A Rare Super-Unstable Hemoglobin Variant, Hb Monroe [β30(B12)Arg→Thr], Found in Two Myanmar Children

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ABSTRACT. During the making of a precise molecular diagnosis of blood from transfusion dependent anemic patients in the Union of Myanmar by the PCR-sequencing technique, we encountered two Myanmar children (My-194 and My-201) with a rare abnormal hemoglobin, Hb Monroe [β30(B12)Arg→Thr], which causes β^0-thalassemia due to missplicing in the course of mRNA processing. One of the children, My-194, was a compound heterozygote for Hb Monroe and a thalassemia mutation of βIVS I-5 G→C, who had a condition of severe β^+-thalassemia resembling a β^0-thalassemia, while the other, My-201, a homozygote for Hb Monroe. However, Hb analyses of My-194 with IEF and DEAE-HPLC revealed a normal pattern, suggesting that it may be due to blood transfusion. Hb analyses of My-201 showed the presence of Hb A to be about 0%, with Hb F being the main Hb component, suggesting that in this case Hb F instead of Hb A may be produced as the main Hb.

Key words : PCR-sequencing — Hb analysis — Union of Myanmar — Hb Monroe — β-thalassemia

β-Thalassemia (thal) is a group of hereditary disorders characterized by a degree of absence (β^0-thal) or reduction (β^+-thal) of the β-globin biosynthesized in blood cells. It presents with a wide spectrum of clinical and hematological features depending on the nature of the β-thal gene.[1] The development of techniques for determining the molecular mutation in thal genes has made it possible to make a more precise genetic diagnosis. There are various kinds of thals with one point mutation which creates a stop or nonsense codon, deletion or insertion of one or two nucleotides in the region of the exon causing a frame shift, or a nucleotide mutation in the consensus sequence in the border between the exon and intron (IVS).[1]

During the making of a precise molecular diagnosis of blood from transfusion dependent anemic patients in the Union of Myanmar,[2,3] we discovered two patients (My-194 and My-201) with a rare hemoglobin (Hb) variant identified as Hb Monroe [β30(B12)Arg→Thr] associated with β^0-thal.

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One of them, My-194, was a compound heterozygote for Hb Monroe and a severe β^+-thal mutation of βIVS I-5 G→C, and the other, My-201, was a homozygote for Hb Monroe.

MATERIALS AND METHODS

Blood samples of transfusion dependent anemic patients (aged 9 months to 12 years old) who visited the Health Care Room of the Children's Hospital, Yangon, Union of Myanmar, were collected into tubes containing heparin and diagnosed briefly as suspected of β-thal from morphological observation of red cells. After separation of the red cell pellet from plasma by centrifugation, they were both frozen and brought to the Department of Biochemistry, Kawasaki Medical School, Okayama, Japan. The hemolysate prepared from a part of the red cell pellet was analyzed by isoelectric focusing method (IEF, pH range 6-9) on a polyacrylamide gel plate containing carrier ampholytes[4] and DEAE-HPLC on DEAE-5PW column (7.5×75 mm, Tosoh Co., Tokyo, Japan)[5] to determine the proportion of Hb components. Separation of the globin chain was performed on CM-cellulose (CM-52, Whatman Paper Co., Kent, UK) by a procedure described previously.[6] DNA was extracted from the red cells with a DNA extraction kit (QIAamp DNA Mini Kit, Quiagen Co.).[7] After the DNA was amplified by use of a specific primer set for the β-globin gene, the DNA sequence was determined by the BigDye terminator method (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits, PE Corp., Tokyo, Japan).[8] Haplotypes at the seven positions of the β-globin gene cluster were determined by 1%-agarose gel electrophoresis in TEA buffer (pH 8.3) of the PCR product digested with the appropriate endonuclease.[9] The combinations of the sites with endonuclease were as follows (site of globin/enzyme) : ε/Hinc II, $^G\gamma$/Hind III, $^A\gamma$/Hind III, in $\psi\beta$/Hinc II, 3'-$\psi\beta$/Hinc II, in β/Ava II, and 3'-β/Bam HI. In amplifying and sequencing of the globin gene, oligonucleotides synthesized by Amersham Pharmacia Biotech Japan were used.

RESULTS

These Hb samples were not expected to show normal electrophoretic and chromatographic elution patterns on IEF and DEAE-HPLC, since they were stored in a freezer for a long time after collection. However, some significant informations from these data indicating Hb components were obtained.

IEF of the hemolysate from My-194 showed no abnormal Hb bands (Fig 1), while the Hb proportions estimated by DEAE-HPLC were Hb A_2 1.5% (slightly lowered than the normal level ; 2.2-3.4%), Hb A 90% and aging Hb 8.5% (Fig 2a). On the other hand, IEF of My-201 showed no presence of the Hb A band, appearance of the thin band of Hb A_2 and the main band of Hb F (Fig 1). Their concentrations as determined by DEAE-HPLC were Hb A_2 1%, Hb F 92%, and other Hb 7% (Fig 2b). From these data, it was concluded that these patients are not carriers of Hb E, although a number of carrier live in Myanmar and in the countries bordering Myanmar.

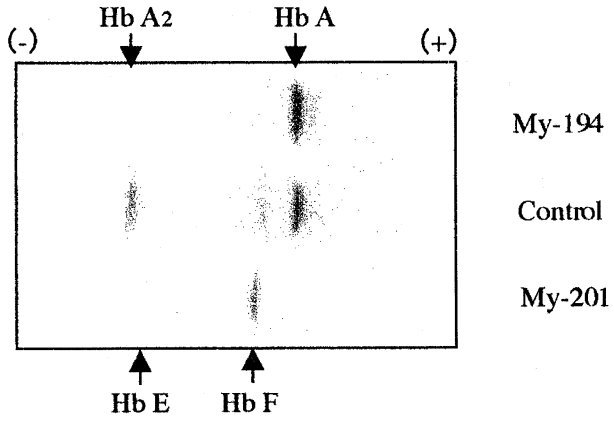

Fig 1. IEF of the hemolysates (pH renge : 6-9) from My-194 and My-201. The hemolysate from a carrier of Hb E and high Hb F was used as a control.

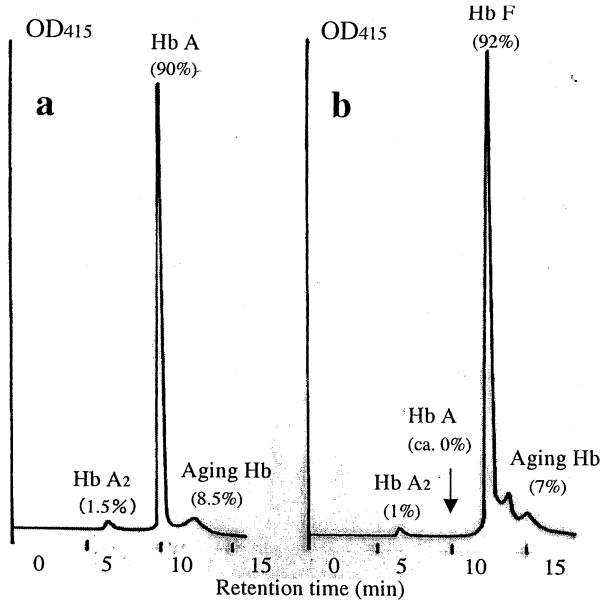

Fig 2. Hb analysis on DEAE-HPLC. a : The case of My-194. b : The case of My-201.

Globin from these patients was analyzed by CM-cellulose column chromatography, but no abnormal globin chains without the normal α, β, and γ chains appeared, suggesting that the main Hb bands observed on IEF and DEAE-HPLC were Hb A in My 194 and Hb F in My 201.

The nucleotide sequencing of the amplified β-globin gene of My-194 showed two mutations of G→C at the fifth position to intervening sequence 1 of the β-globin gene (βIVS I) and G→C at the adjacent position to the donor splice site of βIVS I (indicated by βIVS I-(-1)), indicating a compound heterozygote. The former nucleotide change is considered to be a severe type of β^+-thal[10] and the latter to be an abnormal Hb identified as

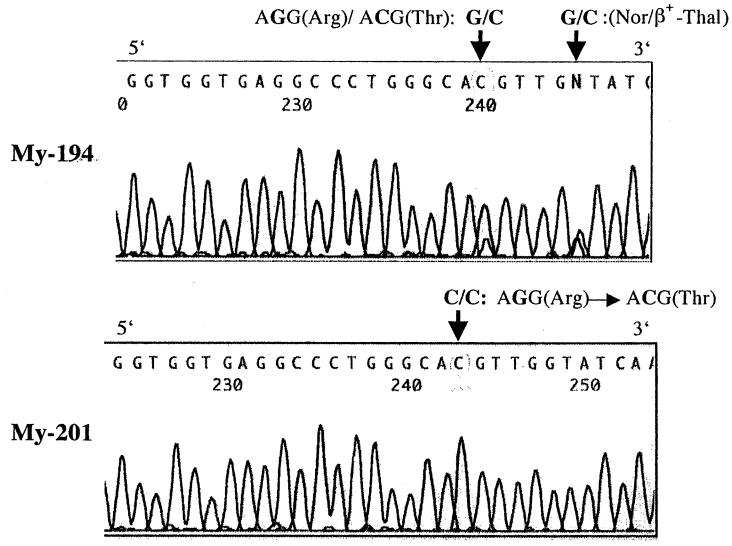

Fig 3. DNA sequencing of the region splice junction between the exon 1 and intron 1 (IVS I) of the β-globin gene.

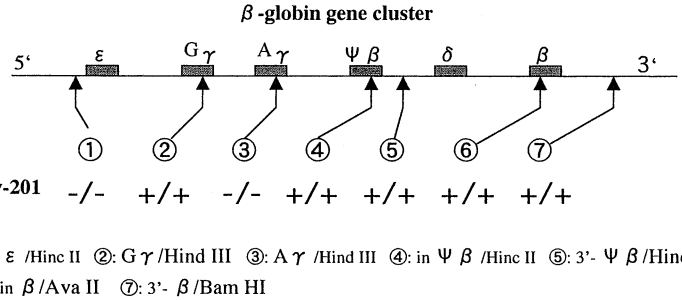

Fig 4. Haplotype at seven position of the β-globin gene cluster in the case of My 201 (the presence of the restriction endonuclease site was indicated by (+) and the absence of it by (−)).

Hb Monroe [β30(B12)Arg→Thr][11] (Fig 3). Hb Monroe has a nucleotide change, one of the consensus nucleotide sequences of the donor splice site, which causes severe β-thal that resembles β^0-thal.[11] The nucleotide sequence of My-201 showed only a mutation of G→C at βIVS I-(-1) or the second nucleotide of the codon 30 AGG, suggesting that this patient was homozygous for Hb Monroe (Fig 3). The haplotype at the seven positions of the β-globin gene cluster of My-201 showed only one type (− + − + + + +), indicating the patient was homozygous for Hb Monroe (Fig 4).

DISCUSSION

The patients investigated here were suspected to have β-thal from a morphological study of their peripheral red cells, but no hematological or clinical findings prior to transfusion dependence were available. However, a Hb study of their hemolysates on IEF and DEAE-HPLC provided some

informations ; namely, one patient, My-194, had a lower level of Hb A_2 and the other, My-201, thad a high Hb F value (ca. 92%) as the main Hb and a lower level of Hb A_2. The separation of globin on CM-cellulose chromatography showed no abnormal globin chains without the normal α, β, and γ-globin chains, indicating they are not carriers of abnormal Hb, or Hb E, which is commonly observed in this area.

Nucleotide sequence analysis of DNA extracted from the red cells of My-194 showed nucleotide changes at two positions, one of which was from G to C adjacent to the donor splice site of the first intron [βIVS I-(-1)] of the β-globin gene and another from G to C at β-IVS I-5. The former change results in an amino acid substitution of Arg→Thr in Hb Monroe because of the nucleotide change of AGG→ACG, a variant associated with β-thal, corresponding to a super-unstable Hb variant, which can not be recognized in the peripheral blood.[11-13] The latter corresponds to β^+-thal, being considered a severe type of β-thal.[10] Hb Monroe [β30(B12)Arg→Thr] should be compared with Hb Tacoma [β30(12)Arg→Ser], which was discovered in European family and characterized in 1969,[14] and was also observed in Japanese female in 1985.[15] Heterozygotes for Hb Tacoma have a normal hematology with Hb A_2 and Hb F levels in the normal range without any signs of β-thal, although Hb Tacoma is slightly unstable and occurs in high quantities (about 40%) in heterozygotes. However, codon 30 (for arginine) is interrupted between the second and third nucleotide by the βIVS I of 130 nucleotides (Fig 5).[11-13] Mutations of the consensus sequence of the donor splice site of IVS I (CAG/GTTGGT) will affect the process of splicing[16] (Fig 5). For instance, a G→T or a G→A substitution[10,17] and a T→C of a T→G substitution at the βIVS I-1 and βIVS I-2[18,19] will abolish splicing and lead to a β^0-thal. Mutations at βIVS I-5 (G→C, G→T, G→A)[20-22] result in greatly decreased splicing and a severe β^+-thal. In Hb

β-globin gene

	Exon 1		IVS I														Exon 2
Codon	29	30	1	2	3	4	5	6	-	-	-	-	-	130			31
Normal sequence	GGC	AG	G	T	T	G	G	T	-	-	-	-	-	AG		G	CTG
Amino acid	(Gly)	(Ar														g)	(Leu)
IVS I-1 (G→T)	GGC	AG	T	T	T	G	G	T								G	
IVS I-1 (G→A)	GGC	AG	A	T	T	G	G	T								G	
IVS I-2 (T→C)	GGC	AG	G	C	T	G	G	T								G	
IVS I-5 (G→C)	GGC	AG	G	T	T	G	C	T								G	
IVS I-5 (G→T)	GGC	AG	G	T	T	G	T	T								G	
Consensus sequence	C/A	AG	G	T	A/G	A	G	T									
Hb Monroe [β30(B12)Arg→Thr]	C	AC	G	T	T	G	G	T								G	
Hb Tacoma [β30(B12)Arg→Ser]	C	AG	G	T	T	G	G	T								C or T	

Fig 5. Comparison of the 5' consensus splice sequence and the donor splice site of the IVS I of the β-globin gene. Several nucleotide substitutions leading to β^0- or β^+-thal and those resulting in the formation of the β chains of Hb Monroe and Hb Tacoma.

Monroe, the G→C mutation occurs at a nucleotide position adjacent to the required GT dinucleotide, and this substitution can be expected to cause a greatly decreased splicing at this site, resulting in a severe type of β^+-thal. In the case of Hb Tacoma heterozygotes, however, a G→C or a G→T mutation at the third position of codon 30 will not affect splicing at the normal donor site, as this nucleotide is located 3' to the βIVS I (Fig 5). The codon ACG is specific for Hb Monroe and its associated severe β^+-thal. The patient My-194 was a compound heterozygote for Hb Monroe and mutation of βIVS I-5 G→C, with both mutations associated with severe β-thal. As an inevitable consequence, the patient might need blood transfusion. The patient My-201 was diagnosed as a homozygote for Hb Monroe, which was also confirmed by determination of the haplotype at the seven positions of the β-globin gene cluster. The patient must also have some signs of a severe β-thal condition, but a higher production of Hb F might reduce severe anemia such as observed in the condition of hereditary persistence of fetal hemoglobin (HPFH). However, in this case, more precise hematological and clinical observations should be made in the future.

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