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IDENTIFICATION OF A DOMINANT AND CYTOADHERENT *VAR* GENE EXPRESSED IN *PLASMODIUM FALCIPARUM* ITG PARASITES

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Abstract

Var gene is a multicopy family gene and encodes the antigenic protein, *Plasmodium falciparum* infected-erythrocytes membrane protein-1 (PfEMP-1). Here we identified expressed *var* gene transcripts and their cytoadherent characteristics. Random cloning and sequencing analysis of DBL-alpha domain of *var* gene transcripts expressed in matured parasites revealed that approximately half of all cDNA clones were derived from a single gene, *var*-1/ItG. Immunofluorescence assay (IFA) showed that approximately 40% of ItG parasites expressed PfEMP-1 encoded by *var*-1/ItG-1 (PfEMP-1/ItG-1). Parasitized RBCs (PRBCs) selected for binding to C32 cells (ItG/C32), where the PfEMP-1/ItG-1-positive PRBC population increased to 78%, showed an increased cytoadhesion to C32 cells (241 PRBCs per 300 C32 cells) compared to originally cultured PRBCs (9 PRBCs per 300 C32 cells). To evaluate the contribution of PfEMP-1/ItG-1 in C32 adherence, PfEMP-1/ItG-1-enriched fraction (ItG/8A) was prepared by limiting dilution. In ItG/8A fraction, 83 % of the PRBCs were PfEMP-1/ItG-1 positive and only *var*-1/ItG was detected in the sequence analysis. PRBCs in ItG/8A fraction also showed higher cytoadhesion to C32 cells (153 PRBCs bound per 300 C32 cells). These results demonstrated that PfEMP-1/ItG-1 is expressed on ItG parasite-infected RBCs and contributes to the cytoadherent phenotype of ItG parasites to C32 cells.

Key Word: *Plasmodium falciparum*-infected erythrocyte membrane protein-1 (PfEMP-1), cytoadhesion, *var* gene,

Introduction

Human malaria is a significant infectious disease caused by four protozoan species of the genus *Plasmodium*. Among them, *Plasmodium falciparum* is responsible for the severe malaria, including cerebral malaria. Sequestration is thought to be central pathological feature in the severe malaria due to adhesion of parasitized-red blood cells (PRBCs) to endothelium of host tissue [12].

Sequestration is mainly mediated by *P. falciparum*-infected erythrocyte membrane protein-1 (PfEMP-1) encoded by the large multigene family, *var* [2,25,27]. The cytoadherent characteristics of PfEMP-1 is determined by the expression of *var* genes and by the binding ability to several receptors on the surface of endothelial cells, including CD36 [18], intercellular adhesion molecule 1 (ICAM-1) [6], vascular cell adhesion molecule 1 (VCAM-1) [17], thrombospondin (TSP) [20], P-selectin [4], and CD31 [4]. In placental malaria, chondroitin sulfate A (CSA) plays a major role in the cytoadherence [9]. The binding domain of PfEMP-1 for these host cell surface molecules have been investigated, revealing that Duffy

binding-like (DBL)-1 domain was a binding region for heparan sulfate [1], gamma type DBL was for CSA [7], DBL-beta-C2 tandem was for ICAM-1 [26], and cystein-rich interdomain region-1 alpha (CIDR1-alpha) was for CD36 [3], respectively. Although it had been studied on the interaction of PfEMP-1 with their single receptor molecules, the studies on the binding properties of PRBCs in the presence of multiple receptors was a little.

P. falciparum ItG parasites, which derived from Brazilian isolate, *P. falciparum* It (Ituxi084), are cytoadherent population selected with its cytoadherence to an amelanotic melonoma line cells, C32 cells [30]. C32 cells had been used as *in vitro* models for sequestration because of the expression of various surface molecules in similar composition to endothelial cells [24]. Therefore, it is expectative that the binding properties of ItG parasites could reflect that of PRBCs in patients in the presence of multiple cellular receptors for PRBCs.

In this study, the expression pattern of *var* gene transcripts in mature ItG parasites selected with high cytoadherent ability to C32 cells were examined. In addition, the cytoadherent characteristics of the major PfEMP-1 of ItG parasites were determined.

Materials and methods

Parasites and cells: *P. falciaprum* ItG parasites were maintained in culture with daily change of medium as described by Trager and Jensen [28] with human group O RBC. ICAM-1-transfected CHO cells (CHO/ICAM-1) was obtained from American Type Cell Collection (Cell No. CRL-2093, Virginia, USA), and CD36-transfected chinese hamster ovary (CHO) cells (CHO/CD36) was kindly gifted from Dr Minami T (Yokohama City University, Japan). These cells and C32 melenoma cells were maintained in RPMI 1640 (Invitrogen, Tokyo, Japan) supplemented with 10 % fetal bovine serum (FBS). Nontransfected CHO-K1 cells were obtained from RIKEN Cell Bank (Cell No. RCB0285, Ibaragi-ken, Japan) and cultured in HamF12 Nutrient Medium (SIGMA, Tokyo, Japan) supplemented with 10 % FBS and 0.02 M L-gultamine.

RT-PCR, cloning and sequencing analysis of var gene expressed in ItG parasites: Trophozoite- and schizont- stage parasites collected by magnetic cell sorter (MACS, Milteny Biotec Gmbt, Gladbach, Germany) [13] was ruptured with 0.2 % sodium chloride solution and followed by the standard acid guanidinium thiocyanate-phenol-chloroform extraction method. Using RNA PCR kit (AMV) ver 1.1 (TaKaRa, Tokyo, Japan), 1 μg total RNA was reverse-transcribed and DBL-alpha domain of *var* gene was amplified. Oligonucleotide primers were based on the sequence alignment described by Su X et al. [2]: prvar-2; 5'-CCA (A/T)C(G/T) (G/T)A(A/G) (A/G)AA TTG (A/T)GG-3', prvar-3F; 5'-CCA T(A/T)(C/T) AGA CGA TTA CAT (C/G)TA TG-3'. Reverse-Transcribing condition was: 10 min at 30^0C and 1 hr at 42^0C followed by 5 min at 99^0C. The cDNA was treated for 5 min at 94^0C followed by 40 cycles of 30 sec at 94^0C, 20 sec at 40^0C, and 1min at 65^0C. Amplified cDNA was ligated to pCR2.1 (Invitrogen) or pBluescript KS (+) plasmid vectors (Strategene, La Jolla, CA). Randomly selected cDNA clones were sequenced by BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan). FASTA research was performed on DDBJ web site (DNA Data Bank of Japan, National Institute of Genetics, Japan).

Immunofluorescence assay with antiserum specific for predominant PfEMP-1: An oligopeptide based on the amino acid sequence specific for predominant *var* transcript was synthesized. Five BALB/c male mice

(Nihon CLEA, Tokyo, Japan) were immunized with 20 μg of KLH-conjugated peptide (SIGMA GENOSYS, Japan, Tokyo) mixed with Freund's complete adujavant (DIFCO LABORATORIES, Detroit, MI) and boosted with incomplete adjuvant. Immunofluorescence assay (IFA) was performed with 10 μl of PRBC. PRBCs were fixed with 1 % paraformaldehyde (PFA) (Wako Pure Chemical Industries, Tokyo, Japan) for 10 to 15 min at 4^0C and incubated with the antiserum at a dilution of 1:100 in PBS(-) for 1 hr at 4^0C and washed for three times with PBS wash. PRBCs were incubated with FITC-conjugated AffiniPure goat anti-mouse IgG and IgM (H+L) (Jackson ImmunoResarch, West Grove, PA) at a dilution of 1:50 for 1 hr at 4^0C and were stained with 0.1 μg /ml of 4',6-diamidino-2-phenylindole (DAPI) for 5 to 10 min at 4^0C. PRBCs were suspended in 2.3 % 1,4-diazabicyclo-2,2,2-octane (DABCO) in PBS (-) to inhibit from discoloring and were observed with microscopy. Expression rate of the predominat *var* gene was calculated as the proportion of FITC-positive PRBCs in 100 PRBCs with more than two DAPI-positive nuclei.

Selection of PRBC with C32 cells and cytoadherence assay: Selection on C32 cells and cytoadhesion assay of ItG parasites were performed following the methods described by Marsh K et al. [10] with a little modification. Five mililiter of trophozoite-rich PRBCs suspension having at least 3% parasitemia at 2.5% hematocrit were co-incubated with 5 x 10^5 C32 cells per 25 cm^2 culture flask at 37^0C for 90 min with agitation at 15 min interval. After co-incubation, unbound PRBCs or normal RBCs were removed by washing with culture medium. Bound parasites were maintained by reinfecting to new normal human erythrocytes at 5% hematocrit. This procedure was repeated eight times. To measure the binding ability to C32 cells and CD36, 500 μl of parasites suspension were also added to 2 x10^4 C32 or CD36/CHO cells seeded onto coverslips. The coverslips were washed by PBS, fixed with methanol, and stained with Giemsa's solution. The cytoadherence was evaluated by the number of bound PRBC per 300 target cells.

Limiting dilution for isolation of PRBC: To fractionate PRBC population presenting the predominant *var* gene, ItG parasites were limiting-diluted. Ring stage PRBCs were negatively separated from crude culture with MACS and diluted at the rate of 0.3 PRBC per well and cultured in a 96-well plate. Wells were stained with Giemsa's solution to detect PRBCs. From the PRBC-positive well, PRBCs were transferred into 6-well plate and cultured. Each parasite-positive wells were examined for the expression of a dominant PfEMP-1 by IFA.

Results

Var gene transcripts in *P. falciparum* ItG parasites

A total of 23 cDNA clones were randomly selected, sequenced, and aligned. They were classified into one major group and four minor groups (Table 1). The major group was designated as *var*-1/ItG, in which 13 clones were included. The other four groups, *var*-2/ItG to *var*-5/ItG, included a few cDNA clones. The nucleotide sequences of these clones within each group were almost same except for several mutations. Since the positions of mutations within the groups were random, they were considered as PCR errors. Amino acid sequences of representative clones within each group were aligned in Fig. 1. FASTA research on the DBL-alpha domain of *var*-1/ItG to *var*-5/ItG showed that *var*-1/ItG and *var*-3/ItG were identical to that of A4tresVAR (accession no. AF193424) and ItG var (accession no. U31083), respectively.

Expression of PfEMP-1 encoded by the major *var* gene

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                10        20        30        40        50        60        70        80        90        100
var-1/ItG       PYRRLHVCDRNLEQIDPA-KITTT--HNLLVDVLLAAKHEGESIIDNYPSDHHNKE------GICTALARSFADIGDIIRGKDLYRGNKQEKD---KLQD
var-2/ItG       PYRRLHVCDRNLEEIYP-DKITNT--NNLLVDVLLAAKYEGESIRNEYDQKEDDYKL-----GLCTALARSFADIGDIIRGKDLYRRDSRTD----KLEE
var-3/ItG       PYRRLHVCSHNLESIQTNNYDSSKAKHNLLAEVCYAAKFEGESIVKNYEQLGHHTTE-----GICTALARSFADIGDIIRGKDLYLGNPQESARRKQLED
var-4/ItG       PYRRLHVCDQHLSHMNAGKTNTTD---NLLLEVCLAAKYEGESLVEKHKEFEKTHNDSN----ICTILARSFADIGDIIRGKDLYRGGGRGRK---QLEE
var-5/ItG       PYRRLHVCVRNLENISALDKINKDT---LLADVCLAALHEGQSITQDYPKYQAQYASSVSPSQICTMLARSFADIGDIIRGKDLYRGNNGKD----KLEE
                110       120       130       140       150       160       170       180       190       200
var-1/ItG       QLKKYFKELENNLES-------EAKNYYNDDTK-KFFKLREDWWNANRLDIWKAIICTAPGTAQYFRNACGGEHTMTYGQCRCVNG-EPPTNFDYIPQFLRW
var-2/ItG       NLKVIFGNIYKELTATSGKN-VALRDRYQKDGP-DYYQLREDWWEVNRQQVWNAITCKAEQNDKYFREKNSNGNTCTVNKCKCATG-DVLTNFDYVPQFLRW
var-3/ItG       NLRKIFEKIYKELTSSRNGKTNGAEERYKDGSG-NYYKLREDWWNANRLDIWKAMICKAPGNAQYFRNTCSNGEKPTGEKCQCIDG-TVPTNLDYVPQFLRW
var-4/ItG       NLQKIFGNIYNELT-TSGKNVDKAKERYKGDADNNYFQLREDWWDANRHTVWEAITCKAEQNNKYFRDACSTG-TPTHEKCRCVNRADVPTYFDYVPQFFRW
var-5/ItG       NLKRIFQQIHKEVTSTSCKNKDALKTRYQNDTD-NYFQLREDWWNANRKMVWYAITCGAGQIGKYFRNACSNNTTETDKKCRCAIG-TVPTYFDYVPQFLRW
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Fig.1 Alignment of amino acid sequences of *var* cDNA clones derived from ItG strain

The positions and sequences of the primers used for RT-PCR analysis are indicated in lowercase letters. Box shows amino acid sequence specific for *var*-1/ItG. Dashed lines represent gaps.

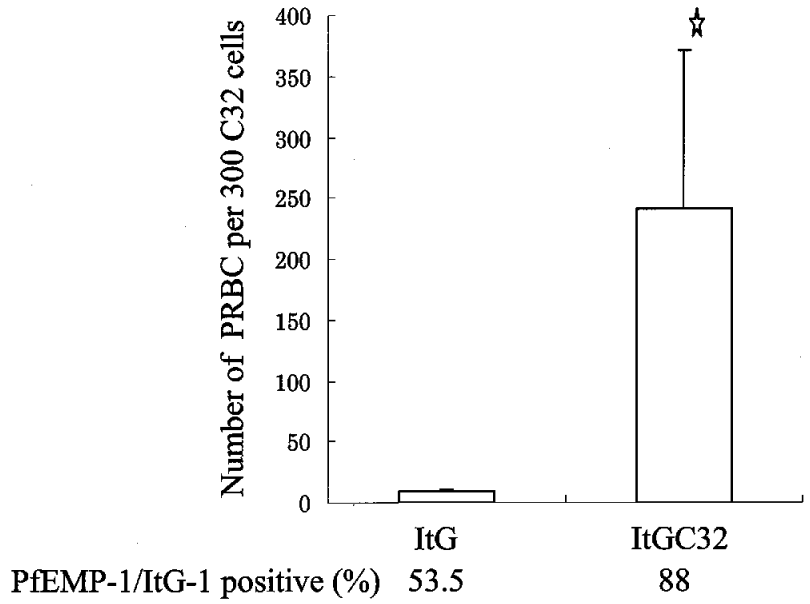

Fig.2 Selection of *P. falciparum* ItG parasites to C32 cells

P. falciparum ItG parasites were selected for cytoadherence to C32 cells and the expression of the PfEMP-1 encoded by a predominant *var* gene (PfEMP-1/ItG-1) was detected by IFA using anti-PfEMP-1/ItG antiserum and pre-serum as described in Materials and Methods. The cytoadherent ability of PRBCs after selection is shown as the number of PRBCs per 300 C32 cells. Lower panel shows the percentage of PfEMP-1/ItG-1-positive PRBCs in ItG or ItG/C32. $P < 0.05$ compared to ItG parasites before selection.

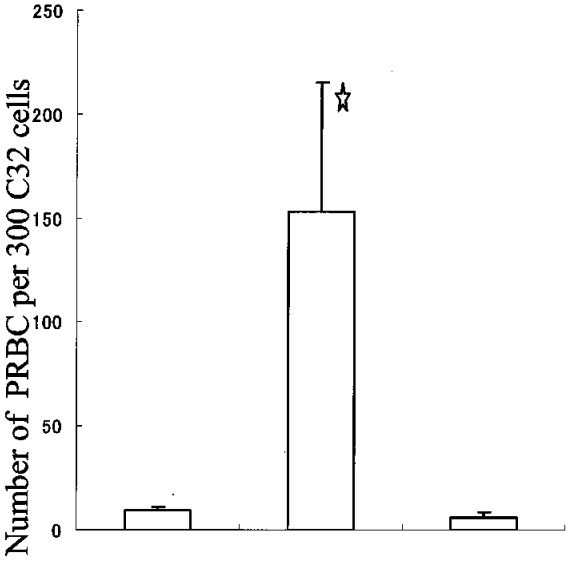

Fig.3 PfEMP-1/ItG-1 expression and the C32 cells cytoadherence

To enrich PfEMP-1/ItG-1 expressing PRBCs, ItG parasites were fractionated by limiting dilution. IFA was performed and PfEMP-1/ItG-1-positive and -negative PRBC populations, ItG/8A and ItG/7B, respectively were picked up. The percentage of PfEMP-1/ItG-1-positive PRBCs in each population is shown under the graph. The cytoadherent ability of each population to C32 cells, which was described as the number of bound PRBCs per 300 C32 cells, was tested. ☆$P < 0.05$ compared to ItG.

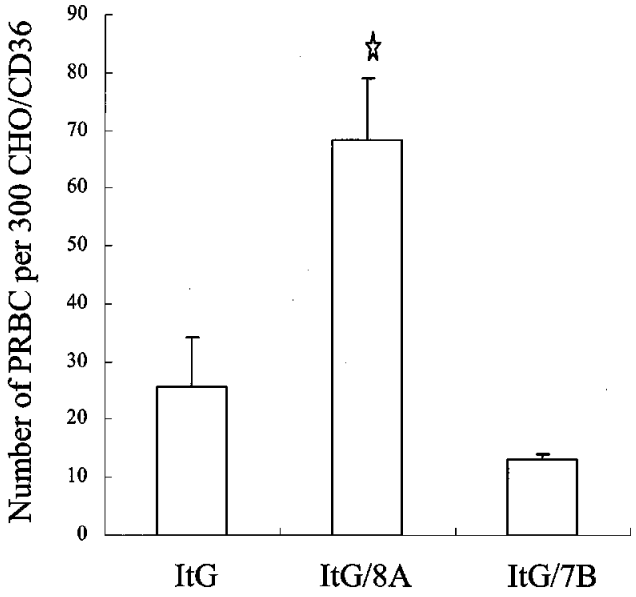

Fig.4 Binding ability of PfEMP-1/ItG-1 to CD36

The binding ability of ItG/8A and ItG/7B to human CD36, which was described as the number of bound PRBCs per 300 CD36/CHO, was tested. ☆$P < 0.05$ compared to ItG.

In order to determine the ratio of the PRBC positive for the major PfEMP-1 encoded by *var*-1/ItG (PfEMP-1/ItG-1), antiserum against PfEMP-1/ItG-1 were generated. Five BALB/c mice were immunized with KLH-conjugated synthetic peptide based on the amino acid sequence specific for DBL-alpha domain of PfEMP-1/ItG-1 (Fig.1). *P. falciparum* ItG parasites was examined by IFA using the antiserum. The signal was detected at the PRBCs surface and more intensively in the parasitophorous vacuole membrane in 53.5 ±1.1 % of *P. falciparum* ItG parasites before any selection (data not shown).

Table 1 *var* cDNA clones expressed in *P. falciparum* ItG strain ItG/8A and ItG/7B

	number of cDNA clone		
Gene	ItG (23)†	ItG/8A (23)†	ItG/7B (26)†
var-1/ItG	13	24	1
var-2/ItG	4	0	2
var-3/ItG	3	0	1
var-4/ItG	2	0	0
var-5/ItG	1	0	1
var-8/ItG	0	0	21

†value of total number of sequenced cDNA was shown in bracket.

The cytoadherent phenotype and expression of var-1/ItG

PfEMP-1/ItG-1 expression was examined in the *P. falciparum* ItG parasites selected by panning PRBCs on C32 cells eight times (ItG/C32). While 9 bound PRBCs per 300 C32 cells were observed in *P. falciparum* ItG parasites before panning, 241 bound PRBCs per 300 C32 cells were counted in ItG/C32 parasites (Fig.2). IFA showed that 88 % of ItG/C32 parasites were positive for PfEMP-1/ItG-1, which was 1.6 times higher than that of ItG parasites before selection (53.5 %).

In order to isolate PRBC presenting PfEMP-1/ItG-1, *P. falciparum* ItG parasites were limiting-diluted, fractionated PfEMP-1/ItG-1-positive or negative PRBCs populations and tested to the cytoadhesion assay. In the 24 examined wells, 6 wells were parasite-positive, and IFA was examined 12 of them, resulting that PRBCs in 1 of 12 wells, subline ItG/8A, was PfEMP-1/ItG-1-positive and 4 of them wells were negative parasites. Both parasites were examined the binding ability to C32 cells. In PfEMP-1/ItG-1-positive fraction (ItG/8A), which included 83 % of PfEMP-1/ItG-1-positive PRBCs, 153 PRBCs attached per 300 C32 cells (Fig.3) and 68 PRBCs per 300 CD36/CHO (Fig.4), respectively. On the other hand, one of PfEMP-1/ItG-1-negative fraction (ItG/7B), of which 3.5 % of PRBCs were PfEMP-1/ItG-1-positive, 6 PRBCs attached per 300 C32 cells and 13 PRBCs bound per CD36/CHO.

To ensure PfEMP-1/ItG-1 expression of ItG/8A parasites, the *var* gene usage of ItG/8A was analyzed by RT-PCR-cloning of DBL-alpha domain. All of total 24 cDNA clones were identical to that of *var*-1/ItG, except for a few PCR errors.

Discussion

Cytoadherence of mature PRBCs to vascular endothelium of several organs, which is called as sequestration, plays a major role in the pathology of severe malaria. It is important to understand how the PRBCs interact with their host receptors. We studied here the expression pattern of PfEMP-1 encoding *var* genes in ItG parasites and demonstrated that the predominant PfEMP-1, PfEMP-1/ItG-, was functionally selected and contributed to C32 cytoadherence of ItG parasites.

Var is a multicopy gene of approximately 50 to 100 copies per haploid. While many different *var* genes are transcribed at the ring stage, one or two of them will be transcribed at mature stage, which defines the cytoadherent phenotype [8, 23]. In this study, *var* transcripts corresponding to 5 *var* genes were isolated from ItG parasites at the mature stage. That may result from many reasons, such as ring-stage parasite contamination [23], co-existance of PRBC expressing another cytoadherent PfEMP-1, and co-expression of PfEMP-1/ItG-1 and other PfEMP-1 [15]. However, no contamination in the *var* transcript profile of ItG/8A (table 1) and others (data not shown), isolated from matured PRBC with the same procedures, denies the first possibility. ItG parasites should have many C32 adherent *var* genes other than Var-1/ItG, and some ItG parasite may express those genes by chance. Alternatively, as shown in recently study by R. Noviyanti et al. [15], multiple *var* gene transcripts may be expressed in a single matured PRBC selected to C32 cells. However, these *var* genes should also be expressed after C32 selection. Single var-1/ItG expression in ItG/C32 (data not shown) and ItG/8A, which was positively selected for PfEMP-1/ItG-1 expression by limiting dilution (Table 1), also denies the second and third possibilities, respectively. A *var* gene is registered in NCBI database (accession no. U31083) which has the same DBL-alpha sequence as our *var*-3/ItG clone (Fig. 1). It is possible that the registered *var* gene is identical to *var*-3/ItG and is one of cytoadherent var genes. However, at least in our system, *var*-3/ItG expression was not positively selected by C32 cytoadherence (Tab. 1). It was reported that expression of *var* genes was switched at the rate of 2.4 % per generation [20, 21]. We considered that multiple *var* gene detection in ItG parasites may be due to the co-existence of PRBCs with the randomly switched *var* gene expression. These results indicated that *var*-1/ItG was functionally cytoadherent transcripts in our ItG parasites.

Since most laboratory *P. falciparum* strains and field isolates bound to CD36 [14, 16, 21], we also examined the binding ability of PfEMP-1/ItG-1-positive and PfEMP-1/ItG-1-negative population to CD36. In our cytoadhesion assay, the binding ability of ItG/8A to CD36 alone increased 2.6 times as compared with that of ItG parasites (Fig.5). However, considering that the binding ability of ItG/8A to C32 cells increased 16.6 times as compared with that of ItG parasites (Fig.4), C32 surface receptors other than CD36 may also affect the cytoadherence of ItG parasites to C32 cells.

FASTA research indicated that the nucleotide sequence of DBL-alpha domain of *var*-1/ItG was identical to that of A4tresVAR. A4tres was derived from A4 clone, which was isolated from IT 4/25/5 by selection on ICAM-1 [25] and bind to both CD36 and ICAM-1. ICAM-1 on C32 cells can be the 2nd receptor for PfEMP-1/ItG-1. In this study, only DBL-alpha domain region was isolated. Full length cDNA, especially, DBL-beta-C2 tandem region, which was responsive for ICAM-1 binding, should be cloned. It was reported that co-expression of ICAM-1 with CD36 on the surface of host cells contributed to increase the cytoadherent ability of PRBCs [11]. PfEMP-1/ItG-1 may also need co-expression of ICAM-1 and CD36 on host cells in order to increase the binding ability to C32 cells.

The current study showed that a dominant *var* gene expressed in *P. falciparum* ItG parasites appeared to adhere to C32 cells, and suggested that it might not be dependent on only CD36 but also other receptors. ICAM-1 is one candidate because it was expressed on the surface of C32 cells [24]. ICAM-1 plays an important role in sequestration of PRBC in brain capillary vascular [14, 28] and in placenta [5, 22], therefore, the binding ability of PfEMP-1/ItG-1 to ICAM-1 should be examined.

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