Adult Onset, Chronic Anemias of Undermined Etiology, Myanmar

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ABSTRACT. Qualitative and quantitative analysis of hemoglobin (Hb) was done by DEAE-HPLC and IEF in six Myanmar patients who had been diagnosed clinically and treated as chronic refractory anamia, most probably thalassemia (Thal) or hemoglobinopathy. All of them were having microcytic hypochromic type of anemia for a long duration varying from two years to more than 15 years. Hb A was detected in all cased and abnormal Hb, Hb E, was associated in four cases. Hb A_2 was measured in two cases in whom Hb E was not associated and found to be 1.5% and 1.1%, respectively. Hb E was not detected in three cases but it was markedly increased in other three cases (18.4%; 21.3%; and 48.1%, respectively). Amplification refractory mutation system (ARMS) was applied for DNA analysis using five primer sets for common Thal mutations of Myanmar. Restriction enzyme assay of PCR products confirmed the associated Hb E in four cases. Compound heterozygosity of Hb E and β^0 or β^+ Thal allele was observed in three cases. Direct sequencing of PCR products or ARMS using additional primers sets for the Thal mutations of near by countries was suggested to obtain a better coverage of detection of Myanmar Thal mutations.

Key words: chronic anamia — thalassemia mutations — Myanmar — Hb analysis — DNA analysis

There are high prevalence of hemoglobin E (Hb E) and various types of β and α thalssemias (Thal) in Southeast Asia region including Myanmar.[1-3] We have initiated a multi-purpose Myanmar-Japan collaborative study on Myanmar thalassemia. There, six cases are found to have chronic refractory anemias but accurate diagnosis has not been established. They have been diagnosed clinically as abnormal hemoglobinopathy or Thal intermedia. Since laboratory facility is limited, the diagnosis is made mainly on clinical ground supported by some hematological and blood film examinations. Further characterization of Hb abnormalities and globin gene mutations has performed in Japan. Blood sampling is accomplished before transfusion and treatment has started for that hospital admission.

After the preparation of hemolysate from red call pellet, DEAE-high performance liquid chromatography (DEAE-HPLC) on DEAE-5PW (7.5×7.5

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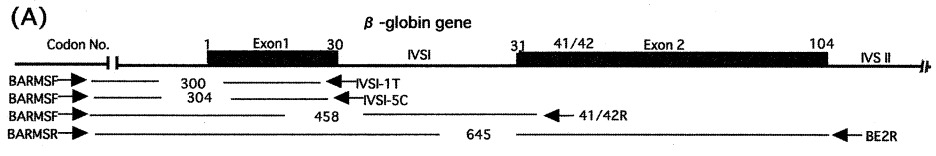
mm, Tosoh Co. Ltd., Tokyo, Japan)[4] and isoelectric focusing (IEF) on polyacrylamide gel plate containing carrier ampholytes (pH range: 6-9)[5] have performed for the qualitative and quantitative assessment of Hbs. DNA is extracted from blood cells by using QIAamp DNA Miki Kit[6] and used in ARMS.[7] Five primer sets are used in this study (Fig 1). These are selected for the screening of common mutations of Myanmar β-Thal patients[8] such as CD 41/42 -TCTT, IVS I-5 (G → C), CD 17 (A → T), IVS I-1 (G → T), and IVS II-654 (C → T). The presence of β^E-gene was confirmed by restriction enzyme assay (Mnl I) of PCR products as described previously.[9]



Detection	Primer set	Sequence of primer	PCR size (bp)
Internal Control	BARMSF BE2R	5'-CACGGCTGTCATCACTTAGACCTCACCCTG-3' 5'-ATCAAGGGGTCCCATAGACTC-3'	645
IVSI-1(G -> T)	BARMSF IVSI-1T	5'-TTAAACCTGTCTTGTAACCTTGATACCAAA-3'	300
IVSI-5(G->C)	BARMSF IVSI-5C	5'-CTCCTTAAACCTGTCTTGTAACCTTGATAG- 3'	304
41/42 (-TCTT)	BARMSF 41/42R	5'- GGAGTGGACAGATCCCCAAAGGCTCAACCTC-3'	458

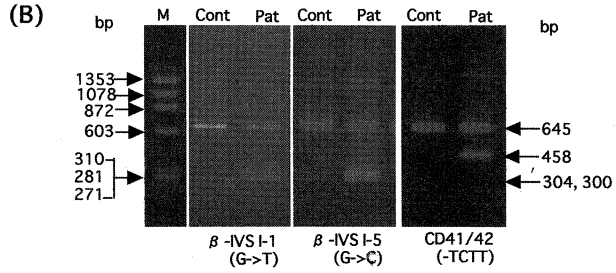


Fig 1. Selection of primer sets for ARMS
(A) A portion of β-globin gene showing position of primers for detection of mutant.
(B) ARMS positive patients observed agarose gel electrophoresis [from left to right: Case 3: β-IVS I-1 (G → T); Case 5: βIVSI-5 (G → C); and Case 6: codon 41/42 (-TCTT deletion)].
M: Molecular maker. Cont: Control. Pat: Patient. bp: base pair.

Case 1:

A 30 year old male was suffering from easy fatigue and lethargy for two years duration. He got five units of blood within the last three months for anemia (total Hb concentration 5.1 g/dl). Peripheral blood film revealed hypochromia, microcytosis, a few target cells, low grade anisopoikilocytosis and occasional nucleated red cells. DEAE-HPLC showed Hb A_2 (1.5%) at a lower content, Hb A (93%) and aging Hb (5.5%). IEF demonstrated a marked Hb A band and afaint Hb A_2 band. DNA analysis detected neither Hb E nor β-Thal mutation.

Therefore structural abnormality of Hb was unlikely but possibility of other type of alleles could not be excluded yet in this case including α-Thal.

Case 2 :

A 24-year old single, university teacher, was presented with a pallor and a tinge of jaundice which was associated with dyspnea on exertion off and on for two and a half year duration. Blood for complete picture showed predominantly hypochromic microcytic anemia and Hb F was raised to 24%. So she was clinically and provisionally diagnosed as thalassemia intermedia and conservative treatments were initiated. She gave no history of blood transfusion until this sample collection.

DEAE-HPLC showed Hb A_2+Hb E (76.2%). Hb A (2.49%) and Hb F (21.3%). In the IEF, there were prominent band of Hb E, a clear band of Hb F and a faint slow moving like band (oxidized Hb E ?). These findings were consistent with Hb E-β^0-Thal. However, DNA work documented the presence of β^E-gene only. ARMS was negative for common mutations of Myanmar β-Thal.

Further ARMS using additional primer sets and/or direct DNA sequencing might demonstrate the β-Thal mutation in this patient.

Case 3 :

A 30-year old, Muslim male, was transfused infrequently (five units within 12 years) for the attacks of general weakness and easy fatigue caused by anemia. Hematological investigations showed hypochromia and microcytosis and raised Hb F level to 15%. Since Hb electrophoresis was not done, abnormal Hb could not be elicited.

DEAE-HPLC provided the presence of Hb A_2+Hb E in (28.8%), Hb A (52.7%), and Hb F (18.4%). The Hb types seen in IEF were also the same to HPLC. This Hb phenotype was suggestive of Hb E-β^+-Thal. DNA analysis showed β^E-gene, and IVS 1-5 (G $\rightarrow$ C) mutation.

IVS 1-5 (G $\rightarrow$ C) was a splicing mutant causing β^+-Thal. In this patient Hb A content was higher than that of expected, probably the donor's blood. Hb F level was expectedly increased. Although it was interesting to know the Hb A_2 content, its quantitation was very difficult when Hb E was associated.

Case 4 :

A 47-year old Buddhist male, a geologist with a history of government service in many malaria endemic areas for several years duration, suffered from attacks of pallor and jaundice associated with tiredness off and on since about 15 years ago. He was treated as chronic malaria during the attacks for the history of traveling to malaria endemic areas. After radical cure treatment of malaria clinical malaria attacks like fever with chills and rigor, loss of weight etc. subsided. However. signs and symptoms of anemia were persisted and repeated peripheral blood film examinations revealed hypochromic microcytic type of anemia. Other special hematological examinations like paroxysmal nocturnal hemoglobinuria (PNH) screening, blood for malaria parasites, coombs test, showed negative. Cellulose acetate Hb electrophoresis did not show Hb F and abnormal hemoglobin. So the definite diagnosis could not be established yet. Meanwhile he was under conservative treatment and a total of 18 units of blood have been given.

DEAE-HPLC demonstrated Hb A_2 (1.1%), Hb A (79.1%), and aging or denatured Hb (19.1%). A thick Hb A, very faint Hb A_2, and a clear fast

moving bands were noted in IEF. All the Hb fractions were apparently normal and detailed analysis of Hb and molecular analysis of globin gene showed neither abnormal Hb nor thalassemic mutations. Since Hb A_2 content is low, the possibility of α-Thal can not be excluded. DNA sequencing should confirm the presence or absence of Thal mutation in this case.

Case 5 :

A 30-year old lady gave a history of pallor and tiredness since about 10 years ago. She lived in a remote area and was admitted frequently to a near by hospital to undergo some symptomatic treatments. Some routine hematological tests available there showed features of hypochromic microcytic anemia. No improvement was obtained with vitamin supplements and hematinics. Transfusion therapy was started irregularly since eight years ago with a total of 18 units at the time of this study. Neither special hematological investigation nor Hb electrophoresis had been done before.

DEAE-HPLC showed two high peaks for Hb E and Hb F (46.8% and 48.1%, respectively) and a small peak of degraded Hb (?) with a concentration of 5.1% at the usual site for Hb A. The marked bands for Hb E and Hb F were observed in IEF. These findings were consistent with Hb E-β^0-Thal. Restriction enzyme analysis showed β^E globin gene and ARMS was positive for IVS 1-1 (G $\rightarrow$ T) mutation.

This mutation in the GT dinucleotide abolishes normal splicing and no normal mRNA is formed causing β^0 Thal. It is also common in Asian Indians[10] but the detailed and exact ethnicity of this patient is not known.

Case 6 :

A 19-year old girl had six units of blood within last two years as a symptomatic treatment for weakness, tiredness, and scanty menstruation. The investigations done were few and less reliable since she lived in a village where a station hospital was accessible. After the admission to Yangon General Hospital some special hematological tests could be done and the results indicated to diagnose her as a case of Thal intermedia. She had no history of malaria, G-6PD deficiency and metabolic diseases. DEAE-HPLC and IEF elicited an abnormal looking Hb peak in and around the area of Hb A_2 (and/or Hb E) with a relative concentration of 55.0%, Hb A (42.2%), and an aging Hb (2.8%). No Hb F was detected. Thus Hb phenotype was consistent with Hb E carrier state and a discrepancy with clinical diagnosis was occurred. The presence of β^E globin gene was established by restriction enzyme analysis. ARMS showed codon 41/42 (-TCTT) four nucleotides deletion of β globin gene. Thus the genotype of this case was Hb E-β^0-Thal.

If this case was considered as Hb E-β^0-Thal, we could not exclude the possibility of recent transfusion with the blood of Hb E carrier or thalassemia trait because Hb A cencentration was too high to be a β^0-Thal. It was important to be assured that blood sample must be taken after a minimum of four months of last transfusion. Hb E as well as thalassemia traits was highly prevalent among the general population in Myanmar.[11]

This report was first document for application of ARMS in the molecular study of Myanmar Thal. All cases presented here were of adult onset chronic refractory anemia and clinical and hematological features are heterogeneous.

With a support of limited laboratory facility, clinicians concerned could only be diagnosed these cases as chronic hemolytic anemia probably Thal intermedia and/or hemoglobinopathies on the clinical ground. From this study we could provide a definitive molecular diagnosis in three cases as Hb E-β-Thal compound heterozygotes and the presence of Hb E could be confirmed in four cases. As mentioned, we used the five sets of primers for ARMS which might cover than 90% of mutations occuring in β-Thal of Myanmar.[8] Some more primer sets should be added to ARMS to get a better coverage in the study of Myanmar Thal mutation. Additional primers for CD 8/9 +G, −28 Cap (A → G), codon 19 (A → G), 619 bp deletion, were suggested. Hence these mutations are common in neighbouring countries like China, India, Bangladesh and Thailand.[12]

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