Hemoglobinopathies and Malaria Infection in Myanmar

Aung Myint Than, Teruo HARANO, Keiko HARANO* and Shigeru OKADA**

Department of Biochemistry, Kawasaki Medical School, Kurashiki 701-0192, Japan
**Department of Clinical Nutrition, Faculty of Medical Professions, Kawasaki University of Medical Welfare, Kurashiki 701-0193, Japan*
***Department of Pathological Research, Faculty of Medicine, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan*

Accepted for publication on September 9, 2002

ABSTRACT. Screening for the incidences of hemoglobinopathies (Hb E, α-thalassemia (thal) and β-thal) and malaria infection was done in 131 healthy volunteers with no clinical symptoms in Shan State in Myanmar. The group consisted of 71 females and 60 males ranging in age from 16 to 46 years old. The analysis of hemoglobin was carried out using high performance liquid chromatography (HPLC) and isoelectric focusing (IEF), and DNA analysis was done by polymerase chain reaction (PCR)-electrophoresis. The incidences of carriers of Hb E, α-thal and β-thal were 17.6%, 7.6% and 0.8%, respectively. The thal genotypes observed were $-\alpha^{3.7}$, which is an α-thal-2 mutation, and CD17A→T, which is a β^0-thal mutation. Both are common mutations in Myanmar. There were only three (2.3%) carriers of two malaria parasites, *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*); namely one female carrier of *Pf* and two male carriers of *Pv*. Although it was suspected that the incidence of subjects infected with malaria would be high, there was comparatively small number of cases. This may be the reason why the blood sample collection from the volunteers was done during the dry season which is not the breeding season for malaria mosquitos.

Key words : Malaria parasites — Hemoglobin E — α-Thalassemia — β-Thalassemia — Polymerase chain reaction (PCR)

Myanmar is a South Asian country in which hemoglobinopathies [abnormal hemoglobin (Hb), especially Hb E, and α- and β-thalassemias (thal)] and malaria parasites are prevalent.[1] In our previous papers concerning molecular diagnosis of transfusion-dependent anemic Myanmar patients, we reported that high incidences of carriers of Hb E and various kinds of α- and β-thals were observed.[2-4] Eighteen types of β-thal mutations, including four main mutations (codon (CD)17 A→T, β-intervening sequence (βIVS) I-1 G→T, βIVS I-5 G→C and CD41/42 TTCTTT→TT) and two deletion types of α-thal mutations (the $--^{SEA}$ genotype of α-thal-1 and the $-\alpha^{3.7}$ genotype of α-thal-2) were detected. We also described the

Aung Myint Than, 原野昭雄, 原野恵子, 岡田 茂
e-mail : haranot@bcc.kawasaki-m.ac.jp

methodology for highly sensitive detection of *Plasmodium falciparum* (*Pf*) and *P. vivax* (*Pv*), and its application to abnormal Hb patients of the Solomon Islands.[5]

At this time, we had the opportunity to screen for carriers of hemoglobinopathies (abnormal Hb E, α- and/or β-thals) and malaria parasites (*Pf* and/or *Pv*) among inhabitants of Shan State in Myanmar.

MATERIALS AND METHODS

Blood samples from 131 healthy volunteers, 71 females (JF series) and 60 males (JM series), ranging in age from 16 to 46 years old living in Shan State in Myanmar were collected in tubes with EDTA-2Na as an anticoagulant in the period from the middle of February through the end of March, 2002. Red cells, which had been separated from plasma by means of centrifugation, were brought to the Department of Biochemistry, Kawasaki Medical School, Kurashiki, Japan. The hemolysates prepared from 200 μL of red cells and 400 μL of distilled water were analyzed using anion exchange high performance liquid chromatography (DEAE-HPLC) on a DEAE-5PW column (7.5$\times$75 mm, Tosoh Corporation, Tokyo, Japan) to detect the presence of abnormal Hbs; e.g., Hb E, and to determine the proportions of Hb components (Hb A_2, Hb A, Hb F and any additional abnormal Hb).[2-4] Isoelectric focusing (IEF, pH range: 6-9) was also used for the analysis of Hb components. DNA was extracted from the red cells by a simple method using a Qiagen DNA Extraction Kit and the method of Poncz *et al*[6] with slight modification. Detection of *Pf* and *Pv* was done using the same procedure, namely the multiplex PCR method, as described previously.[5]

Confirmation of Hb E was done employing the same procedures as described previously; i.e., amplified DNA digested with the restriction enzyme *Mnl* I was electrophoresed on 5% polyacrylamide gel and silver stained, and the appearance of a DNA fragment of 231 bp signified presence of the β^E-globin gene.[2,7] α-Thal mutations, especially the α-thal-2 ($-\alpha^{3.7}$) and α-thal-1 mutations ($--^{SEA}$ or $--^{Med}$), were detected in all subjects by the multiplex PCR method.[4,8] DNA from subjects with relatively high values of Hb A_2 from 2.8 to 5.32% was analyzed for the common Myanmar β-thal mutations (CD17 A$\rightarrow$T, βIVS I-1 G$\rightarrow$T, βIVS I-5 G$\rightarrow$C and CD41/42 TTCTTT$\rightarrow$TT) using the amplification refractory mutation system (ARMS) described previously.[3]

RESULTS

The chromatographic (HPLC) and electrophoretic (IEF) behaviors of Hb E are similar to those of Hb A_2 (Fig 1). The distributions of the values obtained by DEAE-HPLC analysis for Hb A_2 ranged from 1.0 to 5.5%, while that for Hb E plus Hb A_2 ranged from 17.5 to 35%. These data are shown in Fig 2, where the mean values for Hb A_2 and Hb E plus Hb A_2 were 2.17% and 26.0%, respectively, and the standard deviations were 0.49 and 4.70. The lower value of the Hb E plus Hb A_2 than the normal (generally the stable abnormal Hb content of a subject heterozygous for the

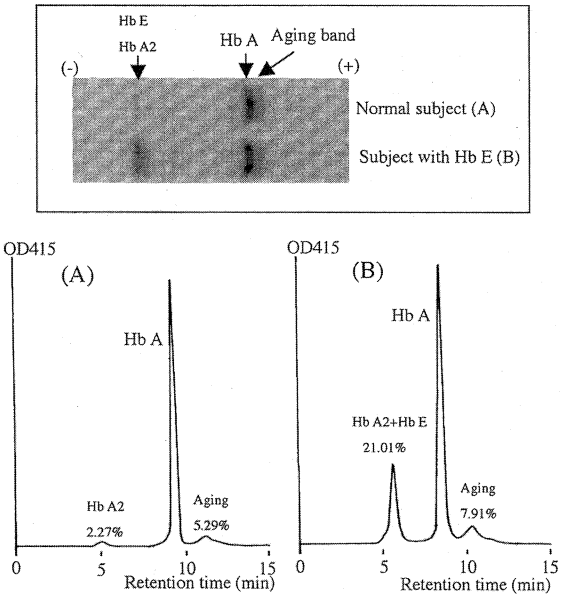

Fig 1. IEF patterns and chromatograms of DEAE-HPLC obtained from hemolysates. A : Normal subject. B : Subject with Hb E.

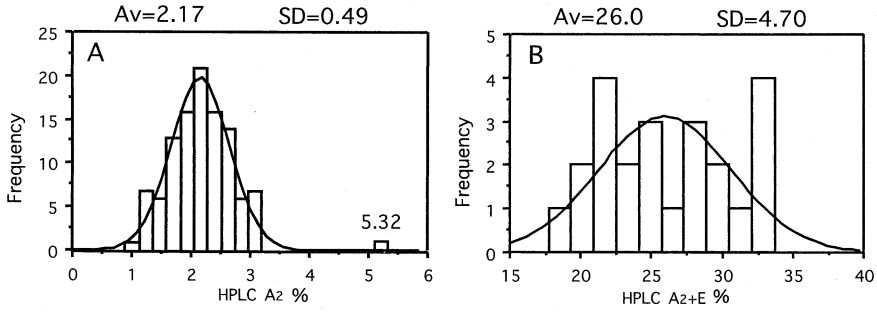

Fig 2. Histograms of subjects having Hb A_2 values of less than 5.5% (A) and with Hb E plus Hb A_2 values between 15% and 35% (B). Av : Mean value. SD : Standard deviation.

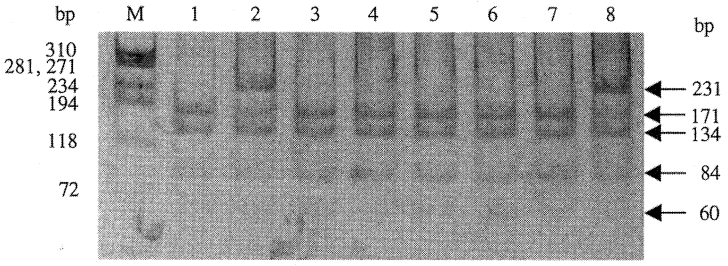

Fig 3. Detection of β^E-globin gene by electrophoresis of the PCR product digested with *Mnl* I on 5% polyacrylamide gel and silver staining. M : Molecular marker. 1 and 3-7 : JM-32 and JM-34-38 (normal cases). 2 and 8 : JM-33 and JM-39 (heterozygous cases for β^E-globin gene).

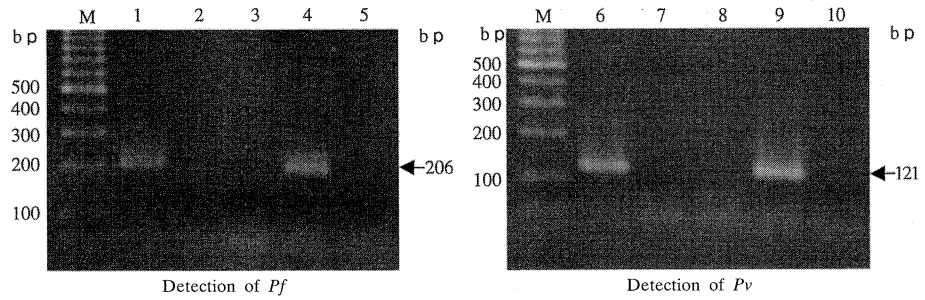

Fig 4. Detection of the malaria parasites, *Pf* or *Pv*, by electrophoresis of the product amplified by the multiplex PCR method on 2% Nusieve gel. M : Molecular marker. 1 : control with *Pf*. 2, 3 and 5 : JF-1, JF-2 and JF-70 (negative cases of *Pf*). 4 : JF-69 (positive case for *Pf*). 6 : Control with *Pv*. 7, 8 and 10 : JM-11, JM-12 and JM-14 (negative cases for *Pv*). 9 : JM-13 (positive case for *Pv*).

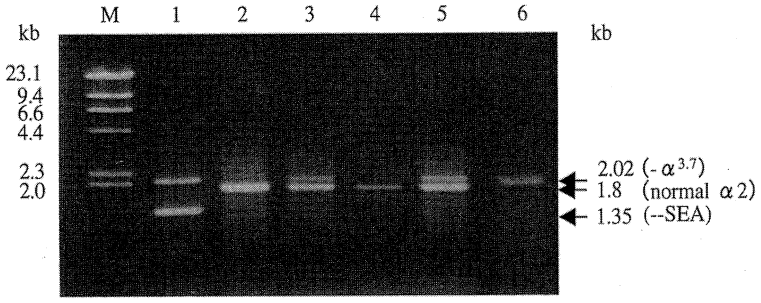

Fig 5. Detection of the $-\alpha^{3.7}$ genotype of the α-thal-2 mutation by electrophoresis of the DNA product amplified by multiplex primer sets on 1% agarose gel. M : Molecular marker. 1 : Control with genotype $-\alpha^{3.7}/-^{SEA}$. 2 and 4 : JF-1 and JF-3 (normal cases). 3 and 5 : JF-2 and JF-4 (cases with genotype $-\alpha^{3.7}/\alpha\alpha$). 6 : JF-13 (case with genotype $-\alpha^{3.7}/-\alpha^{3.7}$).

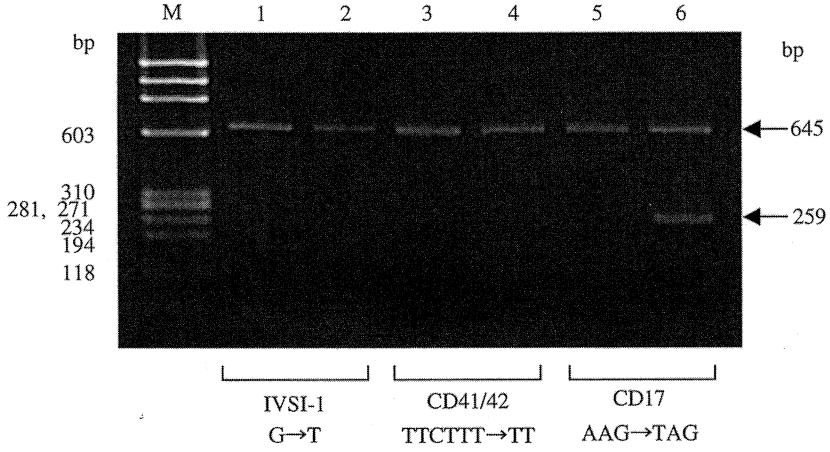

Fig 6. Detection of the CD17 A→T β^0-thal mutation by electrophoresis of the DNA produced by the ARMS method. M : Molecular marker. 1, 3 and 5 : JM-5. 2, 4 and 6 : JM-6.

β-globin chain anomaly is estimated to be approximately 45%) is suspected to have been caused by a β^E-globin gene with a β-thalassemic property.[9] The Hb A_2 value of one subject (JM-6) was 5.32%, when it led us to suspect that the subject might have a β-thal mutation. These results were comparable to the IEF findings. There were 23 Hb E heterozygote carriers; 11 females and 12 males, estimated to be 17.6% of the total subjects.

The DNA of 23 subjects with a high Hb A_2 value was amplified and electrophoresed after digestion with *Mnl* I. Combination of the 171 bp band with the 60 bp band resulting in a 231 bp band with loss of the restriction site of *Mnl* I was found in 23 subjects (Fig 3), indicating to be the Hb E carriers.

Pf and/or *Pv* were detected with a multiplex primer set or a specific primer set in three subjects (about 2.3%). *Pv* was found in two males (JM-2 and JM-13), and *Pf* in one female (JF-69). These findings were reconfirmed using a specific primer set to detect individual *Plasmodium* (Fig 4).

We detected α-thal in 10 subjects (JM-14, JM-20, JM-56, JM-60, JF-2, JF-4, JF-13, JF-26, JF-62, and JF-70) by the PCR method using multiplex primer sets. The genotypes found in them were all $-\alpha^{3.7}$ of the α-thal-2 mutation. No genotypes $--^{SEA}$ and $--^{MED}$ of the α-thal-1 mutation were found (Fig 5). Except for JM-56, they had a relatively lower level of Hb A_2. Among them, JF-13 was homozygous for genotype $-\alpha^{3.7}$. JM-20 and JM-60 were also carriers of Hb E, being compound heterozygotes of Hb E and α-thal-2, $-\alpha^{3.7}$ genotype. The total percentage of α-thal carriers was about 7.6%. The Hb A_2 values of the carriers of α-thal ranged between 1.2 and 2.4%, which were relatively lower values.

TABLE 1. Results of the detection of Hb E, α- or β-thal mutation, and/or malaria parasite, *Pf* or *Pv*.[*1]

Subjects	Hb A_2+Hb E (%)	Hb E (+ or −)	*Pf* or *Pv*	Thal mutations	
				α-thal	β-thal
JM-2	2.5	(−)	*Pv*	(−)	(−)
-6	5.3	(−)	(−)	(−)	CD17 A→T
-13	2.6	(−)	*Pv*	(−)	(−)
-14	2.3	(−)	(−)	$-\alpha^{3.7}$	(−)
-20	32.5	(+)	(−)	$-\alpha^{3.7}$	(−)
-56	3.0	(−)	(−)	$-\alpha^{3.7}$	(−)
-60	20.6	(+)	(−)	$-\alpha^{3.7}$	(−)
JF-2	2.4	(−)	(−)	$-\alpha^{3.7}$	(−)
-4	2.2	(−)	(−)	$-\alpha^{3.7}$	(−)
-13	1.6	(−)	(−)	$-\alpha^{3.7}$ (homo-)	(−)
-26	1.3	(−)	(−)	$-\alpha^{3.7}$	(−)
-62	2.2	(−)	(−)	$-\alpha^{3.7}$	(−)
-69	1.2	(−)	*Pf*	(−)	(−)
-70	1.2	(−)	(−)	$-\alpha^{3.7}$	(−)

[*1]Carriers of the Hb E heterozygote were excluded from this Table. Percentages of Hb A_2 plus Hb E were calculated from DEAE-HPL chromatograms.

In JM-6 and the subjects having higher Hb A_2 values than 2.8%, four common β-thal mutations in Myanmer (CD17 A→T, βIVS I-1 G→T, βIVS I-5 G→C and CD41/42 TTCTTT→TT) were detected by the ARMS. As suspected, JM-6 had a β^0-thal mutation of CD17 A→T (Fig 6). In this study, therefore, the rate of β-thal carriers was 0.8% in total. Among the carriers of Hb E and/or thal mutations, none was infected with *Pf* or *Pv*. The data are summarized in Table 1.

DISCUSSION

Myanmar is considered as an Asian country with a prevalence of malaria parasites and hemoglobinopathies (Hb E, α-thal and β-thal).[1] The incidence of both in rural and mountainous regions tends to be higher than in urban areas. In this study, peripheral blood samples from 131 healthy volunteers with no clinical symptoms who were in Shan State, a mountainous region, were examined. The blood samples were collected in the period from the middle of February through the end of March, which is the dry season in Myanmar, and, therefore, not the breeding season for malaria mosquitos.

In this study, although 17.6% of the volunteers were Hb E carriers, the numbers of α- and β-thal (7.6% and 0.8%, respectively) and malaria parasite carriers (2.3%) (Table 1), were comparatively fewer than expected before this study. However, as expected, there was a high frequency of the $-\alpha^{3.7}$ genotype of the α-thal-2 mutation.[4,10] Unexpectedly, for the β-thal genotype, only a CD17 A→T of β^0-thal mutation was encountered, although it is one of the most common Myanmar β-thal.[3,10] Additionally, only 2.3% of the subjects were carriers of the malaria parasite, *Pf* and/or *Pv*. No carriers with both hemoglobinopathy and malaria parasite were detected in the subjects, but some hemoglobinopathies (Hb E, α-thal or β-thal) were detected in 32 subjects without *Pf* or *Pv* infection. The remaining 99 subjects (75.6%) were free of hemoglobinopathies and malaria parasites. This might have been the reason why they were healthy and had no clinical symptoms. Although it has been generally supposed that carriers of hemoglobinopathies, Hb E or thal, are resistant to malaria parasites, this study could not make clarify that supposition.

ACKNOWLEDGMENTS

This study was partly supported by Grants-in-Aid (12672259 and 14572193) from the Ministry of Education, Culture, Sports, Science and Technology, Japan and also by Research Project Grants (13-104 and 14-102) from Kawasaki Medical School.

REFFERENCES

1) Steinberg MH, Forget BG, Higgs DR, Nagel RL: Disorders of Hemoglobin, Genetics, Pathology, and Clinical Management. Cambridge, Cambridge University Press 2001, pp831-894
2) Harano T, Ne Win, Harano K: Prevalence of hemoglobin E among the children taking regular blood transfusion at the day care room, Yangon Children Hospital, Myanmar. Kawasaki Med J **26**: 149-154, 2000

3) Harano K, Ne Win, Harano T: Molecular aspects of transfusion dependent thalassemic children in Myanmar: Analysis of common β-thalassemia in Myanmer by amplification refractory mutation system (ARMS). Kawasaki Med J **26**: 161-164, 2000
4) Harano T, Ne Win, Harano K: Molecular aspects of α-thalassemia in Myanmer. Kawasaki Med J **27**: 33-36, 2001
5) Harano K, Aung Myint Than, Suetsugu Y, Kawabata M, Harano T: Detection and differentiation of malaria parasites in DNA extracted from blood samples by the polymerase chain reaction (PCR). Kawasaki Med J **27**: 83-89, 2001
6) Poncz M, Solowiejezyk D, Harpel B, Mory Y, Schwartz E, Surrey S: Construction of human gene libraries from small amounts of peripheral blood: Analysis of β-like globin genes. Hemoglobin **6**: 27-36, 1982
7) Handa A, Mibukura T, Aida T, Takahashi T, Hirashima K, Inoue T, Harano K, Harano T: Abnormal hemoglobinopathy (Hb E) diagnosed from microcytic hypochromic red blood cells in a 31-year old Bangladeshian male. J Clin Hematol **39**: 146-149, 1998
8) Chong SS, Boehm CD, Higgs DR, Cutting GR: Single tube multiplex-PCR screen for common deletional determinants of α-thalassemias. Blood **95**: 360-362, 2000
9) Orkin SH, Kazazian HH, Antonarakis SE, Ostrer H, Goff SC, Sexton JP: Abnormal RNA processing due to the exon mutation of β^E-globon gene. Nature **300**: 768-769, 1982
10) A syllabus of thalassemia mutations. Huisman THJ, Carver MFH, Baysal E. (eds). The Sickle Cell Anemia Foundation, Augusta, GA, USA, 1997, pp235-295