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Mutational Screening and Prenatal Diagnosis of B-Thalassemia in Pakistani Families

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Abstract

Beta-thalassemia is one of the most common autosomal recessive disorders worldwide. Prevention by carrier screening and prenatal diagnosis is needed in populations with high prevalence of the disease. Keeping this in mind, this study was aimed at analyzing βthalassemia disorder which is inherited in an autosomal recessive fashion through mutant alleles from parents to their children. Blood and fetal samples of two families were collected at MINAR hospital and sent to NIBGE. DNA was extracted from blood and CVS by phenol-chloroform method and quantified using Nanodrop. Then DNA was amplified by ARMS-PCR followed by horizontal gel-electrophoresis. Results showed the presence of two most prevalent beta-thalassemia mutations IVS 1-5 and FSC 8-9 in Pakistani families. Family A segregating β-thalassemia was found to have IVS 1-5 mutation and parents were carrier for this mutation. Fetal sample of Family A was homozygous of the parental mutation. FSC 8-9 was the mutation found in blood samples of Family B. Parents and fetus both were carriers of this mutation. Genetic testing and prenatal diagnosis can reduce the frequency of β-thalassemia disorders in Pakistan.

Keywords: Beta-thalassemia; ARMS-PCR; Human Genome Project; mutations IVS 1-5 and FSC 8-9

Introduction

The detection of genomic variations is important in studying the relationship between causative agents and diseases [1]. Genetic information is vital in understanding normal and abnormal development of humans. This enables medical practitioners to pre-diagnose the disease before the symptoms appear. It also makes possible to prevent the onset of the disease. Genetics lead to "medical genetics" which include diagnosis and treatment strategies like gene therapy, personalized and preventive medicine [2]. In the past decade, medical genetics became an emerging field in medical care with the successful completion of Human Genome Project (HGP). Era before the HGP does not provide solutions for diseases caused by mutations in many genes like polygenic disorders etc. HGP paves the path from genetics to genomics and likewise genetics to medical genetics [3]. Role of family history is also very important in bridging the gap between genetics and genomics [4]. It shows the existence of shared genes between different family members, shared environment in a family inheriting same cultural and social values and monogenic and polygenic disorders running in a family [5]. A genetic disorder is caused by a change in DNA sequence of an individual that may alter the sequence of amino acids in the proteins or change non-coding regions of DNA. DNA is always at risk of damage by both endogenous and exogenous factors. Development of new technologies has made possible to accurately diagnose the genetic disorders including pre-implantation genetic diagnosis (PGD), prenatal diagnosis (PND), pre-symptomatic testing, conformational diagnosis and forensic/identity testing [6]. Types of genetic disorders include monogenic, polygenic and chromosomal disorders. These disorders are caused by mutations in one gene and run in families. Examples include beta-thalassemia, xeroderma pigmentosum and sickle-cell anemia etc. Pedigree analyses makes possible to determine these disorders in larger families having many affected children. Monogenic disorders are called Mendelian disorders because they run in families in Mendelian pattern of inheritance. Many of these disorders are chronic in nature [7]. Thalassemia represents a group of inherited diseases which arises from the reduced level of

hemoglobin in red blood cells (RBCs). Disorders of hemoglobin are believed to be the most prevalent single-gene disorders. The word "thalassemia" is a Greek word meaning "sea blood" which was coined by George Whipple [9,10]. Thalassemia is basically classified into two groups, i.e., α-thalassemia and βthalassemia. Alpha- thalassemia is caused by the reduced or absent production of the alpha globin chains [11]. Beta-thalassemia is due to a defect in the synthesis of the beta-globin chains, leading to alpha/beta imbalance, ineffective erythropoiesis and chronic anemia. β-thalassemia has three different categories which include β-thalassemia major, β-thalassemia minor and β- thalassemia intermedia (TI). β- thalassemia is one of the major health problems in Pakistan. Carrier rate is 5% [12]. A study was conducted to determine distribution of βthalassemia mutations in various ethnic groups in Pakistan. IVS 1-5(G-C) was the most common mutation identified in Southern Punjab. It was observed in 40.89% of the samples and its frequency varied in different ethnic groups. Fr 8-9 was found in 15.7% of allele pool being the second most common mutation in Pathans. Deletion 619 bp was found to be the third most common and constituted 11.11% of allelic pool. IVS 1-1 was detected as fourth most common mutation in Sindh [13].

Materials and Methods

Before the beginning of the study, formal approval was obtained from the Institutional Review Board (IRB) of National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan.

Subjects study and clinical investigation

Two families with β-thalassemia were investigated in the present study. Families were identified by collaborators in Multan Institute of Nuclear Medicine and Radiotherapy (MINAR), Multan. After taking proper tests, medical history was recorded before molecular investigation. Both families showed strong evidence of autosomal recessive mode of inheritance and consanguinity. After approval for the study by local ethical committee and a written consent from all families, pedigrees were constructed from all available information using standard method introduced by Bennett. Cyrillic (Cherwell Scientific Publishing Ltd, Oxford, UK) software version 2.1.3 was used to draw all extensive pedigrees. Different symbols are utilized in this software to explain architecture of families. Males and females are represented by different symbols i.e., males by squares and females by circles. Numbers enclosed in these symbols represent number of individual/siblings.

Description of families

Families were identified from southern areas of Punjab and at least 1-3 affected member(s) of each family was subjected to genotypic examination. Parents of both families have consanguinity and were found as clinically healthy. Genetic screening of thalassemic families was performed for known mutations prior to prenatal diagnosis. CVS samples were collected at 12th week of the pregnancy.

Family A: Blood sample of three family members (IV: 1, IV: 2, V: 2) along with CVS sample (V: 3) were collected from MINAR hospital for mutational screening of β-thalassemia. This family was genotyped for most frequent mutations of *β*-thalassemia in Pakistan. Individuals represented by IV: 1 and IV: 2 are the parents, V: 2 is sick child (proband) and V: 3 is CVS.

Family B: Blood samples of mother and father (III: 1 and III: 2) along with CV sample (IV: 3) was sent from MINAR hospital to HMGL. They were genotyped for 5 most prevalent mutations of β- thalassemia.

Samples collection

Peripheral venous blood was taken after the consent of the individuals or their legal guardians at MINAR, Multan after proper diagnosis. Non-infectious 5cc syringes were used to take blood which was then transferred into anti-coagulant EDTA vacutainers for storage. After all the investigations, blood was transferred to Human Molecular Genetics Laboratory (HMGL), NIBGE, Faisalabad. Genomic DNA was extracted from blood samples using phenol chloroform method.

DNA Extraction from CVS

DNA is extracted from CVS samples using the following protocol. The CVS sample was separated from normal saline and transferred into 1.5 ml micro centrifuge tube and lysed with 500 µl of CVS lysis buffer. It was then incubated after adding 50 µl of proteinase K and 5.0 µl of 10% SDS at either at 60˚C for two hours or at 37˚C overnight. After incubation, 500 µl of solution C+D was added, vortexed briefly and centrifuged for 5 minutes at 13,000 rpm. The upper aqueous layer was separated in new 1.5 ml micro centrifuge tube. This step was repeated thrice in order to get highly purified DNA. Then 250µl of solution D alone was added, centrifuged it for two minutes at 13,000 rpm, and the aqueous layer was separated. Then 250 µl of chloroform was added and centrifuged for 2 minutes at 13,000 rpm and the aqueous layer was separated. Then 250 µl of 3 M Na-acetate and 500 to 600 µl of isopropanol (stored at - 20˚C) was added and tubes were gently inverted to precipitate DNA. It was then centrifuged for 10 minutes at 13,000 rpm. Supernatant was removed without disrupting DNA pellet, which was then washed with 350 µl of 70% ethanol and dried in incubator at 37˚C. After evaporation of residual ethanol, the DNA was dissolved in 30-50 µl of TE buffer or double distilled PCR water.

Quantification of DNA

Concentration of genomic DNA was determined by Thermo Scientific Nano Drop 8000 UV-VIS Spectrophotometer. Samples were diluted 10/100 in sterile distilled water and absorbance was measured at 260nm.

Agarose gel electrophoresis (Horizontal)

DNA samples mixed with bromophenol blue (tracking dye) were loaded into the wells preformed in the gels. Then electrophoresed in 0.5X TBE buffer at 90 volts (55mA) for 45 minutes on 1% (w/v) agarose gels stained with 4.5µl ethidium bromide (10mg/ml).The DNA fragments stained with ethidium bromide were visualized under UV on a UV transilluminator (Uvitec, UK) and photographed.

Amplification Refractory Mutation System (ARMS) PCR

ARMS PCR was used for genetic screening of thalassemia families. An ARM has also been termed allele-specific PCR or PCR amplification of specific alleles (PASA). It is a modification of PCR and requires two oligonucleotide primers identical in sequence except for the terminal 3' nucleotides. One of which has its 3' terminal nucleotide complimentary to the changed sequence (Mt ARMS primer) and other to the normal DNA sequence (N ARMS primer), both used as reverse primers [9]. Common C was used as a complimentary forward primer for both mutant and normal templates to screen a specific mutation. In this system a second pair of primers was always included in the reaction mixture to simultaneously amplify an unrelated DNA sequence, which serves as an internal control to test that both reaction mixture and thermal cycler is working optimally. The ARMS analysis was performed in a reaction mixture of 20 µl. PCR product was then visualized on 1.8% agarose gel. Amplified PCR products were resolved on 1.8-2% w/v agarose gel. Samples mixed with bromophenol blue were loaded onto gel and electrophoresis was performed at 90 volts (55mA) for 45 minutes in 0.5X TBE (running buffer) filled electrophoresis tank.

Results

Mutational analysis

Two families of β-thalassemia i.e. Family A and Family B were screened for mutation screening via an improvised PCR called as amplification refractory mutation system (ARMS). Parents of both families were first diagnosed for thalassemia minor and then screened for most frequent mutations in Pakistani population in order of the prevalence of these mutations. First trimester prenatal diagnosis was carried out by chorionic villus sample DNA to screen pregnancy at risk of β-thalassemia.

Family A: Proband of this family is receiving regular blood transfusion every month. After DNA was extracted from the blood of these samples, DNA was quantified using Thermo Scientific Nano Drop 8000 UV-VIS Spectrophotometer. Then it was followed by ARMS PCR. ARMS PCR was run for the most frequent mutation IVS-I-5 (G-C) in HBB. Homozygous (affected), heterozygous (carrier) and negative controls were also loaded along with family samples for confirmation of results. Results were checked on 1.8% agarose gel electrophoresis by comparing intensity and sharpness of the DNA bands with control samples (Figure 1-5).

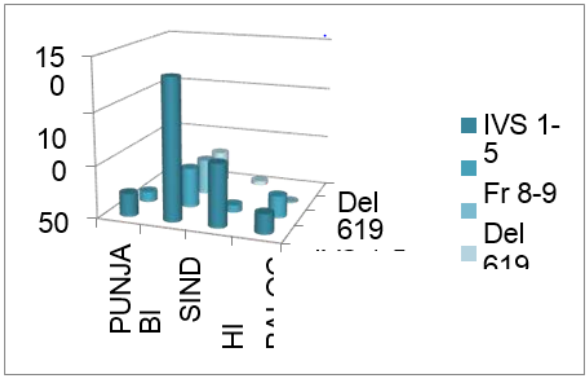

Figure 1: *Distribution of β-thalassemia mutations in Pakistan's various ethnic groups. The figure is reproduced from [13].*

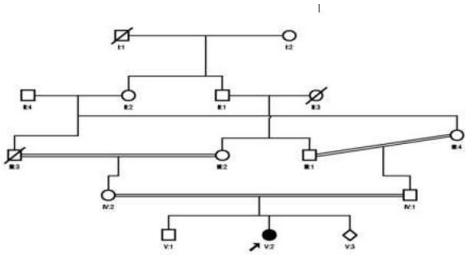

Figure 2: *Pedigree of Family A showing autosomal recessive inheritance. Generation numbers are represented in Roman numbers while Arabic numbers denote individuals in a generation. Fetus is symbolized as diamond i.e. V:3 in this pedigree.*

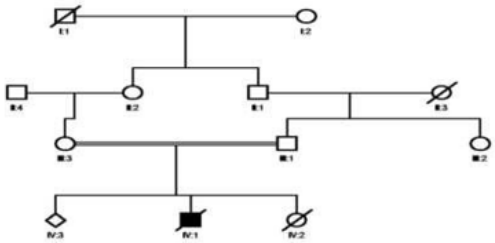

Figure 3*: Pedigree of Family B showing fetus as diamond i.e. IV: 3 in this pedigree. Generation numbers are represented in Roman numbers while Arabic numbers denote individuals in a generation. III: 1 and III: 2 are parents and IV: 3 is CVS.*

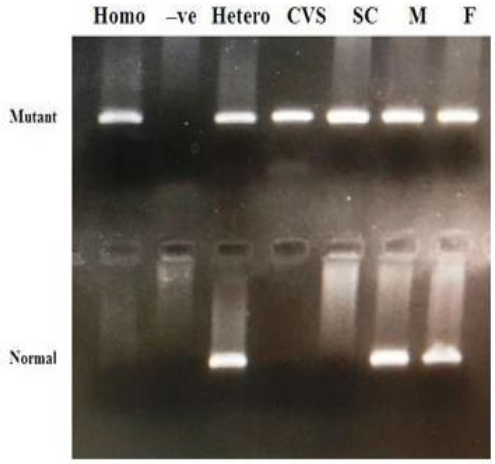

Figure 4: *Agarose Gel electropherogram of Family A showing IVS 1-5 mutation. The upper bands show the mutant allele, while lower one denotes normal allele. Mother (IV: 1) and father (IV: 2) are carrier/ heterozygous for IVS1-5.Proband and CVS are homozygous for IVS 1-5.*

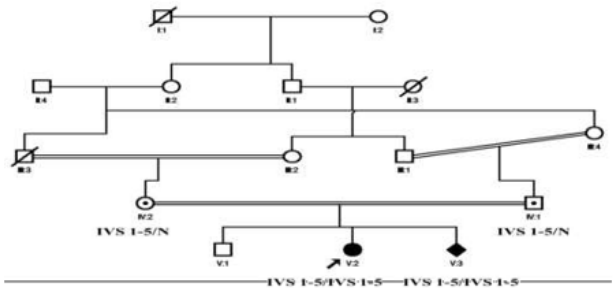

Figure 5: *Genogram for Family A segregating β-thalassemia with an autosomal recessive mode of inheritance. Father (IV: 2) and mother (IV: 1) are heterozygous i.e. IVS 1-5/N. CVS and sick child (proband) are homozygousas IVS 1-5/IVS 1-5.*

Both parents were carrier (heterozygous) while the sick child in the family (proband) was homozygous (inherit both mutant alleles, one from each parent) for this mutation. CVS specimen was checked for parental mutations. The ARMS-PCR results showed that fetus is homozygous for IVS1-5 mutation (IVS 1-5/IVS1-5).

Table 1*: DNA quantification of Family A using Nanodrop.*

No.	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)
3	4269	356.8	ng/µl	7.136	3.904
4	4270	236.9	ng/µl	4.739	2.700
5	4271	246.8	ng/µl	4.936	2.689
6	3272	131.8	ng/µl	2.635	1.440
7	3273	198.6	ng/µl	3.972	2.205

Table 2: *Nanodrop results of Family B.*

No.	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)
1	4266	196.7	ng/µl	3.934	2.145
2	4267	582.0	ng/µl	11.640	6.222
3	4269	356.8	ng/µl	7.136	3.904
4	4270	236.9	ng/µl	4.739	2.700

Table 3: *Summary of genotyping results of β-thalassemia families.*

Family ID	Diagnostic approach	Prenatal genotype MF	Fetal genotype	Fetal phenotype
Family A	ARMS PCR	M IVS-I-5/N F IVS-I-5/N	IVS 1- 5/IVS1-5	Affected
Family B	ARMS PCR	M FSC 8-9/N F FSC 8-9/N	FSC 8-9/N	Carrier

Family B: The genomic DNA of all the extracted samples was of good quality (Table 1-3).

Then Family B was first screened by ARMS PCR for IVS-I-5. Results showed that family is negative for this mutation. These samples were then screened for second most prevalent mutation

FSC 8-9, for which they were found carriers. Chorionic villus sample was idenfigutified as carrier for this particular mutation.

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