The use of the Amplification Refractory Mutation System (ARMS) as an effective and economical tool for prenatal diagnosis of β-thalassaemia in Malaysian subjects

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Abstract

Molecular characterization of 155 β-thalassaemia genes was carried out using the Amplification Refractory Mutation System (ARMS) and comprised of 101 chromosomes from the Chinese, 52 from the Malays and 2 from the Indians. A total of 14 mutations along the β-globin gene complex was analysed using the ARMS: -29 (A→G), -28 (A→G), Cap (+1) (A→C), Cd 8/9 (+G), Cd 15 (G→A), Cd 17 (A→T), Cd 19 (A→G), Cd 26 (Hb E) (G→A), IVSI #1 (G→T), IVSI #5 (G→C), Cd 41-42 (-TCTT), Cd 71-72 (+A), IVSI1 #654 (C→T) and the 619 bp deletion at the 3'-end of the β-gene. Our results indicate that using the ARMS at these 14 sites, prenatal diagnosis can be achieved in 96% of the Chinese couples and 84.6% of the Malay couples at risk for a β-thalassaemia major child. In the Chinese, 5 out of the 14 β-mutations (Cd 41-42, IVSII #654, -28 TATA Box, Cd 17 and Cd 71-72) accounted for 92% of β-thalassaemia in this ethnic group. The β-mutations in the Malays were found to be more heterogeneous. Three β-mutations (Hb E, IVSI #5 and IVSI #1) accounted for 73.08% of β-thalassaemia and 6 other β-mutations (Cd 41-42, IVSII #654, Cd 8/9, Cd 19, CD 17, Cap (+1)) made up the remaining 11.52% of the identified mutations in this ethnic group. The ARMS, an allele-specific DNA amplification system which employs direct amplification of a gene sequence followed by simple agarose gel electrophoresis, was found to be an accurate, rapid and economical protocol for prenatal diagnosis for β-thalassaemia in the Malaysian population.

Key Words: β-thalassaemia; prenatal diagnosis; ARMS; ethnic groups

Introduction

Beta thalassaemia is a genetic disorder of haemoglobin synthesis. Affected individuals express complete suppression of β-globin chain synthesis or are deficient in globin production. Beta-thalassaemia patients have chronic anaemia but with regular blood transfusions and removal of iron with chelating agents, the disease is now compatible with prolonged survival (Giardini, 1997).

In Malaysia, β-thalassaemia is common in the Malays and Chinese with a carrier rate of about 3% in each (George, 1995). Clinical and health complications in β-major patients pose a heavy load on blood transfusion and paediatric services with the only "cure" being a successful bone marrow transplant. An alternative approach in addressing this health problem is to offer efficient and economical prenatal diagnosis measures for couples at risk of producing a β-major child. Molecular protocols for prenatal diagnosis of β-thalassaemia have progressed from the laborious restriction fragment length polymorphism (Old *et al.*, 1986) and allele-specific oligonucleotide hybridization (ASO) (Lindeman *et al.*, 1991; Tan *et al.*, 1993) techniques to more direct DNA amplification techniques using reverse dot blot (RDB) (Thong *et al.*, 1996) and Amplification Refractory Mutation System (Old *et al.*, 1990).

The implementation of a successful and effective prenatal diagnosis programme for β-thalassaemia in a country or region depends very much on the diversity of the ethnic groups and types of different β-mutations in the area as over 160 β-mutations have been identified. The Malaysian population is very heterogeneous with the Malays, Chinese and Indians forming the main ethnic groups and over 10 β-mutations have already been reported to be responsible for β-thalassaemia in the Malays and Chinese (Thong *et al.*, 1996). Our aim was to establish and evaluate the ARMS technique as a rapid, effective and economical prenatal diagnostic tool for the large heterogeneity of β-thalassaemia mutations in Malaysia.

Materials and Methods

Patients

Sixty-eight families with one or more β-thalassaemia major children, 9 couples with β-thalassaemia trait who requested prenatal diagnosis and one β-thalassaemia carrier were studied. β-thalassaemia trait was confirmed by haematological and electrophoresis methods. All patients attended the Obstetrics and Gynaecology or Paediatrics Department, University Hospital, or were referred from private hospitals and private clinics. Blood (10 mls) was collected in sodium-EDTA tubes and frozen at -70°C until required for DNA extraction. Chorionic villi (CV) were obtained by transabdominal placental biopsy at 8-10 weeks gestation.

DNA extraction and purification
DNA was extracted overnight at 37°C using proteinase K and sodium-dodecyl-sulphate. DNA in solution was purified using phenol-chloroform-isoamyl alcohol extractions. Purified DNA was solubilized in double distilled water and stored at -70°C. CV samples were collected in saline with sodium EDTA. CV was cleaned in several washes of saline to remove any maternal contaminating tissue.

Amplification Refractory Mutation System (ARMS)
The primers for the ARMS were synthesized in 2 forms - the normal β-sequences and the mutant β-sequences which contain one or a few nucleotide changes at the 3'end (Old *et al.*, 1990; Varawalla *et al.*, 1991). In each ARMS reaction, amplification of an 861 bp internal control from the 3'end of the β-globin gene was included to check amplification efficiency and DNA purity. In the case of the β-mutation at IVSII #654 the internal control was a 323 bp fragment.

DNA amplification protocol
DNA amplification was performed using the enzyme *Taq* polymerase (Gibco BRL Life Technologies, USA) and the accompanying 10 X buffer and $MgCl_2$ in a Perkin Elmer GeneAmp PCR System 2400. The concentration of $MgCl_2$ was used at 1.5 mM for all primers except for the β-mutation at Cap (+1) which was carried out using $MgCl_2$ at 1 mM. The primer concenrrations used were between 5-20 pmol depending on PCR conditions and efficiency. PCR was carried out in thin wall rubes and consisted of 30 cycles at 93°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min followed by a final extension step at 72°C for 3 min. All primers were annealed at 65°C except for IVSII #654, which was annealed at a lower temperature of 60°C. Amplified DNA was electrophoresed on 1.5% agarose gels and directly visualized after staining with ethidium bromide.

Results

DNA amplification using the ARMS is a sensitive and specific technique as amplification of each β-thalassaemia mutation produces a different molecular weight fragment together with an internal control band (Fig. 1). A total of 155 β-thalassaemia genes were studied using the ARMS. In the Chinese, 5 β-mutations (Cd 41-42, IVSII #654, -28, Cd 17 and Cd 71-72) common to this ethnic group were responsible for 92% of β-thalassaemias (Table 1). Four other β-mutations at Cd 26 (Hb E), Cd 15, Cap (+1) and -29 made up the remaining 4% of the characterized mutations. Taken together, these 9 β-mutarions (5 common and 4 rare) caused 96% of β-thalassaemia in the Chinese families studied. The ARMS was not able to characterize 4% (4/101) of β-mutations in the Chinese families.

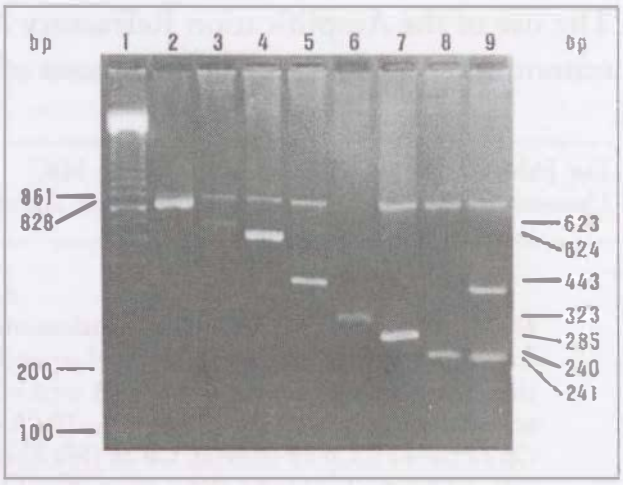

Fig. 1. Agarose gel electrophoresis of amplified DNA after Amplification Refractory Mutation System. Lane 1: 100 bp ladder; lane 2:IVSII #654 β-band (828 bp); lane 3: -29 β-band (624 bp); lane 4: -28 β-band (623 bp); lane 5: Cd 41-42 β-band (443 bp); lane 6: 323 bp internal control band; lane 7: IVSI #5 β-band (285 bp); lane 8: Cd 17 β-band (240 bp); lane 9: Cd 71-72 β-band (241 bp). Lanes 3, 4, 5, 7, 8 and 9 contain the 861 bp internal control band.

Table 1. Distribution of β-thalassaemia mutations in the Chinese in Malaysia

Bera mutation	No. chromosomes affected (%)
Cd 41-42	44 (43.56)
IVSII #654	29 (28.71)
-28	16 (15.84)
Cd 17	2 (1.98)
Cd 71-72	2 (1.98)
Hb E	1 (1.0)
Cd 15	1 (1.0)
Cap (+1)	1 (1.0)
-29	1 (1.0)
Unidentified	4 (3.96)
Total	101

The β-mutations in the Malay families studied were found to be more heterogeneous than the Chinese. The number of chromosomes in the Malays studied was 52 compared to 101 chromosomes from the Chinese families but the number of β-mutations detected in the Malays was equivalent to that detected in the Chinese, that is 9 mutations (Table 2). The mutations causing β-thalassaemia in the Malays were found to be quite different in the types and frequencies when compared with the Chinese. Three common Malay β-mutations (Hb E, IVSI #5 and IVSI #1) caused 73.08% of the β-thalassaemias. A further 6 β-mutations (Cd 41-42, IVSII #654, Cd 8/9, Cd 19, Cd 17 and Cap (+1)) accounted for the remaining 11.52% of the characterized β-mutations in the families studied. The ARMS was

Table 2. Distribution of β-thalassaemia mutations in the Malays in Malaysia

Beta mutation	No. chromosomes affected (%)
IVSI #5	17 (32.69)
Hb E	16 (30.77)
IVSI #1	5 (9.62)
Cd 41-42	1 (1.92)
IVSII #654	1 (1.92)
CD 8/9	1 (1.92)
Cd 19	1 (1.92)
Cd 17	1 (1.92)
Cap (+1)	1 (1.92)
Unidentified	8 (15.38)
Total	52

not able to identify 15.38% (8/52) of the Malay chromosomes studied.

In our study there was only one Indian family with β-thalassaemia. DNA from the β-major child showed a β-mutation at IVSII #5 with the other β-mutation uncharacterized by ARMS.

Discussion

Molecular characterization of β-thalassaemia genes in the Malaysian population has been reported using a multi-modal approach consisting of ASO and RDB (Thong *et al.*, 1996). Preliminary results using a selection of the 6 most common ASOs for mass screening followed by RDR assays for the more uncommon mutations gave a 91.7% mutation detection rate. The authors have suggested the careful selection of 4 ASO primers common to a particular subpopulation followed by RDB assays to obtain a 100% and 80% coverage in the Chinese and Malay subpopulations respectively. Both ASO and RDB techniques are accurate and specific for mutation detection, however the use of two different protocols for mutation detection is neither practical nor economical for prenatal diagnosis and molecular screening in a multiracial population.

The ARMS was adopted for molecular analysis and prenatal diagnosis for β-thalassaemia in Malaysia (George *et al.*, 1993; Ainoon *et al.*, 1994). Six common β-mutations at -28 TATA Box, Cd 17, IVSI #1, IVSI #5, Codon 41-42 and IVSII #654 were detected with the ARMS protocol used. In our study we expanded the number of β-mutations detectable by ARMS to 14 sites along the β-globin gene. The ARMS protocol was expanded to include the β-mutations in the Asian Indians (Cap (+1), Cd 8/9, Cd 15, 619 bp deletion in the 3'end), rare β-mutations found in the Chinese (-29, Cd 71-72) and Malays (Cd 19). At present rare β-mutations in most laboratories are characterized by genomic sequencing. Genomic sequencing is an accurate protocol for directly locating unknown mutations but it can be expensive when used for routine prenatal diagnosis and the technique involves staff who require specialized training.

The ARMS protocols established in our laboratory was developed with primers sharing similar PCR and gel electrophoresis conditions. This was designed intentionally to minimise error and for easy follow-through by laboratory staff who may not be specifically trained in molecular biology protocols. The only difference in PCR conditions is the annealing temperature for the β-mutation ar IVSII #654 which was reduced to 60°C as no amplification occurred at the higher temperature of 65°C. In addition $MgCl_2$ concentrations were also maintained at 1.5 mM for 13 β-mutations except for Cap (+1) where $MgCl_2$ concentration was reduced to 1 mM for more specific amplification. Using these PCR conditions a standardized ARMS protocol was developed with changes only in the primer concentrations.

Reagents used in molecular analysis techniques have always been expensive. To reduce the costs of prenatal diagnosis, the ARMS was developed using a PCR volume of only 25 ml. This strategy has allowed the use of lower concentrations of dNTPs and *Taq* polymerase. We reduced the concentration of *Taq* polymerase from 2.5 Units in previously published ARMS protocols (Old *et al.*, 1990; Varawalla *et al.*, 1991) to 0.5 U per PCR reaction in our laboratory. In addition detection of DNA amplified products is routinely carried our using agarose gels instead of the more expensive recommended 3% Nusieve-Seakem gel combination (Varawalla *et al.*, 1991; Old 1993). By using thinner agarose gelsat 1.5% concentration, maximum visibility of PCR products was obtained.

The large heterogeneity of β-mutations present often results in foetuses that are compound heterozygotes. In our study of the 78 β-thalassaemia families, 64% (50/78) of the β-major children were found to be compound heterozygotes. The ARMS will serve as a rapid prenatal diagnosis protocol especially in these families. Two β-mutations (Cd 41-42, IVSII #654) accounted for the majority of β-thalassaemias in the Chinese families (72.3% in this study) and this was also reported in previous investigations (Thong *et al.*,1996; George *et al.*, 1993). The rare Chinese β-mutations at Cd 71-72 and -29 uncharacterized in the above previous studies were found in the Chinese in this study. In addition 2 of the families were found to carry the β-mutation at Cd 15 and Cap (+1) which has been reported only in the Malays and Indians (Varawalla *et al.*, 1991; Ariffin *et al.*, 1996). The β-mutation Hb E was detected in 30.77% of the Malays studied compared with the lower reported frequencies of 19.5% and 2-4% in the Malays in Northeast and West Coast of Peninsular Malaysia respectively (Lie-Injo *et al.*, 1971; Vella, 1962). The much higher percentage of Hb E reported in this study

is probably due to the much larger sample size and molecular detection technique compared with Hb electrophoresis techniques used in the previous studies. Three β-mutations (Hb E, IVSI #5, IVSI #1) accounred for 73.08% of β-thalassaemia in the Malays studied. The ARMS was able to detect the rare Malay β-mutation at Cd 19 (1/52 chromosomes; 1.92%). In addition rhe β-mutations at Cd 8/9 and Cap (+1) which were previously reported in Asian Indians (Kazazian *et al.*, 1984; Wong *et al.*, 1987) were detected at low frequencies (1.92%) in the Malays.

In conclusion the ARMS protocol for the detection of 14 β-mutations described in this study offers a sensitive, specific, effective and economical technique for molecular screening and prenatal diagnosis of β-thalassaemias in Malaysia.

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