GEKEKRKQLDKKLKT	101
Patient G-03	GACAP <mark>FRELHL</mark> CDOHLEHIKHDEITRHNLLADVCLAAKFEGESLKNYRAOYOKTNSDVNINICTMLARSFADIGDITRGKDLYLGDKKEKQKLODNLKD	99
FCR3S1. 2var-1	FKKT  HDN⊑KDKEAGKRYNGDED-PNF YK⊑REDWWTÄNRE TVWGÄMTGSKELDNS-SYFRATGNDTG0-GPS0THNKGRGDKDKGANAGKPKAGDGDVTI↓VPTYFDYVPDY	215
FCR3S1.2var-1 Patient A-01	[[FKKT HDN]_KDKEAOKRYNGDED-PNFYKLREDWWTANRE[TVWGAW]TGSKELDNS-SYFRATGNDTGO-GPS0[THNKGRGDKDKGANAGKPKAGDGDVTIVPTYFDYVPOY LFKKLYKELTKEEENGTAIKSRYTKDD-PDFY0]REDWWALNRDOWWKAITGEAOGNTYFHATGNGGKSTPNKGRGDKTSDGNGDVNIVPTYFDYVPOY	215 204
Patient A-01	ĹFKKĹYEELTKEEENGTAIKSRYTKDD-PĎFYÖLREDWWAĽNRDÖVWKATTCEROGNTYFHATCNGGKSSTPNKCRCDDKTSDGNGDVNIVPTYFDYVPOY	204
Patient A-01 Patient A-02 Patient A-03	LFKKLİYELİTKEEENGTAIKSRYTKDD-DÖFYÖLREDWIMALINRÖÖVIKÄATITERAGGTRYFFMÄTÖKGGKSSİPMİXCRÖDKTSDGNGDVNI (VPTYFDYVPOY 1FGNI YELİTKKRRIGO-LERRYGODG-ONFYOLREDWIMYÄNRITVIKAITICMÄQGFMYFRPTÖSREPLSODKOROVNLGDOVNI (VP 1FGKI HDALLEENRTNGOLEERCEGBEA-NÖFKI KERDWI I ANRATITÜKEAITIC ÖVSSGNKYFRATÖGSISPS-SPSMARDKOROKDEKFTKE	204 196 209
Patient A-01 Patient A-02 Patient A-03 Patient A-04	LFKKLŸEŁITKEEENGTAIKSRYTKOD-PÖFŸÖLREDWWALNRÖQVWKAITCERÄOGNTYFHATCINGGRSSTPNKCRCIODKTSDGNGDVNIVPTYFDYVPOY 1FGHTYEŁI <u>TKKRRNG</u> O-LEARYGODG-ONFYOLREDWWYÄNRHTYWKAITCINÄOGOWYFRPTCISRFDISODKCRCIVNLGDDYFTNFDYPPOY 1FGKIHDKLLEENRTNGOIEERCEGDEA-NDFFKLREDWWIANRHTYWKAITCONKSGNKYFRATGG-DST-SPSMARDKCRCIKDEKFTKEIV-TDAVPTYFDYVPOY 1FGTIVDKLNGKENYKDES-RNYFRGIERDWWIANRHTYWKAITCONKGOTYFRNTCISGEKOSNITTACKORONNODTDAVPTYFDYPOY	204 196 209 188
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05	LFKKLÜVELLTKEEENGTAIKSRYTKDD-DÖFYÖLREDWINALDNRÖÖWIKÄLT CERAGGTYFFKÄTCIGGGKSSTÜPUKCROD KTSDGNGDVNINPTYFDYVPOY TFGKTYELTKKRNGGO-IEARYGODG-ONFYLGEDWINYÄNRHTYWIKAITCINÄGGFMYFKPTCSRKSSTÜPUKCROVNLGDVPTNFDYVPOY IFGKTHDKLLEENKTNGOIEERCEGDEA-NDFFKIREDWINIANRATTWEÄNTGOVSG-GNKYFRÄTCIG-DST-SPSMARDORCKDEKFTKEDVPTYFDYVPOY IFGKTYDULNGALOARYKDES-RNYFDIREDWINJANRETYWKÄITCIDÄAGCHYFRNTCIGSEKGSATTOKCROPNGNDDVPTPDYPOY IFGKTSVISVOTSGRTKVALOARYDDT-NKFKERLEDWINTANNTWIKÄNTGOVSKLKD-NRYFRPTCIGSEKGSATTOKCROPNGNDDVPTPDYPOY	204 196 209 188 211
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06	LFKKLİYELİTKEEENGTAIKSRYTKÖD-ÖĞFYÖLREDWIMALDRÖÖVIKÄTİ TÇEROGNTYFFMATCINGGKSSİİPMACRCİDDKTSDGNGDVNI VPTYFDYVPOY İFGKIİNBALLEENKINGILERCEGBA-NDFFKI.REDWI INARKIİ I CANAGGFMYFFRATCISRPLSODCRCİVNI.GD I FGKIİNBALLEENKINGILERCEGBA-NDFFKI.REDWI INARKIİ I CANAGGINYFFRATCISABLSAİİTGKCRCİNDD I FGI I YDMLMGI	204 196 209 188 211 188
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-07	LFKK[VELTKEEENGTAIKSRYTKDD-DÖFYÖLREDWINALTNRÖÖWIKÄTTÖERAGGTKYTKPTÄÄTÖKGGKSST[PNIKCRÓDDKTSDGNGDVNIVPTYFDYVPDY  TGGHTYELTKKERNGO-LERKYGDG-ONFYOLREDWINALANTITUKAITONAGGFWYTKPTCSGSSESDOKCRÓVNLGDENVPTKPTDYVPDY  TGGITYDNLNGVPTKPTKDE-A-NDFFKLREDWINIANRAHTIMEAITODXKSGNKYFRATGG-DST-SPSMARDKCRÖKDEKFTKEVPTKP  TGGITYDNLNGKENTKDE-A-NDFFKLREDWINIANRAHTIMEAITODXKSGNKYFRATGG-DST-SPSMARDKCRÖKDEKFTKEVPTKP  TGGITYDNLNGKENTKDE-A-NDFFKLREDWINIANRAHTIVKANITODXAGFNYFRPTGSDLKOSATTOKCRÖKDEKFTKEVPTKPTDYPDY  TGGITYDNLNG	204 196 209 188 211 188 206
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-07 Patient A-08	LFKKLÜVELLTKEEENGGUVIN VETVEDOFVÖL REDWIN LÜNRÖÖWIKÄLT CERGGKSSTPHIK CRODKTSDGNGDVNI VPTYPDYVPOY TFGKT VELTKKRNG	204 196 209 188 211 188 206 201
Patient A-01 Patient A-02 Patient A-03 Patient A-03 Patient A-04 Patient A-06 Patient A-06 Patient A-08 Patient A-09	LFKKLÜVELLTKEEENGGUVIN VETVEDOFVÖL REDWIN LÜNRÖÖWIKÄLT CERGGKSSTPHIK CRODKTSDGNGDVNI VPTYPDYVPOY TFGKT VELTKKRNG	204 196 209 188 211 188 206 201 199
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-07 Patient A-08 Patient A-10	LFKKLVYELITKEEENGGDVNIVPTYFDYPOPY [FGKIYEELITKKRNGG	204 196 209 188 211 188 206 201 199 188
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-07 Patient A-08 Patient A-09 Patient A-10 Patient A-11	LFKKLVELTKEEERGENTAIKSRYTKDD-DÖFYÖLREDWINALDRÖÖWIKÄTTÖERGGTKYFRPTÖKGGKSSTPUKCRÖDKTSDGHGDVNIVPTYDVYDOY IFGKTIVELTKKERMGO-LERCEGDEA-NDFFKLREDWINARRITWEKAITOGAGFWYFRPTÖKSG-SDOKCRÖVNLGDVPTRPTÖYDVYDOY IFGKTIVDNLNGVPTRPTÖKGGOST-SPSMARDKCRCKDEKFTKE	204 196 209 188 211 188 206 201 199 188 204
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-07 Patient A-08 Patient A-09 Patient A-10 Patient A-11 Patient A-12	LFIKKLİVELİTKEEENGKISRYTİKDD-DÖFYÖLREDWINALİNRÖQWIKAI'TICENGGKISTİPHİKICİGGOLKSOĞUDKISDGMOPTINPTYPOY IFIGKIYELİTKERRIG	204 196 209 188 211 188 206 201 199 188 204 188
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-07 Patient A-07 Patient A-08 Patient A-10 Patient A-11 Patient A-12 Patient B-01	LFKKLVELTKEERNG	204 196 209 188 211 188 206 201 199 188 204 188 204 188 194
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-05 Patient A-07 Patient A-08 Patient A-09 Patient A-10 Patient A-11 Patient A-12 Patient A-12 Patient B-01 Patient B-02	LFKKLVELITKEEENG	204 196 209 188 211 188 206 201 199 188 204 188 194 194
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-07 Patient A-08 Patient A-09 Patient A-10 Patient A-11 Patient A-12 Patient A-12 Patient B-01 Patient B-02 Patient C-01	LFKKLVELITKEEENG	204 196 209 188 211 188 206 201 199 188 204 188 204 188 194 194 178
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-05 Patient A-07 Patient A-09 Patient A-10 Patient A-12 Patient A-12 Patient A-12 Patient B-02 Patient C-01 Patient C-02	LFKKÜVELTKEEENG	204 196 209 188 211 188 206 201 199 188 204 188 204 188 194 194 194 178 189
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-06 Patient A-07 Patient A-09 Patient A-10 Patient A-11 Patient A-12 Patient A-12 Patient B-01 Patient C-01 Patient C-03	LFKKLÜVELITKEEENG	204 196 209 188 211 188 201 199 188 201 199 188 204 188 194 194 178 189 189
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-07 Patient A-07 Patient A-09 Patient A-09 Patient A-09 Patient A-11 Patient A-12 Patient B-02 Patient B-02 Patient B-02 Patient C-02 Patient C-02 Patient C-03 Patient D-01	LFKKLVELTKEEENG	204 196 209 188 211 188 206 201 199 188 204 188 204 188 194 178 189 189 189
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-06 Patient A-07 Patient A-09 Patient A-10 Patient A-11 Patient A-12 Patient A-12 Patient B-01 Patient C-01 Patient C-03	LFKK[VELITKEEENG	204 196 209 188 211 188 201 199 188 201 199 188 204 188 194 194 178 189 189
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-07 Patient A-07 Patient A-09 Patient A-09 Patient A-09 Patient A-11 Patient A-12 Patient B-02 Patient B-02 Patient B-02 Patient C-02 Patient C-02 Patient C-03 Patient D-01	L FKKL VELTKEEENG	204 196 209 188 211 188 206 201 199 188 204 188 204 188 194 178 189 189 189
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-06 Patient A-07 Patient A-09 Patient A-10 Patient A-11 Patient A-11 Patient A-12 Patient B-02 Patient B-02 Patient C-03 Patient C-03 Patient C-03 Patient C-03 Patient C-03 Patient D-01 Patient D-02	L FKKL VELTKEERNO	204 196 209 188 211 188 201 199 188 204 188 194 194 194 178 189 189 189 197
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-07 Patient A-07 Patient A-07 Patient A-07 Patient A-09 Patient A-10 Patient A-11 Patient B-01 Patient B-02 Patient C-03 Patient C-03 Patient C-03 Patient C-03 Patient D-01 Patient D-03	L FKKL VELTKEERNO	204 196 209 188 211 188 201 199 188 201 199 188 194 194 194 194 194 194 194 194 195 189 189 189 197 209
Patient A-01 Patient A-02 Patient A-03 Patient A-05 Patient A-05 Patient A-06 Patient A-07 Patient A-07 Patient A-07 Patient A-09 Patient A-09 Patient A-10 Patient A-11 Patient B-01 Patient B-01 Patient B-01 Patient C-01 Patient D-01 Patient D-03 Patient D-03 Patient D-03 Patient D-04 Patient D-04 Patient D-04 Patient D-04	L FKKL VELTKEERNO	204 196 209 188 211 188 206 201 199 188 204 188 204 188 194 194 194 194 194 194 194 194 197 209 203 209 203 207 209
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-06 Patient A-07 Patient A-07 Patient A-09 Patient A-10 Patient A-10 Patient A-12 Patient A-12 Patient B-02 Patient B-02 Patient C-02 Patient C-03 Patient D-04 Patient D-04 Patient D-04 Patient D-04 Patient D-04 Patient D-04 Patient D-04 Patient D-04 Patient D-04 Patient D-04 Patient D-04	L FKKL VELTKEERNO	204 196 209 188 211 188 206 201 199 188 204 188 194 194 178 189 189 189 197 209 203 178 88 186
Patient A-01 Patient A-02 Patient A-03 Patient A-05 Patient A-05 Patient A-06 Patient A-07 Patient A-07 Patient A-07 Patient A-09 Patient A-10 Patient A-11 Patient B-01 Patient B-02 Patient B-01 Patient C-01 Patient C-03 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient D-04 Patient E-01 Patient E-03	L FKKL VELTKEERNO	204 196 209 188 211 188 206 201 199 188 204 194 194 194 188 194 194 197 209 189 189 189 189 197 203 178 186 177
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-06 Patient A-07 Patient A-07 Patient A-09 Patient A-10 Patient A-11 Patient A-12 Patient B-01 Patient B-02 Patient B-02 Patient C-02 Patient C-02 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient E-04	L FKKL VELTKEERNO	204 196 209 188 211 188 206 201 199 188 204 188 194 178 189 197 197 197 209 209 209 209 203 178 186 177 186
Patient A-01 Patient A-02 Patient A-03 Patient A-05 Patient A-05 Patient A-06 Patient A-07 Patient A-07 Patient A-07 Patient A-09 Patient A-10 Patient A-10 Patient A-11 Patient B-02 Patient B-02 Patient B-01 Patient C-01 Patient C-03 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient D-04 Patient E-01 Patient E-03 Patient E-03 Patient E-03 Patient E-03 Patient E-03 Patient E-03 Patient E-03	L FKKL VELTKEERNO	204 196 209 188 211 188 201 199 188 204 188 204 194 194 194 194 194 194 197 209 189 189 189 189 189 187 197 203 203 178 186 203 203 203 203 203 203 203 203 203 203
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-07 Patient A-07 Patient A-07 Patient A-09 Patient A-10 Patient A-11 Patient A-11 Patient B-02 Patient B-02 Patient B-02 Patient C-02 Patient C-02 Patient D-03 Patient D-03 Patient D-03 Patient E-02 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient F-02	L FKKL VELTKEERNG	204 196 209 188 211 188 206 201 199 188 199 188 194 178 189 197 209 203 178 189 197 209 209 203 178 186 186 186
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-07 Patient A-07 Patient A-09 Patient A-09 Patient A-09 Patient A-10 Patient A-11 Patient B-02 Patient B-02 Patient C-01 Patient C-03 Patient C-03 Patient C-03 Patient D-04 Patient E-03 Patient E-03 Patient E-03 Patient E-03 Patient E-03 Patient E-04 Patient E-03 Patient E-03 Patient E-04 Patient E-03 Patient E-04 Patient E-03 Patient E-03 Patient E-03 Patient E-03 Patient E-03 Patient E-03	L FKKL VELTKEERNO	204 196 209 188 211 188 201 199 188 204 194 194 194 194 194 197 209 203 178 189 203 178 189 197 197 209 203 178 6 186 202 203 186 186 201 203 186 203 203 203 203 203 203 203 203 203 203
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-05 Patient A-07 Patient A-07 Patient A-07 Patient A-09 Patient A-09 Patient A-11 Patient A-11 Patient B-02 Patient B-02 Patient B-02 Patient C-02 Patient C-02 Patient C-02 Patient D-03 Patient D-04 Patient D-04 Patient E-04	L FKKL VELTKEEENG	204 196 209 188 201 199 188 201 199 188 204 188 188 204 188 188 194 194 197 197 197 197 209 209 209 209 209 209 209 209 209 209
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-06 Patient A-07 Patient A-07 Patient A-09 Patient A-10 Patient A-10 Patient A-12 Patient A-12 Patient B-01 Patient B-02 Patient C-02 Patient D-02 Patient D-03 Patient D-03 Patient D-04 Patient E-03 Patient E-03 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-03 Patient E-04 Patie	L FKKL VELTKEERNO	204 196 209 188 201 198 201 199 188 204 188 204 188 194 194 194 197 197 209 203 178 186 186 203 1777 186 186 202 181 206
Patient A-01 Patient A-02 Patient A-03 Patient A-05 Patient A-05 Patient A-06 Patient A-07 Patient A-07 Patient A-07 Patient A-07 Patient A-09 Patient A-09 Patient A-11 Patient B-01 Patient B-02 Patient B-02 Patient C-03 Patient C-03 Patient C-03 Patient C-03 Patient C-03 Patient D-04 Patient D-04 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient D-03 Patient E-03 Patient E-03 Patient E-03 Patient E-03 Patient E-03 Patient F-04 Patient F-04 Patient F-04 Patient F-04 Patient F-04 Patient G-02	L FKKL VELTKEERNG	204 196 209 188 201 199 188 201 199 188 204 188 188 194 194 194 197 209 203 178 189 197 209 209 209 209 209 209 209 209 209 209
Patient A-01 Patient A-02 Patient A-03 Patient A-04 Patient A-05 Patient A-06 Patient A-06 Patient A-07 Patient A-07 Patient A-09 Patient A-10 Patient A-10 Patient A-12 Patient A-12 Patient B-01 Patient B-02 Patient C-02 Patient D-02 Patient D-03 Patient D-03 Patient D-04 Patient E-03 Patient E-03 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-04 Patient E-03 Patient E-04 Patie	L FKKL VELTKEERNO	204 196 209 188 201 198 201 199 188 204 188 204 188 194 194 194 197 197 209 203 178 186 186 203 1777 186 186 202 181 206

Fig 2- Predicted amino acid sequences of *var* transcripts of FCR3S1.2-var1 (GenBank<sup>™</sup> 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.

## ACKNOWLEDGEMENTS

This study was supported in part by a Grant for International Health Cooperation Research (10A-4 and 13C-5) from the Ministry of Health, Labor and Welfare, Japan. We would like to express our gratitude to the staff of the Davao Regional Hospital, Mindanao, The Philippines, for their help with sample collection. We would also like to thank Prof Kumiko Sato, Gunma University School of Health Sciences, Japan, for his valuable support.

#### REFERENCES

- Chen Q, Barragan A, Fernandez V, *et al.* Identification of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite *P. falciparum. J Exp Med* 1998a;187:15-23.
- Chen Q, Fernandez V, Sundström A, *et al.* Developmental selection of *var* gene expression in *Plasmodium falciparum*. *Nature* 1998b;394: 392-95.
- MacPherson GG, Warrell MJ, White NJ, Looareesuwan S, Warrell DA. Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *Am J Pathol* 1985;119:385-401.
- Magowan C, Wollish W, Anderson L, Leech J. Cytoadherence by *Plasmodium falciparum*infected erythrocytes is correlated with the expression of a family of variable proteins on

infected erythrocytes. J Exp Med 1988;168:1307-20.

Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976;193:673-5.

Thompson, JD, Higgins DG, Gibson TJ, Clustal, W.

Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673-80.

WHO. Severe and complicated malaria. 2nd ed. *Trans R Soc Trop Med Hyg* 1990.