Molecular Aspects of Transfusion Dependent Thalassemic Children in Myanmar: Analysis of Common β-Thalassemia in Myanmar by Amplification Refractory Mutation System (ARMS)

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ABSTRACT. We investigated 112 DNAs from transfusion dependent thalassemic children (68 boys and 44 girls; from 9 months to 12 years of age) of Myanmar, using amplification refractory mutation system (ARMS) for five β-thalassemia (β-Thal) mutations in Myanmar. The β^E-globin gene producing Hb E was detected by PCR-enzyme (Mnl I) analysis and it was positive in 66 cases. ARMS demonstrated (a) 19 cases (17%) were of homozygotes [4 cases of CD41/42 (-TCTT) deletion, 11 cases of IVS I-1 (G → T) mutation and 4 cases of IVS I-5 (G → C) mutation], (b) 62 cases (55.4%) were of compound heterozygotes [58 cases of Hb E/β^0 or β^+-Thal (93.5%) and 4 cases of β^0/β^0 or β^+-Thal (6.5%)] and (c) 18 cases (16.1%) of heterozygotes [8 cases of Hb E, 3 cases of CD 41/42 (-TCTT) deletion, 2 cases of IVS I-5 (G → C), and 5 cases of IVS I-1 (G → T)]. Thus ARMS could diagnose in 81 samples (72.3%) of the cases as either homozygous β-Thal or compound heterozygotes. Thirteen cases with negative ARMS and 18 heterozygotes need further characterization. The application of ARMS was an effective and rapid method for the identification of five β-Thal mutations [(above three and additional two mutations of CD 17 (A → T) and IVS II-654 (C → T)] which were common in Myanmar.

Key words: ARMS — transfusion dependent — β-thalassemia — Myanmar

Diagnosis of thalassemias (Thal) has been enhanced greatly by the advent of DNA diagnostic methods, particularly polymerase chain reaction (PCR). We conducted a study on Thal mutations among transfusion dependent children who attended the day care room of out-patient department, Yangon Children Hospital, Yangon, Union of Myanmar. A modification of PCR, amplification refractory mutation system (ARMS),[1] which is advantageous for the detection of the known mutation gene and analysed by only electrophoresis after PCR reaction, was used for the identification of mutations [IVS I-5 (G → C), CD41/42 (-TCTT) deletion, IVS I-1 (G → T), CD17 (A → T), IVS II-654 (C → T)] which were known as common β-Thal in Myanmar.[2]

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MATERIALS AND METHODS

Report of the study: The study population consisted 112 blood samples of Myanmar children patients (68 boys and 44 girls), aged 9 months to 12 years, who were transfusion dependent for chronic refractory anemia. Various major ethnic groups like Bama, Kayin, Shan, Rakhine, Mon, Kachin, were involved. All of these children were clinically diagnosed as severe Thal either Hb E/ β-Thal or Thal major and were under interval blood transfusion to maintain the Hb level about 6-7 g/dl. The duration of blood transfusion varied from one month to more than 10 years. Then total blood units already transfused also varied from two more than 200 units.

Procedure of DNA Analysis (PCR or ARMS): Genomic DNA was isolated from peripheral blood.[3] The reaction for ARMS or PCR was performed as following procedure: A mixture of 2 μl 10×of PCR buffer, 3.2 μl of 1.25 mmol/l dNTPs solution, 8 pmol each of oligonucleotide primer (2 control primers, 1 mutant primer), 0.2 μl of Gold Taq DNA polymerase (1 U), and 1 μl of genomic DNA (20~50 ng) and distilled water to 20 μl in total volume was set in a GeneAmp PCR System (Model 2400, Perkin-Elmer Applied Biosystems, Japan). After activation of the enzyme at 95°C for 15 min, the reaction mixture was subjected to 25-35 cycles of denaturation at 95°C for 1 min, annealing (55~65°C) for 30 sec, and extension at 72°C for 2 min, with a final extension period for 10 min at 72°C. Fifteen μl of PCR products added with 2 μl of loading buffer (0.25% bromophenolblue-50% glycerol in TE buffer) was electrophoresed on 2% NuSieve 3: 1 agarose gel (Bio Whittaker Molecular Applications, Rockland, ME, USA) at 50 volts for 40 min. Then the gel was stained with ethidium bromide for 30 min and photographed under ultraviolet illumination.

The oligonucleotides used for ARMS and PCR product sizes are listed in Table 1. One primer set, A and B, used as internal control for all mutants (except for IVS II-654 for which primer set C and D was used).

For the detection of homozygosity, another PCR was done in ARMS positive cases using normal sequence primers.

The β^E-globin gene was detected by Mnl I digestion of PCR products as described previously.[4]

TABLE 1. Nucleotide sequence in the primer used for β-globin DNA amplification

Primers	Sequence	Product size
Control A:	5' TAC GGC TGT CAT CAC TTA GAC CTC ACC CTG 3'	
Control B:	5' ATC AAG GGT CCC ATA GAC TC 3'	645 bp
Control C:	5' GAG TCA AGG CTG AGA GAT GCA GG 3'	
Control D:	5' ACC TCT TAT CTT CCT CCC AC 3'	829 bp
Mutant Primers	**Sequence**	**Product size**
CD17 (A → T) :	5' CTC ACC ACC AAC TTC ATC CAC GTT CAC CTA 3'	259 bp
IVS I-1 (G → T):	5' TTA AAC CTG TCT TGT AAC CTT GAT ACC AAA 3'	300 bp
IVS I-5 (G → C):	5' CTC CTT AAA CCT GTC TTG TAA CCT TGA TAG 3'	304 bp
CD 41/42 (-TCTT):	5' GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT 3'	458 bp
IVS II-654 (C → T):	5' GAA TAA CAG TGA TAA TTT CTG GGT TAA GGT 3'	829 bp

RESULTS

Polyacrylamide gel electrophoresis of the DNA fragment digested PCR product with Mnl I gave positive results in 66 samples (58.9%), and were carriers heterozygous with Hb A, (Hb E/Hb A). There was no homozygotes of Hb E (Hb E/Hb E).

The β-Thal mutations obtained by ARMS were shown on Table 2. Nineteen cases (17%) were homozygous for three types of β-Thal mutations [4 cases of CD 41/42 (-TCTT) deletion, 4 cases of IVS I-5 (G → C) and 11 cases of IVS I-1 (G → T)]. Fifty-eight cases (51.8%) were Hb E/β-Thal compound heterozygotes [21 cases with CD 41/42 (-TCTT), 15 cases with IVS I-1 (G → T), 13 cases with IVS I-5 (G → C), 8 cases with CD 17 (A → T), and one case with IVS II-654 (C → T)].

Four cases (3.6%) were compound heterozygotes of the reaction between IVS I-1 (G → T) and CD 17 (A → T) and CD 41/42 (-TCTT) deletion, [2 cases of CD41/42 (-TCTT) / IVS I-1(G → T) and 2 cases of CD17 (A → T) / IVS I-1 (G → T)].

TABLE 2. β-thalassemia mutations in transfusion depedent patients with homozygous β-thalassemia (n=112)

	Type of mutation	No.
Homozygous :		
	CD41/42 (-TCTT) deletion	4
	IVS I-1 (G → T)	11
	IVS I-5 (G → C)	4
Compound heterozygote :		
	Hb E/CD41/42 (-TCTT) deletion	21
	Hb E/IVS I-1 (G → T)	15
	Hb E/IVS I-5 (G → C)	13
	Hb E/CD 17 (A → T)	8
	Hb E/IVS II-654 (C → T)	1
	CD 41/42 (-TCTT)/IVS I-1 (G → T)	2
	CD 17 (A → T)/IVS I-1 (G → T)	2

DISCUSSION

All samples (n=112) were first investigated for DNA diagnosis by ARMS, using specific primers for five out of the six common β-Thal mutations [except -28Cap (A → G)] previously identified in Myanmar[2] and by PCR-enzyme (Mnl I) digestion analysis for β^E-grobin gene which synthesize Hb E. At the time of this study the control DNA for -28Cap (A → G) (β^+-Thal) was not available and it was, therefore, impossible to include in this protocol. The initial investigation allowed to identify compound heterozygosity, either Hb E/β^0 or Hb E/β^+ or β^0/β^+-Thal in 81 out of the 112 samples (72.3%). Remaining 31 children (27.7%) were also transfusion dependent, and so, their genotypes were expected to be either homozygosity (β^0 or β^+-Thal) or compound heterozygosity (either

with Hb E or with β-Thal mutations each other). Thus, their PCR products were subjected to DNA sequencing to meet expected genotypes.

The spectrum of β-Thal mutations in Myanmar seems to be more heterogeneous than reported previously,[2] which was considered as the blood samples collected were from different racial and ethnic groups. Hb E-β-Thal is found to be the majority of transfusion dependent thalassemic children in Myanmar, reaction with CD 41/42 (-TCTT) deletion is most commonest followed by IVS I-1 (G → T) and IVS I-5 (G → C) mutations, respectively (Table 2). Although the carriers of β-Thal mutation genes were found in high frequency, β-Thal homozygosity was observed only in 17% of the cases, and may be due to less common marriage consanguity in Myanmar.

In this study, we investigated only five common mutations and abnormal Hb, Hb E, in 112 transfusion dependent children. According to the review of literature α-Thal mutation is more common than β-Thal mutation[5] and thus any from of α-Thal (α-Thal-1, α-Thal-2, Hb H) should be expected to be associated with β-Thal mutations and with Hb E as well. Our study will be continued to that area of research later.

The application of the ARMS to analysis of Thal mutant gene has advantages such as direct identification of any point mutations, the procedure is relatively simple, safe and accessible to every clinical laboratory. ARMS has provided an accurate and rapid diagnosis of the β-Thal mutations occurring in transfusion dependent clinically diagnosed Thal children of Myanmar.

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