Haplotypes of the β^E-Globin Gene Cluster Found in Myanmar

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ABSTRACT. We had the opportunity to study the members of 10 families with Hb E/β-thalassemia and characterized the ethnic features of the population of Myanmar from haplotyping of a β-globin-like gene cluster producing Hb E. Detection of the Hb E and β^E-globin genes was carried out by DEAE-HPLC and isoelectric focusing of the hemolysates, by electrophoresis of DNA fragments which resulted in the digestion of the PCR product with *Mnl*I, and by PCR-direct sequencing. The haplotype of the β-globin-like gene cluster was determined by the presence (+) or absence (−) of the restriction enzyme site in the PCR product including the polymorphic site. Polymorphism or the haplotype were determined at seven sites, 5' to the ε-gene/*Hin*cII, in the $^G\gamma$-gene/*Hin*dIII, in the $^A\gamma$-gene/*Hin*dIII, in the $\psi\beta$-gene/*Hin*cII, 3' to the $\psi\beta$-gene/*Hin*cII, in the β-IVS II/*Ava*II and 3' to the β-gene/*Bam*HI, in the β-globin-like gene cluster. Three types of haplotypes were found in this study ; (a) −+−+++− (five families), (b) +−−−−+− (four families) and (c) +−−+++− (one family). Haplotypes (a) and (b) were identified as one's in the Thai and Cambodian populations in eastern Myanmar. They were considered to be the most common haplotypes in that area and to have been the result of gene confusion or gene flow between ethnic groups. Haplotype (c), on the other hand, was a rare type, suggesting both the specificity and diversity of ethnic groups in Myanmar.

Key words : hemoglobin E (Hb E) — haplotypes — PCR-restriction enzyme — PCR-direct sequencing — Myanmar

Hb E ($\alpha_2\beta^{26Glu\rightarrow Lys}{}_2$) is mildly unstable but this instability does not alter the red cell life span significantly. It is a common Hb variant in some Southeast Asian countries ; i.e. Cambodia, Thailand, parts of China, and Vietnam, where, based on various studies, from 15 to 30% or from 70 to 80% of the population are carriers.[1-3] The clinical condition of Hb E heterozygotes resembles that of someone with a very mild β-thalassemia (thal) trait. Hematologically, homozygotes exhibit more microcytosis, but most cases are clinically asymptomatic. However, the clinical conditions of compound heterozygotes for Hb E and the β-thal gene resembles to that of patients with β-thal intermedia or β-thal major.[4-6] In our previous report,

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most of 113 (about 60 percent) of 186 transfusion dependent anemic Myanmar patients who had been diagnosed as thal intermedia or thal major were heterozygotes of Hb E/β-thal.[7,8] Afterwards, we had the opportunity to study the members of 10 families with Hb E and β-thal, and specifically investigated their haplotypes in the β-globin-like gene cluster producing Hb E to find the ethnic features of Myanmar population.

MATERIALS AND METHODS

The peripheral blood used here was obtained from Myanmar family members of transfusion dependent anemic patients with Hb E and β-thal who visited the Day Care Room of the Outpatient Department of Yangon Children's Hospital from various states (Barmar, Kayin, Mon, Rankhine, Shan and Chin). At that time, 10 families of Hb E carriers were agreed to participate. Red cells separated from the plasma of their peripheral blood and frozen for transport to the Department of Biochemistry, Kawasaki Medical School, Kurashiki, Japan, were used for this study.

The hemolysates were prepared from red cells as follows: 25 μL of red cells, 50 μL of distrilled water and 30 μL of carbon tetrachloride in a 1.5 μL eppendorf tube were mixed well by vortex for several minutes, and then were centrifuged at 12000 rpm for 10 minutes to collect supernatant as the hemolysate. Hemoglobin (Hb) analysis, after 50 μL of hemolysate was treated with 1 μL of 5% potasium cyanide (KCN) solution and 1 μL of 0.1 mol/L dithiothreitol (DTT), was performed by anion exchange high performance liquid chromatography (HPLC) on DEAE-5PW (7.5 $\times$ 75 mm, Tosoh Corporation, Tokyo, Japan)[9] and by isoelectric focusing (IEF, pH rang 6-9)[10] to identify the Hb E carrier. DNA was extracted from the red cells by a simple method using a Qiagen DNA Extraction Kit (Qiagen Japan Corporation, Tokyo, Japan). A portion of the DNA which was amplified with two specific primers included the region of codon 26 (β26) of the β-globin gene it was digested with a restriction enzyme, *Mnl*I, to electrophoretically detect the presence of the mutant gene producing β^{E}-globin.[11] A nucleotide replacement at β26, *G*AG(Glu)$\rightarrow$*A*AG(Lys), was confirmed by direct sequencing of the PCR product amplified as described above. The BigDye termination cycle sequencing method (BigDye Terminator Cycle Sequencing Ready Reaction Kits, PE Applied Biosystems, Chiba, Japan) was used.

Polymorphism (haplotype) was determined at seven polymorphic sites of the β-globin-like gene cluster, 5' to the ε/*Hin*cII, in the $^{G}\gamma$/*Hin*dIII, in the $^{A}\gamma$/*Hin*dIII, in the $\psi\beta$/*Hin*cII and 3' to $\psi\beta$/*Hin*cII, in the β/*Ava*II and 3' to the β/*Bam*HI, by electrophoresis of the PCR product digested with the appropriate restriction enzyme. The synthesis of oligonuclotides as PCR primer sets used here and the PCR conditions followed the method of Winichagoon *et al.*[12] The presence or absence of polymorphic sites is indicated by (+) and (−).

RESULTS

DEAE-HPLC and IEF of the hemolysates treated with KCN and DTT gave the results shown in Fig 1. On the HPL-chromatogram, the first elute

was Hb A_2, followed by Hb A as the main component of Hb, and finally Hb F and/or aged Hb. If Hb E is present in the hemolysate, its peak is eluted at the same retention time as Hb A_2, and if an abnormal Hb without Hb E is present, it is eluted between the Hb A_2 and Hb A peaks or at a longer retention time than that of Hb A, owing to an ionic property brought about by the amino acid substitution (Fig 1). Hb separation on IEF owing to the isoelectric property of the amino acid contained in the Hb molecule is shown in Fig 2. The order of Hb migration from the cathode to the anode was Hb A_2, Hb F and Hb A. If Hb E is present in the hemolysate, it appears at the same position as Hb A_2. Separation of Hb E from Hb A_2 is generally difficult but it is easy to find Hb E carriers since they show an extremely high value of Hb A_2; i.e., more than 15% of the total Hb. Detection of the β^E-globin gene was made by both digestion of the PCR product with restriction enzyme *Mnl* I and by direct sequencing of the PCR product as shown in Fig 3 and 4. The haplotype of the β-globin-like gene cluster was determined on the basis of the presence (+) or absence (−) of an appropriate enzyme site, as shown in Fig 5. On the basis of these results, the haplotypes of the family members having a heterozygote for Hb E/Hb A and a comopund heterozygote for Hb

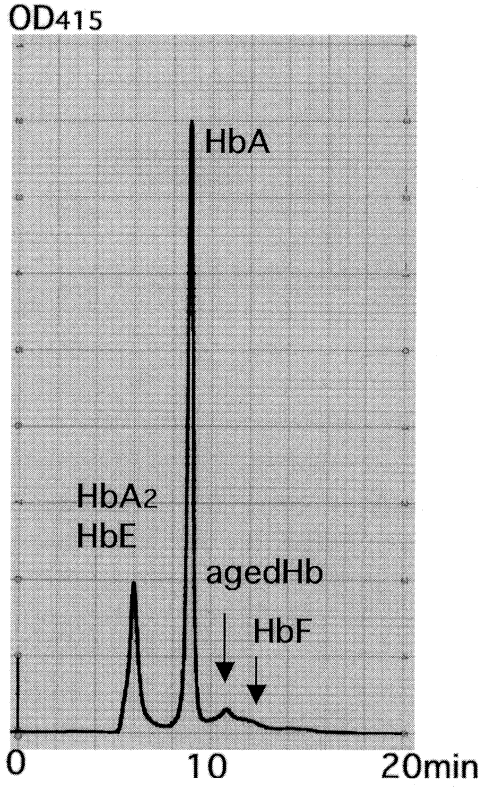

Fig 1. DEAE-HPLC elution pattern of the hemolysates treated with KCN and DTT. The elution positions of Hb F and aged Hb are indicated by arrows. When a high Hb A_2 value is observed (more than about 15% of the total Hb), the patient may be considered to possibly be a carrier of Hb E.

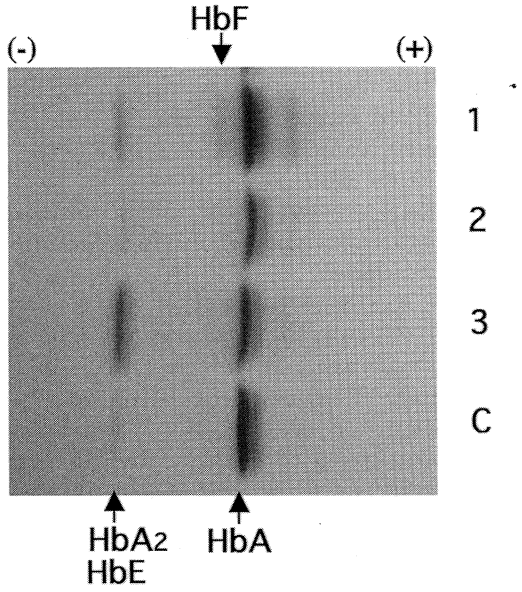

Fig 2. IEF pattern of the hemolysates treated with KCN and DTT. 1 : Subject with high Hb F. 2 : Normal subject. 3 : Subject with Hb E. C : Control

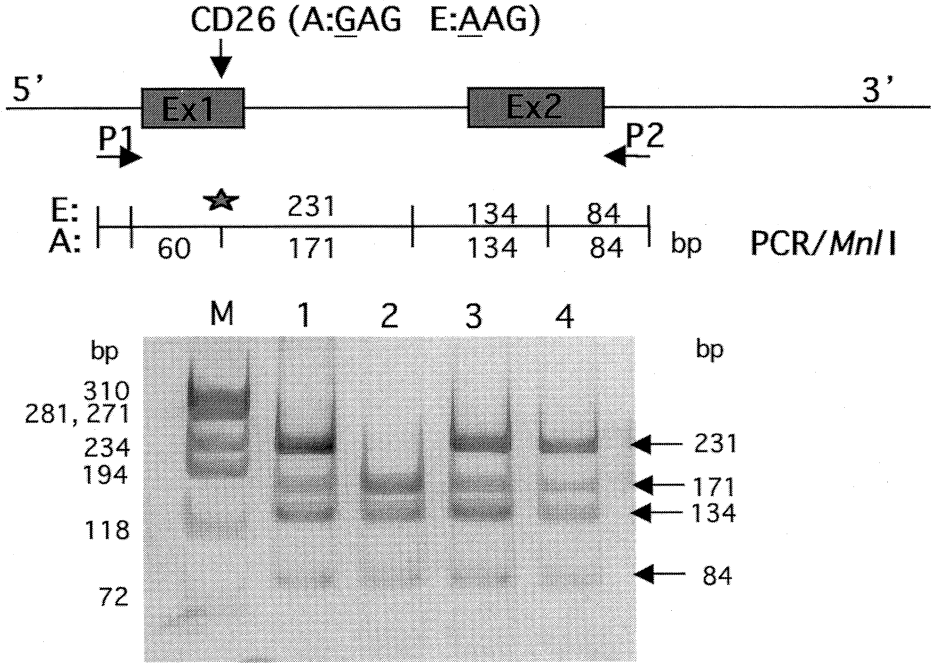

Fig 3. Detection of the β^E-globin gene by electrophoresis of DNA fragments resulted in digestion of the PCR product with the restriction enzyme *Mnl*I. P1 and P2 indicate the position of primers used here. E and A show the fragment size in the case of the β^E- and normal β-globin genes. ★ : indicates the enzyme site of *Mnl*I. M : Molecular marker. 1, 3 and 4 : Subjects with the β^E- and normal β-globin genes. 2 : Normal subject.

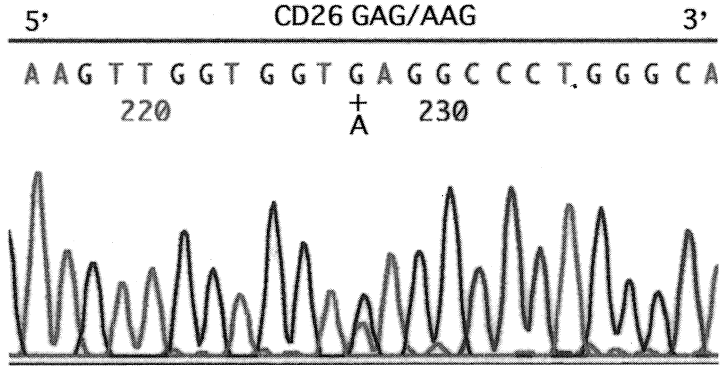

Fig 4. Detection of the β^E-globin gene by the BigDye termination cycle sequencing method. This case is a heterozygote for the β^E-/normal β-genes.

codon	23	24	25	26	27	28	
Normal β-seq. :	5'-GTT·	GGT·	GGT·	**G**AG·	GCC·	CTG-3'	(GAG=Glu)
β^E-seq. :	5'-GTT·	GGT·	GGT·	**A**AG·	GCC·	CTG-3'	(AAG=Lys)

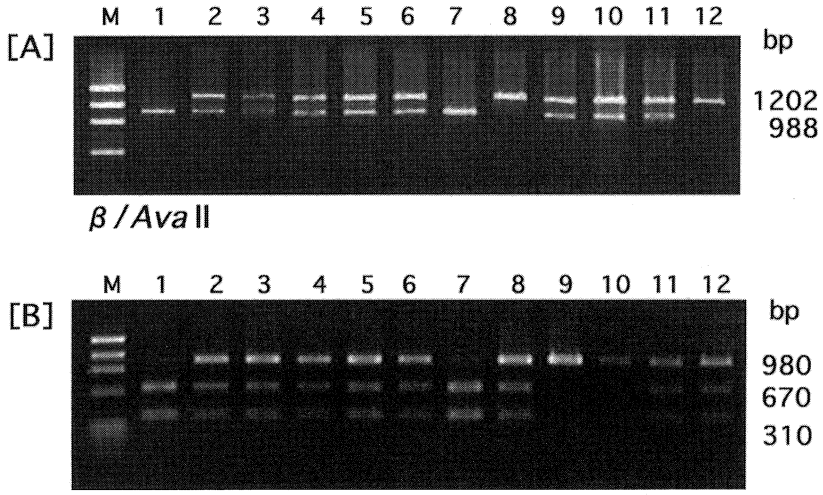

Fig 5. Examples of determination of polymorphism, (+) or (−), performed by the PCR-restriction enzymatic method. [A] : The cases of β/*Ava*II. 1 and 7 lanes : The cases of polymorphism +/+. 2-6 and 9-11 lanes : The cases of +/−. 8 and 12 lanes : The cases of −/−. [B] : The cases of 3'-$\psi\beta$/*Hin*cII. 1 and 7 lanes : The cases of +/+. 2-6, 8, 11 and 12 lanes : The cases of +/−. 9 and 10 lanes : The cases of −/−.

E/β-thal were analyzed, as shown in Fig 6. The haplotypes for the β-globin-like gene cluster possessing the β^E-globin gene were of only three types, (a) : − + − + + + − in five families, (b) : + − − − − + − in four families and (c) : + − − + + + − in one family. The haplotypes of β^0-thal mutations, CD41/42TTCTTT → · · · · TT (4 bases deletion) (F-38), CD17AAG → TAG (F-56), CD35TAC → TAA (F-33) and others were also predicted, and those of the various types of normal β-globin gene clusters were also identified, as shown in Fig 6.

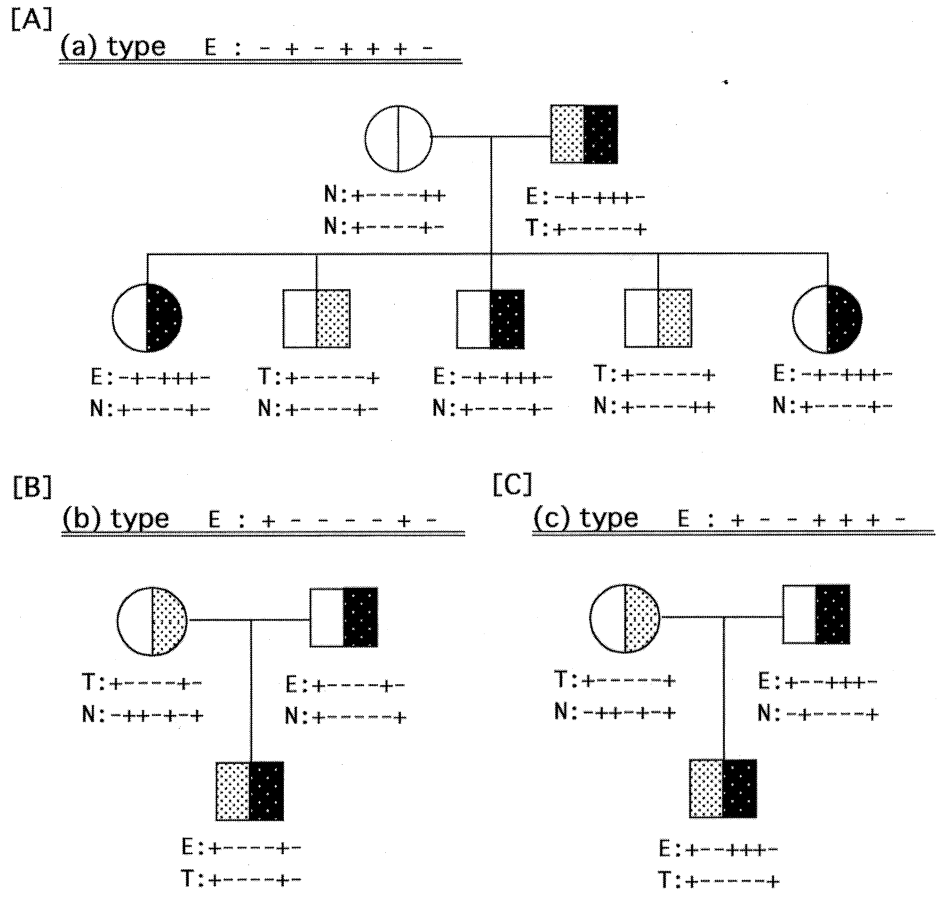

Fig 6. Haplotypes of the β^E- (E), β-thal (T) and normal β-globin gene (N) clusters in the family members. [A] : Family (F-56) with (a) type of β^E-globin gene and β-thal mutant gene, CD17AAG→TAG (Amber codon). [B] : Family (F-38) with (b) type β^E-globin gene and β-thal mutant gene, CD41/42TTCTTT →···· TT (4 bases deletion). [C] : Family (F-33) with (c) type β^E-gene and β-thal mutant gene, CD35TAC → TAA (Ochre codon). Five haplotypes of the normal β-globin gene cluster are seen in these families.

DISCUSSION

Nucleotide replacement in the first exon, especially at codons, β19, β26 and β27, is associated with a mild β-thalassemic mutation, which modifies the DNA sequence of this region making it similar to a splicing site.[13,14] The consequence is an aberrant splicing of precursor mRNA through these activated sites with a decrease in normal splicing. These nucleotide replacements in exon 1 result in production of Hb variants. Hb Malay (β19Asn→Ser or A*A*C→A*G*C) was discovered as a rare one in Southeast Asians, Hb E (β26Glu→Lys or *G*AG→*A*AG) is very common in Southeast Asians, and Hb Knossos (β27Ala→Ser or *G*CC→*T*CC) is in Mediterraneans. These variants have mild β-thalassemic symptoms. A homozygote for Hb E has a mild β-thal intermedia trait and heterozygosity for Hb E and β^0- or β^+-thal results in relatively severe thal*symptoms, thal intermedia or thal

major.[1,15] Hb E, a mildly unstable and mildly β-thalassemic Hb variant, also has been observed with high frequency in Myanmar as well as in Thailand, Cambodia, Laos and a part of China. In these areas, various types of β-thal mutations are often found and, therefore, it is believed that heterozygotes for Hb E and various types of β-thal might appear more frequently than homozygotes for β-thal. The situation in Myanmar is considered to be similar based on our previous report of the discovery of a high frequency of Hb E in transfusion dependent anemic patients suspected of having β-thal intermedia or β-thal major.[8,9] Therefore, based on the haplotype in the β-globin-like cluster producing Hb E, it is considered that there may be various haplotypes. Although at this time only 10 families have been investigated in detail, three haplotypes of the β-globin-like gene cluster possessing the β^E-globin gene have been identified. Two haplotypes were found in nine of the ten families, (a) : $-+-+++-$ (5 families) and (b) : $+-----+-$ (4 families), and were considered to be the common haplotypes in Myanmar, and another one, (c) : $+--+++-$ (one family), was considered as a minor type. In these three haplotypes, two ploymorphisms at the *Ava* II site in the β-globin gene and the *Bam* HI site 3' to the β-globin gene, which are generally called the framework of the β-globin gene, were all same, (+) and (−). This led us to speculate that the mechanisms of haplotypes (a) and (b) might be produced by either meiotic recombination 5' to the β-globin gene between the β^E- and common normal β-globin gene clusters or by point mutation of the first nucleotide of β26 *G*AG in the commonest normal haplotype gene cluster. Comparison with those haplotypes in the Thai and Cambodian populations showed haplotypes (a) and (b) in Myanmar to also be common types in Thiland and Cambodia, suggesting gene confusion or gene flow among ethnic groups.[17-19] However, type (c) seems to be a rare one and might be characteristic of Myanmar. The haplotype, $-+-++-+$, found in high frequency in eastern part of Thailand, especially in the Khmer population, was not discovered.

In this study, although many haplotypes of the β^0-thal and β^+-thal genes were spontaneously identified, the clinical symptoms caused by the combination of the haplotype of Hb E and the genotype of β^0- or β^+-thal need to be further discussed on the basis of hematological findings, red cell morphologies, clinical manifestations and other characteristics.

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