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THE VALUE OF ARMS/PCR AND RFLP/PCR IN PRENATAL DIAGNOSTIC ACCURACY OF β-THALASSEMIA

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ABSTRACT

Background: It is estimated that about 3,000 pregnancies in Iran are at risk for β-thalassemia each year.

Objective: To evaluate the diagnostic accuracy of combination of ARMS/PCR and RFLP/PCR techniques in prenatal diagnosis of β-thalassemia.

Methods: Sixty-seven β-thalassemia carrier families were enrolled in this study. To analyze β-globin gene, amplification refractory mutation system (ARMS) and restriction fragment length polymorphism (RFLP) methods were used. In order to obtain fetal cells for DNA analysis, chorionic villous sampling (CVS) was implemented.

Results: Using the two techniques (i.e. ARMS and RFLP), prenatal diagnosis (PND) was successfully performed in 98.6% of subjects. From 72 cases, 20 fetuses (27.8%) were found to be affected, 32 (44.4%) were heterozygous carriers and 19 (26.4%) were homozygous normal. In 62 families (including 5 twins) the ARMS technique enabled us to trace the mutation in either one or both parents, out of which 46 were further confirmed by RFLP. However, in 16 cases RFLP could not be informative and the diagnosis was based only on ARMS results. In the remaining 10 families, diagnosis was possible only with RFLP.

Conclusion: ARMS coupled with RFLP provides an effective way for prenatal diagnosis of β-thalassemia. Using the two techniques together increases the accuracy of PND and in cases in which ARMS is not sufficient to reach a final conclusion, it can be replaced with RFLP. In addition, using these two methods in parallel increases accuracy, saves time and increases confidence.

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Key Words • Beta-thalassemia • mutation • haplotypes • prenatal diagnosis

Introduction

ß-thalassemia is an autosomal recessive disease characterized by hypochromic and hemolytic anemia, and is dependent on blood transfusion to sustain life.[1] This disease is common in several parts of the world, especially in the Mediterranean region and Southeast Asia.[2] Due to the high morbidity and mortality in ß-thalassemia disease, prenatal diagnosis (PND) has been an important option for couples in whom both are carriers of ß-thalassemia.[3] PND is the most effective means for preventing the birth of an affected child.[2]

Methods used for PND of ß-thalassemia have progressed in recent years. It began with the study of ß-globin chain synthesis, continuing to indirect detection by using DNA polymorphisms (i.e. RFLP analysis) and finally leading to direct detection of mutant alleles.[1]

Using the ARMS technique, mutations can be successfully identified.[4,5] More than 180 mutations affecting almost every known stage of ß-globin gene expression results in a reduction or complete absence of ß-globin synthesis by the affected allele.[3] Fortunately,

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in any given population, there are a few common mutations and some rare ones.[2] In families in which the type of mutation is not known, it is often possible to define the affected prenatal chromosome by RFLP linkage analysis, and then to determine whether the fetus has received both affected chromosomes from its parents or not.[6]

In the present study, we have successfully performed the prenatal diagnosis for 72 at-risk fetuses for ß-thalassemia in Iranian families using the two techniques, ARMS and RFLP.

Materials and Methods

Subjects:

Sixty-seven ß-thalassemia carrier families (including 5 twins, amounting to a total of 72 PND) requested prenatal diagnosis at our genetic unit from March 2000 to March 2001. They were studied by ARMS/PCR in parallel with RFLP analysis. From each family member, including parents and affected child (if present), 5-10 ml of blood was collected in EDTA as an anticoagulant. In childless couples, blood samples were obtained from parents as well as paternal and maternal family members for RFLP analysis. CVS was obtained at 10-12 weeks of gestation by a specialist. All CVS were sorted free of any maternal cells and blood clots by microscopic dissection before preparing DNA. Genomic DNA was extracted by boiling method from the peripheral blood leukocytes.[7]

DNA Analysis:

The DNA was then amplified by polymerase chain reaction (PCR)[8] using Taq DNA polymerase (Sinagene, Iran). In order to diagnose a specific mutation, we applied ARMS technique to detect each known mutation. To this end, we initially used only mutant primers for mutations to be tested. Each sample was initially analyzed for common mutations reported in Iran: [IVSII-1 (G→A), IVSI-5 (G→C) and Fr8/9 (-AA), IVSI-110 (G→A), IVSI-6 (T→C), IVSI-1 (Med.) (G→A), IVSI-130 (G→C), Codon 30 (G→C), Codon 44 (-C), Codon 5 (-CT), IVS1-1 (Ind) (G→T), IVSI 3'end (-25 deletion), IVSII-745, Codon 39 (C→T), Codon 36-37 (-T) and C8 (T→C)].[9-13] Whenever we noticed a band in a given sample reaction, we used ARMS primers for wild type, also in order to confirm its homozygous or heterozygous status.[4]

Haplotype analysis was carried out for the four common ß-globin gene cluster RFLP: HincII/ 3'ψβ, AvaII/ β, HinI/β and RsaI/β. In these situations, it was necessary to undertake family studies to identify which DNA polymorphisms would be informative. In order to save time and money, ARMS primers were designed to analyze these RFLP sites and compare their effectiveness by conventional RFLP using restriction endonuclease enzymes in parallel.

The PCR products were subjected to electrophoresis on 1.5- 2.5% agarose gel, stained with ethidium bromide and visualized under UV transluminator.

Results

We routinely use ARMS and RFLP for PND. In this study, we analyzed the effectiveness of each of these methods. Diagnosis of fetal genotype was possible in 71 out of the 72 cases investigated (Table 1). Nineteen fetuses were normal, 32 were heterozygous, and 20 were found to be affected. We could not obtain complete diagnosis in one fetus. In this

Table 1:Results of Prenatal Diagnoses

Fetal Status	Cases (%)
Normal	19 (26.4)
Heterozygote (carrier)	32 (44.4)
Homozygote/Compound Heterozygote (major)	20 (27.8)
Heterozygote/ Compound Heterozygote	1* (1.4)
Total	72 (100)

* Incomplete PND for one family

Table 2: Informativeness of RFLP and ARMS methods for PND

DNA Analysis	Cases	Percentage
100% ARMS 100% RFLP	23	31.9
100% ARMS 50% RFLP	11*	15.3
50% ARMS 50% RFLP	5	6.9
50% ARMS 100% RFLP	7	9.7
100% ARMS 0% RFLP	16	22.2
0% ARMS 100% RFLP	10	13.9
Total	72	100

*In one family, results obtained from RFLP were not confirmed with ARMS.

case, mutation or informative RFLP was found in the mother. The other parent had homozygous haplotypes and his mutation was unknown. The informative parent had transmitted the mutant ß-globin gene to the fetus. Therefore, this PND was only 50% possible and the fetus was either heterozygous or compound-heterozygous (incomplete PND for one out of 72).

Using allele-specific ARMS technique, we were able to characterize the mutation in 76% of couples. Of these, 60% had one of the four most common mutations: IVSII-1 (G→A), IVSI-5 (G→C), Fr8-9 and IVSI-110 (G→A). Rare mutations were found in 16% of subjects. Notably, the predominant mutation in most provinces was IVSII-1 (G→A) as previously reported.[10,11]

In these cases, 50 fetuses were all diagnosed by ARMS/PCR. In 12 fetuses, the mutations remained unknown in one of the parental members. The PND for 10 families was possible only with DNA linkage study. In 69 samples, RFLP analysis was used for diagnosis. In 2 childless couples (including one twin) haplotype analysis was impossible, as blood samples from paternal and maternal family members were not made available. In 38 families (including 2 twins) both parents had informative haplotypes (100% diagnosis). In 14 families (including 2 twins) one parent had homozygous haplotypes (50% diagnosis). In 13 families both parents had homozygous haplotypes (non-informative). Therefore, diagnosis was only based on ARMS results in 16 cases (Table 2).

Results obtained from each of the two techniques (ARMS and RFLP) could be confirmed with the other. In only one case, there was no agreement between results from RFLP mapping and ARMS. Using ARMS results, this fetus was found to be a carrier of either of the paternal or maternal mutation (both of them had Fr8/9 mutation), but in the RFLP study it was found to be affected by ß-thalassemia.

For the affected fetuses, termination of pregnancy was offered after diagnosis and the families were referred to the National Legal Medicine Organization. No false diagnosis was found during postnatal follow-up.

Discussion

Many different types of mutation can produce ß-thalassemia condition.[1,14] Several previous studies on some 17 mutations prevalent in Iran, have shown that in using ARMS method, only up to 87% of mutations can be detected.[9-12] In prenatal diagnosis, mutation from any of the parents may be unknown. If just the ARMS technique is employed, PND can not be completed. Using RFLP alone, the same difficulty arises; for instance, had we only used ARMS method, 30.5% of PND would not have been completed and for RFLP only, 44.4% would have failed to reach the final conclusion. But using both methods, we could reach the final conclusion in 98.6% cases (i.e. 71 out of 72 cases).

In the RFLP study, one fetus was diagnosed as ß-thalassemia major. This result was not confirmed by ARMS technique in

which the fetus was found to be a carrier. Diagnosis by this method carries a predictable error of about one in 300 to 500 due to the meiotic recombination between the RFLP site and the site of mutation itself.[1]

These results suggest that ARMS coupled with RFLP provides effective prenatal diagnosis for ß-thalassemia. Implementation of both techniques together increases the accuracy of PND and in cases where ARMS is unsuccessful, it can be replaced with RFLP.

In addition, the use of ARMS and RFLP in parallel, reduces the time of PND and is therefore, more cost-effective. It is also more convenient for families; should one method prove inconclusive, the other may be employed with limited disruption.

In conclusion, using the two techniques has several advantages in PND of β-thalassemia, and it is recommended that it becomes a routine procedure in all PND centers in the country.

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