Molecular Genetic Diagnosis of Beta Thalassemia in Pakistan

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Abstract

A set of procedures, based on DNA analysis, has been developed to detect deletions and point mutations causing Beta thalassemia in the Pakistani population. These procedures can be used to analyze the presence of relevant changes in DNA, thus providing a reliable means for screening the high risk families, to provide them genetic counselling and prenatal diagnosis during early pregnancy. We have identified two mutations IVS-1 nt.5 (G--C) and codon 8-9 (+G) in 4 of the 6 families analyzed for these mutations (JPMA 45:66,1995).

Introduction

Thalassemia is an autosomal recessive disorder associ- ated with a reduced or absence of synthesis of globin chains of the hemoglobin molecule, causing severe anemia in homozygotes. Beta thalassemia is the commonest inherited hemoglo- bin disorder in Pakistan[1]. The average gene frequency is estimated to be 5,4%[2], Consanguineous marriages have contributed to the increased incidence of this disease[3]. No study, dealing with the molecular charactenzation of beta thalassemia, has been reported. We have initiated a programme to study the molecularbasis of the disease in order to devise a DNA analysis based diagnosis during early pregnancy. These techniques canbe usedforscreening genetic counselling and prenatal diagnosis to reduce the incidence of beta thalassemia.

Material and Methods

Blood samples were collected in EDTA from 23 thalassemia affected families (father, mother, patient and siblings of the patient if available) of different ethnic groups residing in and around Lahore area. DNA was extracted either from the buffy coat[4] or from whole blood stored at -20°C[5]. "Guthrie Spots" were directly used to amplify beta globin gene by polymerase chain reaction (PCR) [6]. To detect a large deletion or any other mutation that creates or abolishes a restriction site, fragments of DNA from both parents and the affected child, digested with appropriate restriction endonucleases, were resolved by electrophoresis in 0.8% agarose gel. The resolved DNA fragments were transferred to a nylon membrane by Southern blotting technique[7]. The membrane was hybridized with the globin gene probe labeled by using Enhanced Chemiluminescence (ECL), a non-radioactive nucleic acid labeling and detection kit (Amersham International Plc. UK). After hybridization the filters were washed and target nucleic acids were detected by autoradiography. The sizes of various fragments were calculated using a computer programme[8]. The probe was a Bam HI 1.9kb beta globin gene fragment cloned inpBR328 consisting' offirstand secondExon,first intronand 5'flankingsequences. The set of primers consists of (a) nt -158 to nt -138, complementary to the sense strand and (b) codon 89 to codon 83, complementary to the antisense strand. The resulting segment was 608 nucleotides long, canying the point mutations previously described in Asian Indians[9]. For screening known Asian Indian point mutations in our population, oligonucleotides (19-mer) were designed from normal beta globin gene sequence[10], one complimentary to the mutationto be detected and the other homologous to the normal DNA sequence and were synthesized using a DNA synthesizer (Applied Biosystems' Model 380B) according to the

suppliers specifications. The primers and oligonucleotides were purified by a 15% denaturing polyaciylamide gel electrophoresis (PAGE). DNA bands were visualized under short wave UV, eluted overnight from the gel using 0.3M sodium acetate at 37°C, precipitated with 2.5 volume ethanol overnight at -20°C, washed with 70% ethanol, dried and suspended in Tris-EDTA (TE) pH 8.0[11]. Beta globin gene was amplified by using 0.5-1.0 ug genomic DNA, 50 pmol of eachof the PCR primers, 200 umol (fmal concentration) each of the dNTP, 1.0 unit Vent polymerase (New England Biolabs Inc.), Vent reaction buffer, prepared as recommended by the manufacturer and Dimethyl Sulfoxide (DMSO) at a final concentration 10% v/v in 100 ul reaction
volume. The reaction was carried out through 25 cycles that consists of 30 second denaturation at 95°C, 30 seconds annealing at 57°C and 1 minute extension at 72°C on a thennal cycler. In case of "Guthrie Spots" a 4x4 mm square of dried blood on filter paper (Schleicher and Schuell No. 2992) was fixed in methanol, speed Vac to dryness and used as such for amplification of beta globin gene in the PCR reaction[6]. Amplification of the right fragment was determined by 2% agarose gel electrophoresis using PhiX 174 Hae ill digest as marker. Dot blot analysis with ASO probes were used to detect point mutations in the Pakistani population. 5 ug of amplified DNA, mixed with 0.4 N NaoH and 25mM EDTA (final concentration of each) and incubated at 25°C for 20 minutes, was spotted onto a nylon membrane. Oligonucleotide probes were prepared by using DIGOligonucleotide 3' end Labelling Kit (BoehringerMannheim, Germany) in which oligonucleotides are enzymatically labelled at their 3' end with terminal transferase by incorporation of a single digoxigenin- labelled dideoxyuridine-tnphosphate (DIG-ddUTP). These oligonucleotides were then used for ASO hybridization of PCR amplified DNA spotted onto a nylon membrane. Prehybridization was done at 68°C while hybridization temperature was calculated for each probe according to the formula Tm = °2 (nA+nT)+4 LnG±nC)s. The DIG-labelled oligonucleotide probes were detected after hybridization to target nucleic acids, by using DIG-Nucleic Acid DetectionKit (BoehringerMannheim, Germany), which produces an insoluble purple blue precipitate, within 2 hours. Once used labelled probe in the hybridization buffer was stored at -20°C and reused after heating at 95°C for 10 minutes. In this procedure the same DNA blot could be used several times or at least for two oligonucleotide probes, one complementary to the mutation to be detected and the other homologous to normal DNA sequences successively after stripping the blot[12].halassemia is an autosomal recessive disorder associ- ated with a reduced or absence of synthesis of globin chains of the hemoglobin molecule, causing severe anemia in homozygotes. Beta thalassemia is the commonest inherited hemoglo- bin disorder in Pakistan[1]. The average gene frequency is estimated to be 5,4%[2], Consanguineous marriages have contributed to the increased incidence of this disease[3]. No study, dealing with the molecular charactenzation of beta thalassemia, has been reported. We have initiated a programme to study the molecularbasis of the disease in order to devise a DNA analysis based diagnosis during early pregnancy. These techniques canbe usedforscreening genetic counselling and prenatal diagnosis to reduce the incidence of beta thalassemia.

Results

DNA samples from a beta thalassemic family were digested with BamHI, Bg 1 11 and EcoRl, resolved to yield 1.9, 5.2 and 5.5 kb fragments respectively (Figure 1)

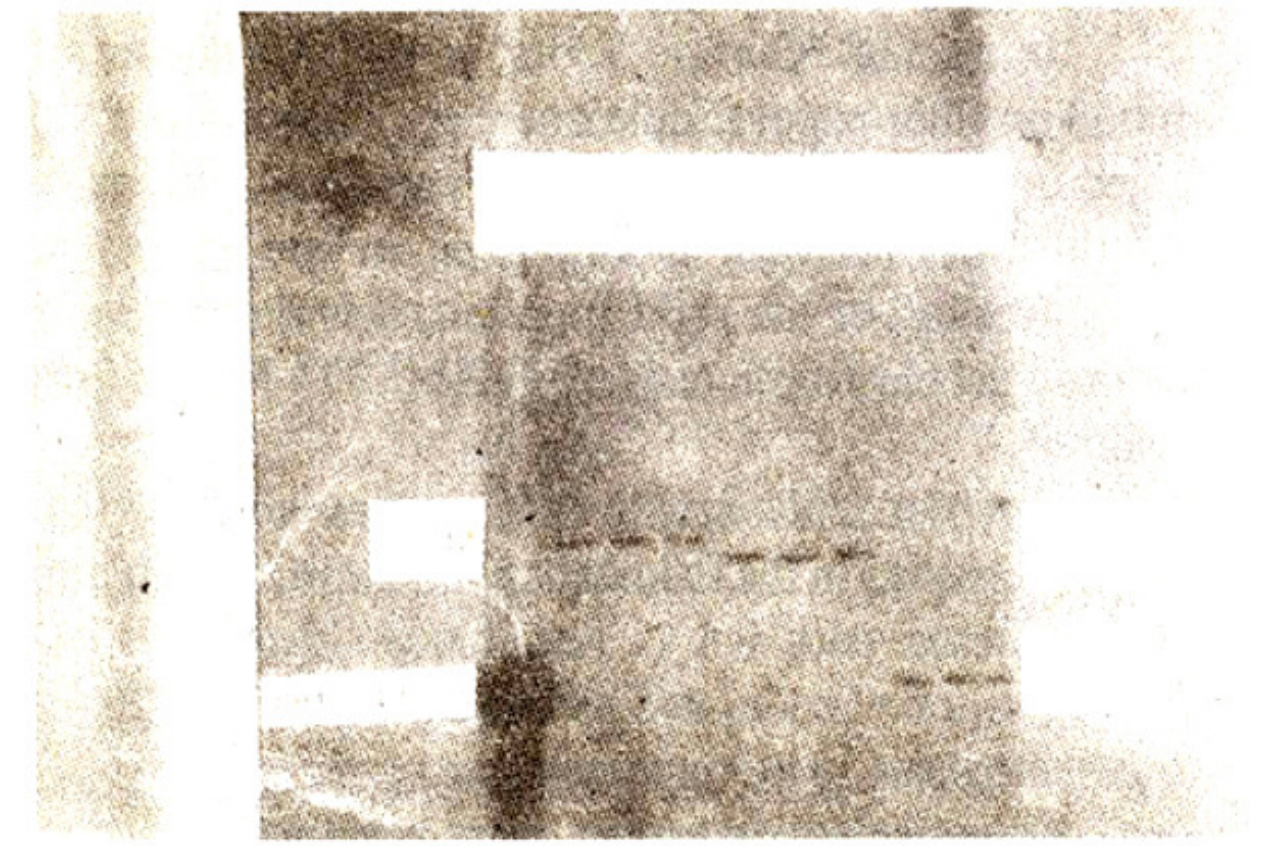
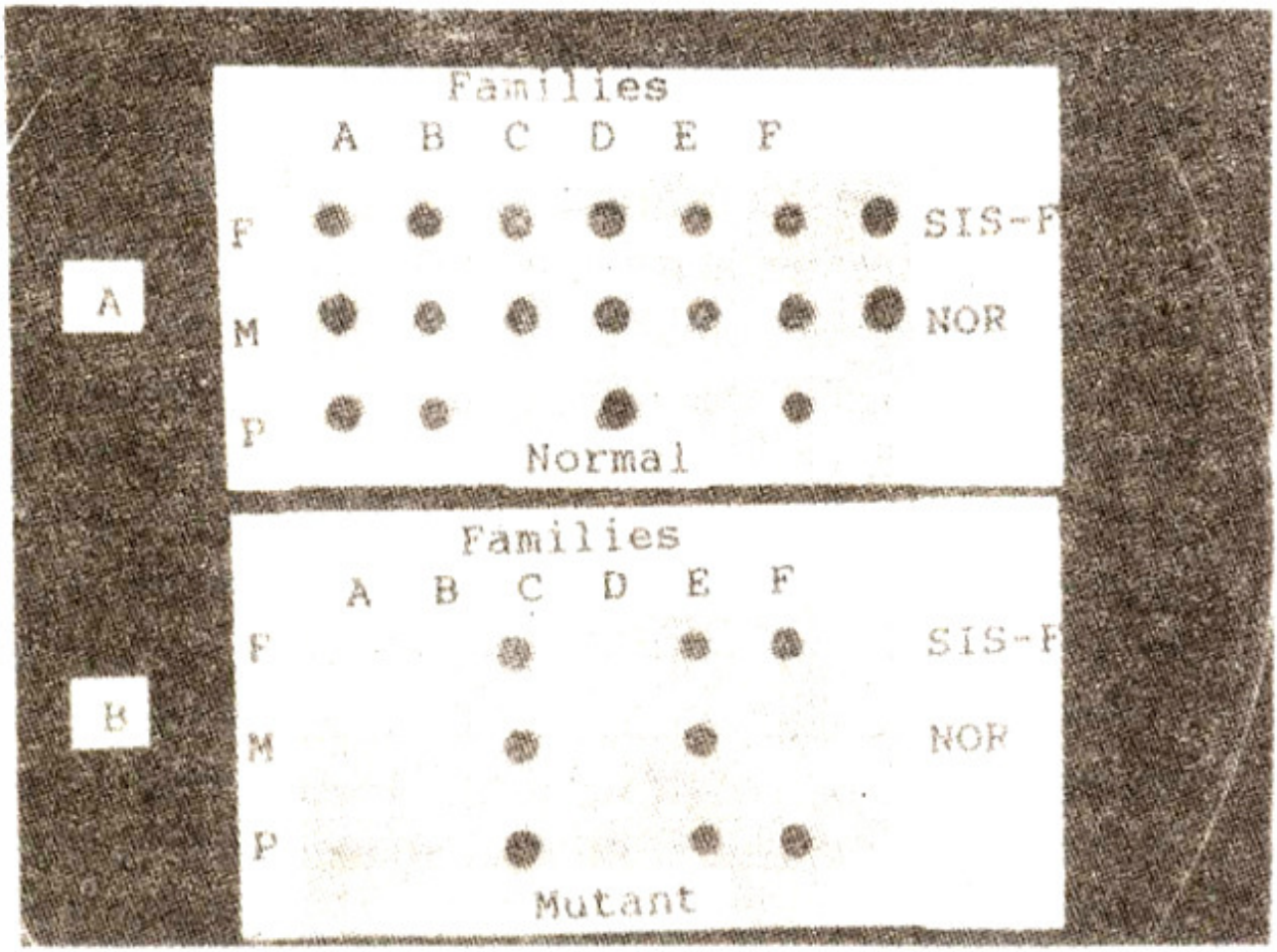


Figure 1. Restriction endonuclease analysis of a thalassemic family. Patient (P). Mother (M) and Father (F)

10 corresponding to the sizes produced by the normal DNA10. These results indicated that the molecularbasis ofthalassemia in the subject family was not due to deletion in the beta globin gene. Amplified DNA from six families and one normal control were hybridized with ASO probes to detect the mutation IVS-1 nt.5 (G-->C) (Figure 2A and 2B).



.Figure 2. Dot blot analysis. A: Detection of normal sequences at IVS-1 nt.5. B: Detection of mutation at IVS-1 nt.5 (G--C). Father (F), Mother (M), Patient (P), Sister (SIS) and Normal Control (NOR)

As families A, B and D gave positive results only with the normal ASO probe, they were negative forthe mutation IVS-1 nt.5 (G-->C) while parents of families C and E gave positive results with both normal and mutation. ASO probes were heterozygous for the mutation IVS-1 nt.5 (G-->C) but both patients only hybridized with the mutation ASO probe and were therefore, gous forthe above mutation. In case of family F both the father and the patient were positive for this mutation while the mother and the sister of the patient gave normal results. Two moreblots comprisingthe same numberof samples were hybridized with ASO probes to detect the mutations between codon 8 and 9 (+G) (Figure 3A and 3B).

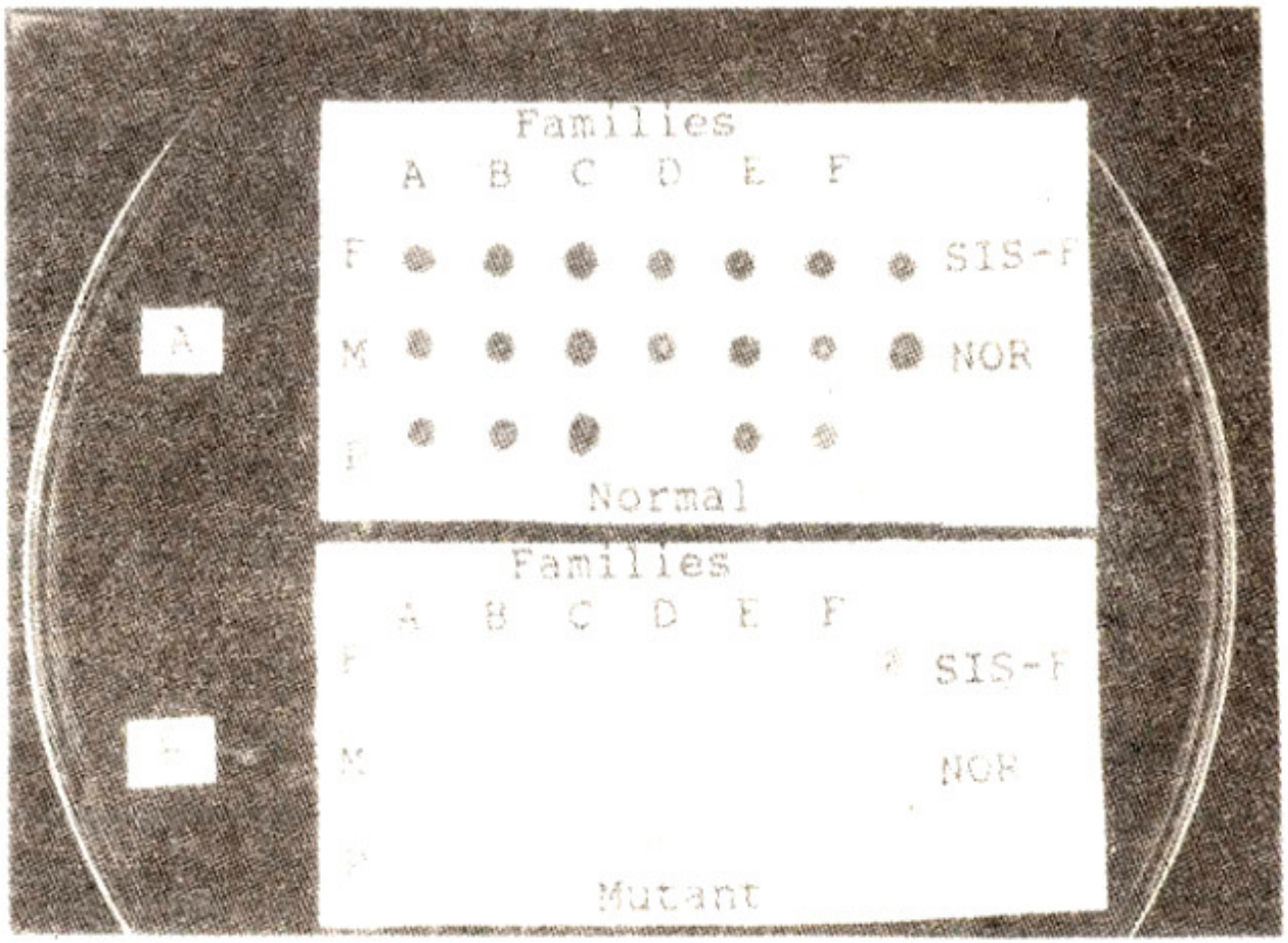


Figure 3. Dot blot analysis. A: Detection of normal sequences between codon 8 and 9. B: Detection of mutation between codon 8 and 9 (+G). Father (F), Mother (M), Patient (P), Sister (SIS) and Normal Control (NOR)

Parents of family D gave positive results with both normal and mutation ASO probes were therefore, heterozygous while the patient hybridized only with the mutation ASO probe and was homozygous for the mutation between codon 8 and 9 (+G). Family F showed that the patient, the mother and the sister were heterozygous for this mutation while the father was normal in this mutation. The normal control gave positive result only with the normal ASO probes and confirms the accumcy and sensitivity of the procedure. Analysis of results shows that families C and E have the mutation IVS-l nt.5 while family D has the mutation between codon 8-9 (+G) as the cause of beta thalassemia. In the case of family F both parents were carrier of the different mutations. The father being carrier of the mutation IVS-1 nt.5 while the mother was the carrier of the mutation between codon 8-9 (+G). Patient was a double heterozygote and inherited both mutations from his parents while his sister inherited the mutation from her mother only and was heterozygous like her mother. Families A and 13 were negative for these two mutations.

Discussion

The deletion of 619 nucleotides[13,17] which involves the 3' region of the beta globin gene accounts for almost 36% of the beta thalassemia genes in Asiatic Indians[13]. According to one study, it has been

demonstrated that this deletion is localized to the Sindhi and Gujrati speaking Muslims and Hindus of the Sindh region in Pakistan and adjacent region of Gujrat in India where it contributes at 56% and42%ofthebeta thalassemia respectively[14]. Keeping the above findings in view, the procedure of restriction endonuclease analysis was standardized to detect deletions or other mutations which might have created or abolished a restriction site. As our subjects were from Lahore area we were not expecting this deletion but the same procedure could be used in linkage analsis. If within a population or family, a disease allele is consistently associated with a particular polymorphism, it is possible to track inheritance of that disease allele within a family without having access to it directly[18]. The different procedures which could be used to detect known point mutations include (I) Dot-blot analysis with allele specific oligonucleotide (ASO) probes[19-22]. (H) Reverse Dot blot[22,23]. (ifi) Amplification Refractoiy Mutation System (ARMS) [24-26] (IV) Fluorescence amplification[27]. In order to detect point mutations causing beta thalassemia in the Pakistani population we have standardized the procedure of Dot blot analysis with ASO probes. The ASO probe anneals to sequences that have a perfect match with it, a single mismatch being sufficient to prevent hybridization under appropriate conditions. To standardize this procedure in our laboratories only six of the 23 thalassemic families were screened. We have identified 2 different mutations IVS-1 nt.5 (G--C) and codon 8-9 (+G) in 4 of them. Since there are so many mutations that produce the same final result that is, reduced or absent beta globin synthesis, there are many combinations of genes which produce the phenotype ofbetathalassemia major. The frequency of genetic compounds i.e. beta thalassemia individuals with two different beta thalassemia alleles is much greater. For example, among beta thalassemics of Italian, Greek and Indian origin 85%, 50% and 50% respectively, are genetic compounds. Thus, genetic compounds, rather than homozygotes, are the rule in this order[13] . Out of four families in which we have identified the mutations, one patient was a genetic compound and earned two mutations IVS-1 nt.5 (G—>C) and codon 8-9 (+G)as each parent was a carrierof different mutation while in other three families two had the mutation IVS-1 nt.5 (G-->C) and the third one was positive for the mutation between codon 8-9 (+G). As the procedure has been standardized we intend to screen rest of our thalassemic families. Beta thalassernia is a common disease in the provinces of Sindh and Balochistan along the Arabian sea coast and the North West Frontier Province (NWFP) where populations from different parts of the world have settled during various periods in history. Carrier rate varies from 1.4%[28] to 9.6%[29]. The latest study done on five hundred apparently healthy adults from northern parts of Punjab and NWFP showed prevalence of 3.2% in Punjabis and 7.9% in Pathans with an overall prevalence rate of 5.4 %2 Pakistan has a population of approximately 120 million. Considering 5.4% earner rate for beta thalassemia applicable to the whole country, the total earner population in Pakistan would be more than 6.0 million and the likely number of children suffering from thalassemia major would be approximately 36,000[30]. Consanguineous marriages are quite common in Pakistan. This social practice may have compounded the problem. The appropriate response is not to try to decrease the frequency of consanguineous marriages but to offer genetic counseling and provide facilities for prenatal diagnosis. In Sardinia, Italy, for example, the incidence declined from 1 per 250 to 1 per 1200 live births, with 90% of the cases effectively prevented[30]. To date over 130 different molecular defects resulting in beta thalassemia have been defined[9]. Despite this marked heterogeneity, however, each population at risk usually has its own (three to five) specific set of mutations thataccount forthe large majority of the molecular defects[22] causing beta thalassemia. For example, in Sardinia, out of 10 known mutations for this population a nonsense mutation at codon 39 is the most frequent beta thalassemia mutation, accounting for 95% of the cases[31]. It is important to ascertain the molecular defects in the various ethnic groups in the Pakistani population to find out the most prevalent type of defects in our population. Such a studs has not been undertaken so far and is badly needed. Parental genotypes prior to fetal sampling should be determined to enhance the ease and speed with which a diagnosis can be made in the fetus. The control

of beta thalassemia by carrier screening and prenatal diagnosis may represent a model for the organization and delivery of preventive programmes for other common autosomal recessive disorders.

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