Recent Studies on the Abnormal Hemoglobins found in Thailand

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Summary: Numerous abnormal hemoglobins (Hb) have been discovered in Thailand, and yet further studies continue to show additional variants not previously described. This paper reviews the discovery of two more variants, as well as additional studies on families with unusual combinations of mutations. One proposita had an abnormal Hb with similar electrophoretic mobility to Hb J Bangkok, but tryptic peptide mapping by h.p.l.c., amino acid analysis and protein sequence analysis revealed an abnormal peptide α-T9, due to the mutation [α61 Lys-Asn], previously found in Hungary and identified as Hb J Buda. Direct DNA sequence analysis confirmed this result, and showed that the mutation occurred in the α1 gene and not the α2-gene. The abnormal Hb in another proposita was shown by peptide mapping, protein sequence analysis, and DNA sequence analysis, to be identical to Hb G Coushatta [β22 Glu-Ala]. In addition to the above variants, unusual combinations of mutations from two other families were studied, namely the combination of Hb E (common in Thailand) with Hb C (common in African populations) and the combination of Hb E/ β^{17}-thalassemia/ β^{41-42}-thalassemia.

Introduction

Several hemoglobinopathies are found in Thailand: these include α-thalassemia, β-thalassemia and the abnormal hemoglobins (Hb). α-Thalassemia [1,2] is generally due to large gene deletions: those removing both α_1 and α_2 genes cause the more severe condition known as α-thalassemia 1, while those resulting in one functional α-gene, produce the milder condition known as α-thalassemia 2. The incidence of α-thalassemia 1 and α-thalassemia 2 in Bangkok are 3.5% and 16.25% respectively [1,2]. β-Thalassemia [1-3] occurs with lower frequency in Thailand, ranging from 1% and 9% in different parts of the country, and is generally caused by base substitutions, or small additions and insertions at various sites. Several abnormal hemoglobins (Hb) have been reported in Thailand [4], and these are mainly due to point mutations, but they also include two C-terminal extensions and a crossing-over mutation, leading to a δ-β hydrid chain. The abnormal hemoglobins so far discovered in Thailand [5-23] are summarised in Table 1, including two novel mutations described in this paper. The commonest hemoglobin variant is Hb E [β26 Glu-Lys], occurring with a frequency of 13% nationally, rising to 50% in the Northeast, followed by Hb Constant Spring, an α-chain extension occurring with a frequency of 1-8%. Most abnormal hemoglobins seem to cause no clinical manifestations, but some such as Hb Constant Spring or Hb Tak have a mild thalassemic effect, while others show greater severity in association with other conditions, e.g. Hb E/ β^0-thalassemia. For these reasons, we are continuing to study the abnormal hemoglobins in Thailand, as well as their interactions with each other and with thalassemia.

Results and Discussion

Hb J Buda

Proposita "L.K." [10] had the following hematological profile at the steady state: Hb 12.7 g/dl; RBC 4.9 x 10^6/ml; Hct 40.0%; MCV 82 fl; MCH 28 pg; MCHC 34 g/dl; Hb F 0.2%. Cellulose acetate electrophoresis of the hemolysate showed a strong band of Hb A, a fainter band at the position of Hb A_2, and a faint smeared band moving slightly faster than Hb A, which was believed to be the abnormal hemoglobin and was present at a level of 29%.

The hemolysate has been fractionated by DEAE-cellulose chromatography and one of the

ABNORMAL HEMOGLOBINS FOUND IN THAILAND

Hemoglobin	%	Position	Mutation	Location	Hematology	Refs
Hb Anantharaj (Hb J Wuming)	rare	α11	Lys-Gln	external	Normal	5,6
Hb Siam (Hb Ottawa)	rare	α15	Gly-Arg	external	Normal	7
Hb Queens (Hb Ogi)	rare	α34	Leu-Arg	$\alpha_1\beta_1$/external	Normal	8
Hb Thailand	rare	α56	Lys-Thr	external	Normal	9
Hb J Buda	rare	α61	Lys-Asn	external	Normal	10
Hb Mahidol (Hb G Taichung)	rare	α74	Asp-His	external	Normal	11
Hb Suan Dok	rare	α109	Leu-Arg	internal	Mild α-thal	12
Hb Constant Spring	4%	αC.t.	elongation		HbH/α-thal	13
Hb C	rare	β6	Glu-Lys	external	Target cells	14
Hb Siriraj	rare	β7	Glu-Lys	external	No anemia	15
Hb Malay	rare	β19	Asn-Ser	external	β-thal-like	16
Hb G Coushatta (G Hsin Chu)	rare	β22	Glu-Ala	external	Normal	10
Hb E	13%	β26	Glu-Lys	external	Microcytosis	17
Hb J Bangkok (J Meinung)	rare	β56	Gly-Asp	external	Normal	18
Hb New York (Kaoshiung)	rare	β113	Val-Glu	internal/$\alpha_1\beta_1$	No effect	19
Hb D Punjab (D Los Angeles)	rare	β121	Glu-Gln	external	Normal alone	20
Hb Dhonburi	rare	β126	Val-Gly	surface crevice	β+-thal-like	21
Hb Tak	rare	βC.t.	elongation		β+-thal-like	22
Hb Lepore Washington-Boston	rare	δ87-β116	fusion		β+-thal-like	23

* References shown are to publications on the discovery of each variant in Thailand, as listed in the References section.

peaks obtained, eluted after HbA, was believed to be the abnormal hemoglobin. An abnormal α-chain peak, eluting out before the normal α^A-peak, was purified by CM-cellulose chromatography in 8 M urea. The abnormal α-chain and normal α^A-chain were studied by tryptic peptide mapping. The normal peptide α^A-T9 had the amino acid composition (Lys 0.95, His 2.81, Asx 6.34, Thr 0.81, Ser 1.68, Pro 1.16, Ala 6.75, Val 2.97, Met 0.73, Leu 3.81) expected for residues 62-90 of α-chain. However, peptide α-T9 from the abnormal α-chain had an amino acid composition (Lys 1.05, His 2.76, Asx 7.38, Thr 0.81, Ser 1.84, Pro 1.28, Ala 6.80, Val 3.14, Met 0.88, Leu 3.91), which indicated the presence of an additional Asx residue compared to residues 62-90 of α-chain.

Sequence analysis of the normal peptide α^A-T9 indicated the N-terminal sequence expected for residues 62-90 of α-chain, namely Val-Ala-Asp-Ala-Leu-Thr-Asn- . However, the N-terminal sequence of peptide α-T9 from the abnormal chain indicated an extra Asn residue at the N-terminus as follows: *Asn*-Val-Ala-Asp-Ala-Leu-Thr-Asn-. These results indicate that the abnormal α-chain contained the mutation Lys-Asn at residue 61 of the α-chain, corresponding to the hemoglobin Hb J Buda (as shown in Figure 1). Peptides α^A-T9 from the normal α^A-chain and $\alpha^{J\ Buda}$-T9 from the abnormal α-chain are therefore actually of different lengths. The normal peptide α^A-T9 stretches from Val 62 to Lys 90, and results from a tryptic cleavage at Lys 61 (shown by arrow ↓). In the $\alpha^{J\ Buda}$ chain, Lys 61 is replaced by Asn 61, so that tryptic cleavage now occurs at Lys 60 (shown by arrow ↑), and the resulting $\alpha^{J\ Buda}$-T9 peptide stretches from Asn 61 to Lys 90.

Further studies were performed by amplification and direct sequence analysis of the proposita's DNA, using probes specific for the α_1-gene and the α_2-gene. The results indicate heterozygosity for the mutation AA<u>G</u> (Lys)→AA<u>T</u> (Asn) at codon 61 of the α_1-gene. The location of the Hb J Buda mutation on the α_1-gene is similar to the α_1-gene location of the Hb Q Thailand mutation, and differs from the α_2-location of the Hb Suan Dok mutation. Hb J Buda has only previously been discovered in a Hungarian family [24], and in some family members, it was found in association with another mutation Hb G Pest. In our studies, detailed investigation of the α-chains failed to reveal any other mutation. The discovery of Hb J Buda in Thailand for the first time shows that other Hb J can be found in addition to the most common one, Hb J Bangkok.

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                60↓              65                  70
α^A             Lys-Lys-Val-Ala-Asp-Ala-Leu-Thr-Asn-Ala-Val-Ala-His-
α^J Buda        Lys-Asn-Val-Ala-Asp-Ala-Leu-Thr-Asn-Ala-Val-Ala-His-
                ↑

                    75              80                  85
α^A             Val-Asp-Asp(Met,Pro,Asn,Ala,Leu,Ser,Ala,Leu,Ser,Asp,
α^JBuda         Val-Asp-Asp(Met,Pro,Asn,Ala,Leu,Ser,Ala,Leu,Ser,Asp,

                    90
α^A             Leu,His,Ala,His)Lys
                Leu,His,Ala,His)Lys
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Fig. 1. Alignment of the α^A-chain and $\alpha^{J\ Buda}$-chain indicating the tryptic cleavages (↓ and ↑ respectively) which give rise to α^A -T9 and $\alpha^{J\ Buda}$-T9.

Hb G Coushatta

Proposita "A.B." [10] had the following hematological profile at the steady state: Hb 9.7 g/dl; RBC 3.8 x 10^6/ml; Hct 33.7 %; MCV 89.0 fl; MCH 26.0 pg; MCHC 30.0 g/dl; Hb F 0.5 %. The abnormal Hb had intermediate electrophoretic mobility between Hb F and Hb A_2, and was present at at level of 39.9 % of total Hb.

The hemolysate was fractionated by DEAE-cellulose chromatography and the abnormal hemoglobin peak, between Hb A_2 and Hb A, was used to prepare globin, which was fractionated on CM-cellulose chromatography in 8 M urea. The abnormal β-chain and normal β^A-chains were digested with trypsin, and the maps were compared by h.p.l.c. Differences were found in the locations of peptide T3 from the normal and abnormal β-chains. The amino acid composition of the abnormal β-T3 peptide (Arg 1.23, Asx 1.82, Glx 1.07, Gly 3.09, Ala 2.35, Val 3.23, Leu 1.00) showed one more Ala and one less Glx than the composition of the normal β^A-T3 (Arg 1.19, Asx 1.91, Glx 2.06, Gly 2.72, Ala 1.15, Val 2.85, Leu 0.95). Edman degradation of the abnormal β-T3 yielded the sequence: Val–Asn–Val–Asp–*Ala*–Val–Gly–Gly–Glu-(Ala–Leu–Gly)–Arg, indicating that the proposita had the mutation Glu-Ala at position 22 of the β-chain.

The protein analysis results were confirmed by direct DNA sequence analysis of the proposita's DNA, which indicated heterozygosity for the mutation GAA (Glu)→GCA (Ala) at codon 22 of the β-chain. This mutation [β22 Glu-Ala] is identical to that found in Hb G Coushatta [25], Hb G Hsin-Chu [26], and Hb G Saskatoon [27], but has not previously been described before in Thailand.

Family with Hb C / Hb E

Hb E is predominantly found in the Southeast Asian region and is typically present at lower levels (25-30% of total Hb) in the heterozygous state, due to abnormal mRNA processing [28]. Its slow electrophoretic mobility is almost identical to that of Hb C [β6 Glu-Lys] [29], which is most frequently found in African and Negro populations. Earlier, we reported the first discovery of Hb C in Thailand in a 29 year-old male "B.D." of Thai origin from Nakorn Srithammaraj [14], raising the question of whether the β^C gene found in Thailand is the same as the β^C genes found in negro populations.

We recently found another Thai family from Nakorn Sawan with Hb C, this time in association with Hb E. The Hb A, Hb E and Hb C were characterised by h.p.l.c. on a BioRad Variant instrument, which gives excellent separation of all three, with elution times of about 2.8 min, 3.7 min and 5.0 min respectively.. The proposita "W.J." was the mother, who was doubly heterozygous for Hb C and Hb E: despite slightly low Hb level at 11.5 g/dl and MCV at 59 fl, she was essentially asymptomatic. The proposita married the father, a β^E/β^A heterozygote (Hb 15.3 g/dl; MCV 83 fl), and they

a. Present Family		A	B	C	D	E	F	G
Father:	β^E	-	+	-	+	+	+	-
	β^A	-	+	+	-	+	+	-
Mother:	β^E	+	-	-	-	-	-	+
(W.J.)	β^C	+	-	-	-	-	-	+
Daughter:	β^E	-	+	-	+	+	+	-
	β^C	+	-	-	-	-	-	+
Daughter:	β^E	-	+	-	+	+	+	-
	β^E	+	-	-	-	-	-	+

b. Previous Family		A	B	C	D	E	F	G
Father:	β^C	+	-	-	-	-	-	+
	β^A	+	-	-	-	-	-	+
Mother:	β^A	-	+	-	-	+	-	+
	β^A	+	-	-	-	-	-	+
Son	β^C	+	-	-	-	-	-	+
(B.D.)	β^A	-	+	-	-	+	-	+

c. Negro Populations	A	B	C	D	E	F	G
β^C Type I	-	+	-	-	+	+	+
β^C Type II	-	-	-	-	+	+	+
β^C Type III	-	-	-	-	-	+	+

Fig. 2: Deduction of Hb C haplotypes in the two families and comparison of the Thai β^C gene haplotypes with the β^C gene haplotypes found in Negro populations (31). Restriction endonuclease cleavage sites are designated as follows: **A**: ε/Hc II; **B**: $^G\gamma$/HdIII; **C**: $^A\gamma$/HdIII; **D**: ψβ/Hc II; **E**: 3'ψβ/HcII; **F**: β/Ava II ; **G**: 3'β/BamHI.

had two daughters: one was a β^E/β^C double heterozygote (Hb 12.9 g/dl; MCV 54 fl), while the other was a β^E/β^E homozygote (Hb 12.1 g/dl; MCV 57 fl).

Haplotype analysis of the β-gene cluster [30] was performed by digesting the globin genome with restriction endonucleases, and detecting the presence of cleavage sites by electrophoresis. Propositus "W.J." in the present family (Fig. 2a) had the same haplotypes for the β^E and the β^C genes, + - - - - - +. Both daughters inherited the β^E gene from the father, but one daughter inherited the β^C gene from the mother, while the other daughter inherited the β^E gene. When the haplotypes of the first family are considered, propositus B.D. inherited the β^C gene from the father, who had the same haplotype for both the β^A and β^C genes (Figure 2b), so that the haplotype of the β^C gene of the propositus must be + - - - - - +.

The two Thai β^C gene haplotypes are identical, even though the two families have their origins in different parts of the country. However, the Thai β^C gene haplotypes differ distinctly from the haplotypes of the β^C genes found in negro populations (Fig. 2c), in particular at the framework region (β/Ava II and 3'β/BamHI sites) in or near the β-gene, being - + instead of + +, suggesting that the β^C genes found in Thailand derive from an independent mutation to the β^C genes found in the Negro population [31].

Family with Hb E/β^{17}-Thalassemia/β^{41-42}-Thalassemia

Proposita "S.S." was a 23 year-old female showing signs of anemia [32]. Her hematological data at the steady state were: Hb 8.6 g/dl; RBC 6.3 x 10^6 /ml; Hct 27 %; MCV 49 fl; MCH 13 pg; MCHC 27 g/dl; Hb F 6.2 %. Cellulose acetate electropho-

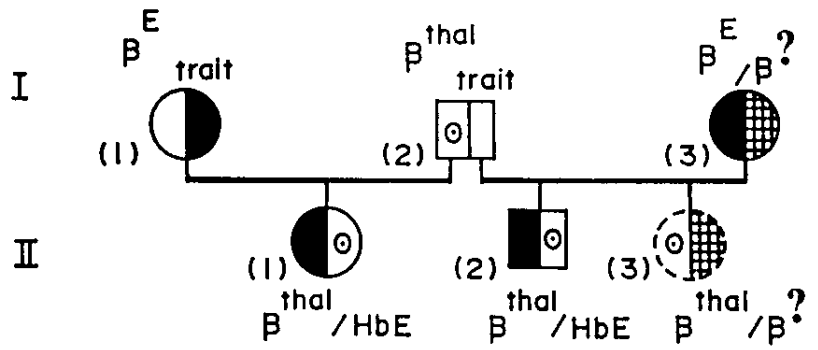

Fig. 3. Family tree of family with β^E (●) , β^{41-42}-thalassemia (⊙), and unknown $\beta^?$ mutation ▨. The propositus I.3 ("S.S.") was further studied and the $\beta^?$ mutation was subsequently shown to be β^{17}-thalassemia.

resis showed one intense band at the same position as Hb E, while the Bio-Rad Variant profile showed peaks due to Hb E and Hb F. The propositus (I.3) married a man (I.2) who was doubly heterozygous for β^{41-42}-thalassemia/ Hb E (Figure 3). The fetus (II.3) was aborted.

Dot blot hybridisation of the fetus (II.3) gave a positive signal to the probe for β^{41-42}-thalassemia, a 4 base pair deletion giving rise to a frameshift mutation at codons 41/42, but gave a negative signal to the β^E mutant probe. This suggests that the fetus has inherited the β^{41-42}-thalassemia gene from the father (I.2). However, the β^E -like gene inherited from the mother appeared not to be β^E . This is in agreement with the dot blot results with the proposita (I.3) , which gave positive signals to both the β^E mutant and the β^E normal probes.

Further studies were performed on the hemoglobins isolated from the proposita. Direct CM-cellulose chromatography of the proposita's hemolysate in 8 M urea was used to prepare β^E-like chains, which were digested with trypsin and subjected to peptide mapping on h.p.l.c. The only difference compared to normal β^A chains was the appearance of a new β^E-T3a peak. This abnormal peptide was collected, hydrolysed with 6 N HCl and analysed. The amino acid composition (Asx 1.93, Glx 0.92, Gly 1.81, Val 3.28, Lys 1.00) was identical to that expected for β^E-T3a (residues 18-26) indicating that the proposita did indeed contain the abnormal Hb E [β26 Glu-Lys]. Attempts to identify other abnormal peptides from the abnormal chains of the proposita were unsuccessful, suggesting that the β^E chain was the only abnormal β-chain that was expressed.

DNA sequence analysis of the proposita's DNA was therefore performed. The results readily indicated that codon 26 of the β-globin gene yielded both AAG (Lys) and GAG (Glu) showing that codon 26 contains both the β^E mutation and the normal β^A sequence, confirming the results obtained by studies with the protein. This indicates that the propositus was not doubly heterozygous for the β^E mutation. Moreover, codon 6 showed no evidence of mutation, indicating that the proposita did not contain the β^C, which we have recently shown to be found in Thailand. More extended DNA sequencing using different primers was repeatedly carried out covering the entire coding region of the β-gene, employing both manual and automated methodologies. These detailed studies indicated the existence of another mutation in the first base of codon 17, where the normal AAG (lysine) has been mutated to TAG, a chain termination codon, giving rise to the well-known β^{17}-thalassemia. This was further confirmed by dot blot hybridisation with a probe specific for the β^{17}-thalassemia mutation. Accordingly, it was concluded that the proposita was doubly heterozygous for HbE/ β^{17}-thalassemia.

In this paper, we have reviewed more recent studies in our laboratory on the abnormal hemoglobins found in Thailand. These have included the

discovery and characterisation of novel variants not previously found in Thailand, namely Hb J Buda [α61 Lys-Asn] and Hb G Hsin-Chu [β22 Glu-Ala]. The latter mutation has been found in several populations, under various names Hb G Coushatta [25], Hb Hsin-Chu [26] and Hb G Saskatoon [27]. In view of the finding of this mutation in Chinese families [26], the ethnic relationship of Thais to Chinese, and Chinese migration to Thailand, it is not surprising that the mutation has now also been found in Thailand. The mutation Hb J Buda is much less common, and was described in Hungary [24]. The family of the propositus appears to have had a Portuguese ancestor, but links to the original mutation are not clear. By using DNA sequence analysis, the mutation has been shown to be located on the α_1 locus: identification of which of the two α-globin genes a mutation occurs on not only has implications on the pattern of inheritance, but is also of important since the expression of the α_1-globin gene is 2-3 fold slower than that of the α_2-globin gene.

Our earlier studies [14, 30] have shown that another slow hemoglobin variant, Hb C, is found in Thailand in addition to Hb E, the major abnormal hemoglobin. Both abnormal hemoglobins have the same amino acid substitution [Glu-Lys], but at different exterior positions on the β-globin chain, and as such show similarities in properties. This raises the question of whether any cases of Hb C have been incorrectly diagnosed as Hb E. To check this, methods are required that will differentiate Hb E and Hb C. As previously noted [32], these methods include citrate-agar electrophoresis and dot blot analysis with a β^E-specific oligonucleotide probe, and in the work described here, h.p.l.c. analysis using the Bio-Rad Variant instrument has been shown to be very effective and rapid. Hb C and Hb E are slow hemoglobins with similar slow electrophoretic mobility, but there are also variants with similar fast mobility. Thus the Hb J Buda [α61 Lys-Asn] described here has a similar fast electrophoretic mobility to the well-known Hb J Bangkok [β56 Gly-Asp], even though the mutation occurs in a different chain.

It was surprising to find Hb C in Thailand, since this variant is very common in African and negro populations. It was therefore interesting to see whether the Hb C mutation found in Thailand was the same as the mutation found in the negro population. This was studied by haplotyping, i.e. comparing the restriction endonuclease digestion patterns in the vicinity of the β-globin gene cluster. The results showed that the β^C genes in the two families found in Thailand had the same haplotype, which differed from the haplotypes reported in the β^C genes reported for the negro population. This suggests that the β^C gene(s) found in Thailand have an independent evolutionary origin to the β^C genes of negro populations, presumably arising from a separate mutation.

Finally, an abnormal hemoglobin may also be associated with another abnormal hemoglobin or with α- or β-thalassemia, and this can affect the clinical manifestations or hematological parameters. In this paper, we describe two families which have more than one hemoglobin mutation. The first family with both Hb E and Hb C stresses the importance of proper diagnosis of these two similar variants, but since both mutations are mild, little was found in terms of pathological changes, except that β^E homozygotes and β^E/ β^C double heterozygotes appear to have rather lower MCV than normal. The second family has the combination of three mutations Hb E, β^{17}-thalassemia, and β^{41-42}-thalassemia. Hb E is the most frequent of the abnormal hemogobins in Thailand, while β^{41-42}-thalassemia and β^{17}-thalassemia are the two most frequent β-thalassemias in many parts of Thailand, so it is perhaps not surprising to find a family with all three mutations. Proposita S.S. was shown to have the β^{17}-thalassemia in double heterozygous association with β^E, and her husband was found to have the β^{41-42}-thalassemia mutation in the heterozygous state. Thus the children born from these parents must have abnormal production of β-chains, with the following genotypes being possible: heterozygous Hb E, doubly heterozygous association of Hb E with β^{41-42}-thalassemia, heterozygous β^{17}-thalassemia, or doubly heterozygous association of the two thalassemia syndromes. After considering the dot blot results, the aborted fetus, is in retrospect likely to have been doubly heterozygous for the two β-thalassemia mutations, β^{17}-thalassemia and β^{41-42}-thalassemia.

In conclusion, novel abnormal hemoglobins have been described not previously reported in the Thai population. Some mutations have similar properties to other hemoglobin variants found in Thailand (e.g. Hb C and Hb E; or Hb J Buda and Hb J Bangkok). Moreover, abnormal hemoglobins may be associated with each other or with α- or β-thalassemia. For proper genetic counseling, correct diagnosis of the hematological defects is essential, and in some cases, this requires detailed analysis at the protein and DNA level, which will allow the detection of genetic abnormalities not only when found separately, but also when found in combination with each other. This confirms the continuing importance of characterising abnormal hemoglobins found in Thailand.

Experimental

The methodology used here essentially follows the procedures described in our previous reports [14]. Venous blood was collected with heparin as anticoagulant and hematologic data were obtained by standard procedures. Electrophoretic analysis of Hb and isolated chains were performed on cellulose acetate strips at pH 9.1 under non-denaturing conditions or at pH 6.5 under denaturing conditions. Identification of Hb variants by h.p.l.c. was performed on a Bio-Rad Variant instrument (Bio-Rad Diagnostics Group, Hercules, CA, U.S.A.). Hbs were isolated by DEAE-cellulose chromatography and globin chains were separated by CM-cellulose chromatography in 8 M urea-mercaptoethanol. Tryptic peptide maps were fractionated by high performance liquid chromatography (h.p.l.c.) on a Waters 510 instrument (Millipore Corp., Milford, MA, U.S.A.): amino acid compositions were determined using the Waters Pico Tag system for hydrolysis and derivatization, followed by h.p.l.c. analysis. Sequence analysis of isolated β chains was performed on a Model 473A protein sequencer (Applied Biosystems Inc., Foster City, CA, U.S.A.). DNA was isolated from peripheral leukocytes by phenol-chloroform extraction and segments of the α-globin and β-globin gene were amplified on a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT, U.S.A.), using appropriate primers. Then an aliquot of the amplified DNA from the first amplification was further amplified to obtain the sense stranded DNA, which was directly sequenced by the dideoxynucleotide chain termination method, using [^{35}S]dATP and T_7 polymerase. Haplotype analysis of DNA polymorphisms on the β-globin gene cluster was performed by amplification of the seven DNA segments containing each of the common polymorphic restriction sites within the β-globin gene cluster, using different sets of oligonucleotide primers. These included the Hinc II site 5' to the ε-globin gene, the Hind III sites within the $^{G}\gamma$- and $^{A}\gamma$-globin genes, the Hinc II sites within and 3' to the ψ β-globin gene, the Ava II site in the β-globin gene, and the Bam HI site 3' to the β-globin gene.

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