Spectrum of Beta-thalassaemia Mutations in Transfusion Dependent Thalassaemia Patients: Practical Implications in Prenatal Diagnosis

E. George, FRCPA*
R. George, FRCP**
W.A. Ariffin, FRCP**
A.B. Mokhtar, DCP*
Z.A. Azman, BSc*
K. Sivagengei, DipMLT*

* *Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur*
** *Currently attached to the Department of Paediatrics, University Hospital, 59100 Kuala Lumpur*

Summary

The study concerned the identification of the beta-thalassaemia mutations that were present in 24 patients with beta-thalassaemia major who were transfusion dependent. The application of a modified polymerase chain reaction, the amplification refractory system (ARMS) was found to be an effective and rapid method for the identification of the beta-thalassaemia mutations. Six different mutations were detected. Seventy five percent of the patients were Chinese-Malaysians and showed the commonly occurring anomalies:

1. frameshift codon 41 and 42 (-TCTT);
2. the C to T substitution at position 654 of intron 2 (IVS-2);
3. the mutation at position -28(A to G); and the nonsense mutation A to T at codon 17.

In the Malays, the common mutations seen were:

1. the G to C mutation at position 5 of IVS-1;
2. the G to T mutation at position 1 of intron 1 (IVS-1); and the A to T at codon 17.

The delineation of the specific mutations present will enable effective prenatal diagnosis for beta-thalassaemia to be instituted.

Key words: Beta-thalassaemia major, prenatal diagnosis, amplification refractory system.

Introduction

Diagnosis of inherited haemoglobin disorders has been enhanced greatly by the advent of DNA diagnostic methods. Particularly helpful has been the use of the polymerase chain reaction (PCR), to obtain from minute quantities of DNA amounts sufficient for analysis by methods such as restriction digestion, linkage analysis and allele specific hybridisation. The wealth of information at the molecular level has provided

an insight into the heterogenecity of clinical presentations of the thalassaemia syndromes. In each ethnic group there are common beta-thalassaemia mutations and a variable number of rare ones[1]. In view of the need to establish prenatal diagnosis for thalassaemia, a systematic study of the beta-thalassaemia mutations among transfusion dependent patients who attended the Medical Specialist Haematology Clinic, Universiti Kebangsaan Malaysia, and the Paediatric Department, General Hospital, Kuala Lumpur, were studied. A modification of the PCR called the amplification refractory mutation system (ARMS) was used to diagnose each mutation[2].

Methods

Patients

The study group consisted of 24 patients (18 Chinese and 6 Malay patients) aged 2 to 11 years who were transfusion dependent.

PCR conditions

Genomic DNA was isolated from peripheral blood cells[3]. PCR was done in a mixture of 1.5 mM/L $MgCl_2$, 5 µl 10x PCR buffer II (Perkin-Elmer Cetus Instruments, Norwalk, CT 06859-0250 USA), 1 µM deoxyribonucleotide triphosphates (dATP, dTTP, dCTP and dGTP), 0.25 µM/L of 4 primers (2 control primers, 1 common primer and either the normal or mutant primer), 1 unit of 'Amplitaq' enzyme (Perkin-Elmer Cetus Instruments) and 0.5 µg to 1 µg of genomic DNA was added last to the PCR mixture in a total volume of 50 µl. The mixture was overlaid with 30 ul of mineral oil and the reaction mixture was subjected to 25 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1.5 mins, with a final extension period for 7 mins at 72°C, in a Perkin-Elmer programmable thermal cycler. Ten µl was then removed, mixed with 3 µl of loading buffer (25% 'Ficoll', 1.25% bromophenol-blue and 10 ml TBE buffer), and 5 µl of this was loaded on a minigel of 1.5% agarose and 1.5% Nusieve agarose (FMC BioProducts, Rockland, ME 04841-2994, USA). After electrophoresis at 100V for 30 mins, the gel was stained with ethidium bromide and photographed under ultraviolet illumination.

Design of ARMS primer

The amplification refractory system (ARMS) requires the terminal 3' nucleotide only of a PCR primer to be allele specific. Thus, the primer is synthesised in 2 forms. The 'normal' form is refractory to PCR on 'mutant' template DNA and the 'mutant' form is refractory to PCR on 'normal' DNA. The sequence of nucleotides for the primers used in this study was provided by Dr J.M. Old of the National Haemoglobinopathy Reference Laboratory, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom (Table I). A control pair of primers was included in each assay. The control primers amplified a region of DNA a short distance from that amplified by the ARMS primers and produced a fragment of a size distinguishable from any produced by the ARMS primers and served as an internal control for the efficiency of amplification. A negative control (blank) containing no DNA was included in each set of amplifications. DNA samples with known genotypes were used to determine the fidelity of the beta-globin amplifications.

Results

The beta-thalassaemia mutations seen in the patients who were transfusion dependent are shown on Table II. Genotypically, 29% were homozygous for the following beta thalassaemia mutations:

1. Fr 41-42, frameshift codon 41-42 (-TCTT);
2. IVS 1-1 (G to T mutation at position 1 of intron 1 (IVS-1); and
3. IVS 1-5 (G to C), the G to C mutation at position 5 of IVS-1.

The remaining were compound heterozygotes, where the most common form (35.2%) was Fr 41-42/IVS 2-654 (the C to T substitution at position 654 of intron 2 [IVS-2]). The control primers (A and B were used with all except IVS 2-654; E and F were used with IVS 2-654) amplified 861 and 421 bp fragments from the 3' end of the beta-globin gene. Three mutations (Fr 41-42, IVS 2-654, and -28 [A to G]) comprise 89.9% of Chinese beta-thalassaemia. In the Malays, 81.25% of the beta-thalassaemia mutations comprised of 3 mutations (IVS 1-5 [G to C], CD 17 [A to T], the nonsense mutation A to T at codon 17 and IVS 1-1 [G to T]). Fig 1 illustrates the results of an analysis in an unknown DNA sample for the frameshift mutation (Fr 41-42) in codon 41 and 42 (-TCTT). Results were available within a day.

Table I
Nucleotide sequence in the primers used for beta-globin DNA amplification

Primer		Sequence
Control A	:	5' CAA TGT ATC ATG CCT CCT TGC ACC 3'
Control B	:	5' GAG TCA AGG CTG AGA AGA TGC AGG A 3'
Control E	:	5' AGT GCT GCA AGA AGA ACA ACT ACC 3'
Control F	:	5' CTC TGC ATC ATG GGC AGT GAG ATC 3'
Common C	:	5' ACC TCA CCC TGT GGA GCC AC 3'
		Mutant
IVS 1-5 (G to C)	:	5' CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG 3'
IVS 1-1 (G to T)	:	5' TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA 3'
Fr 41-42 (-TCTT)	:	5' GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT 3'
IVS 2-654 (C to T)	:	5' GAA TAA CAG TGA TAA TTT CTG GGT TAT GGT 3'
-28 (A to G)	:	5' AGG GAG GGC AGG AGC CAG GGC TGG GCT TAG 3'
CD 17 (A to T)	:	5' CTC ACC ACC AAC TTC ATC CAC GTT CAG ATA 3'

Table II
Beta-thalassaemia mutations in transfusion dependent patients with homozygous beta-thalassaemia (n=24)

	Type of mutation	No
Homozygous	Fr 41-42	4
	IVS 1-1 (G to T)	2
	IVS 1-5 (G to C)	1
Compound heterozygote	Fr 41-42/IVS 2-654	6
	CD 17 (A to T)/-28 (A to G)	3
	IVS 1-1 (G to T)/IVS 1-5 (G to C)	3
	Fr 41-42/CD 17 (A to T)	1
	Fr 41-42/-28 (A to G)	2
	Fr 41-42/IVS 1-1 (G to T)	1
	IVS 2-654/-28 (A to G)	1

18 (75%) of the patients were Chinese and 6 (25%) were Malays. Mutations seen were in keeping with those previously described in the Malays and Chinese.

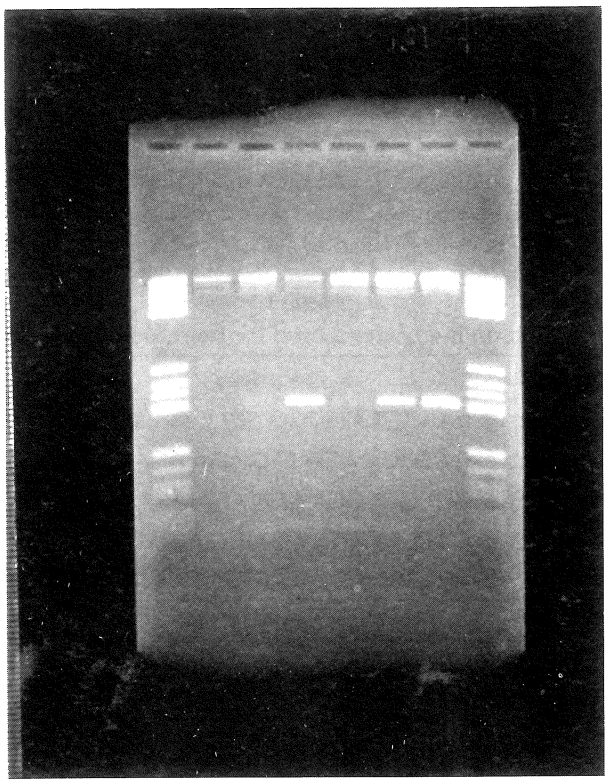

Fig 1: ARMS analysis of an unknown DNA sample for Fr 41-42.
Lanes 1 and 2 are DNA markers; lane 2 = blank (no DNA); lane 3 = unknown foetal DNA with Fr 41-42 normal primer; lane 4 = unknown foetal DNA with Fr 41-42 mutant primer; lane 5= normal DNA with Fr 41-42 mutant primer; lane 6 = paternal DNA; and lane 7 = known case of Fr 41-42 with Fr 41-42 mutant primer.
The upper bands are the 861 bp control bands.
Fr 41-42 is identified by a band of 439 bp.

Discussion

West Malaysia is a multi-racial society. The 3 main races are the Malays, Chinese and Indians. In addition to these, there are the Ceylonese, Indonesians, Pakistanis, Europeans, Eurasians and the Thais. Patients with thalassaemia seen at our clinic since 1984 have been Chinese and Malays, with an occasional patient of Indian origin. The 48 thalassaemia alleles seen in this study were accounted for by 6 mutations. There were differences, depending on their ethnicity. The Chinese-Malaysians had beta-thalassaemia similar to that encountered in Chinese patients in South China[4] and that seen in the Malays was similar to that described in earlier studies on Malays[5].

PCR allows direct identification of point mutations. The application of the amplification refractory mutation system (ARMS) to the detection of individual beta-thalassaemia mutations in heterozygous parents and 'at risk' foetuses has been assessed in Indian and Cypriot immigrant populations in the United Kingdom, where 100 first trimester prenatal diagnoses were done, which entailed the detection of 17 different mutations[2]. The method allowed the determination of the mutations in both parental and foetal DNA on the same day. The main advantage of the ARMS method is that it uses unlabelled oligonucleotides,

which makes it a simple and safe procedure accessible to almost any clinical laboratory. The result can be visualised immediately after gel electrophoresis by viewing the gel under UV light. Differences in the sizes of the fragments obtained from a single PCR determine the presence or absence of certain point mutations on one or both chromosomes. Despite its many advantages, it should be noted that the DNA amplification (PCR) technique requires considerable care, as the sensitivity of PCR is such that minute amounts of contaminating DNA can be amplified to produce false positive results. The inclusion of a blank 'no DNA' negative control is mandatory to detect this type of error. In February of this year, in our first prenatal diagnosis, we successfully identified the beta-thalassaemia mutations, using the ARMS method. The DNA was extracted from foetal blood obtained by cordocentesis done at the Obstetrics and Gynaecology Unit, Universiti Kebangsaan Malaysia, from a Chinese patient at 20 weeks' gestation, where the results were made available on the same day. Currently, we are in the process of establishing the ARMS method for the diagnosis of beta-thalassaemia on foetal DNA to be extracted from chorionic villi obtained by transabdominal placental biopsy. In conclusion, the PCR technique described in this study has permitted accurate and rapid diagnosis of the beta-thalassaemia mutations and an insight to the spectrum of mutations present, which is a prerequisite in the setting-up of a centre for the prenatal diagnosis of beta-thalassaemia.

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