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IDENTIFICATION OF COMMON MUTATIONS IN PATIENTS WITH BETA-THALASSEMIA MAJOR AT LIAQUAT UNIVERSITY OF MEDICAL AND HEALTH SCIENCES JAMSHORO/ HYDERABAD

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Abstract:

OBJECTIVE: *To determine the common mutations in patients with Beta thalassemia major at LUMHS Jamshoro/Hyderabad, Sindh.*

MATERIAL AND METHODS: *This cross sectional study was held in the department of Genetics and Molecular Biology/Pathology department at LUMHS Jamshoro, and Diagnostic and Research Laboratory, Hyderabad, Sindh from: 06-02-2015 to 06-08-2015. All Patients of Beta thalassemia major diagnosed on the basis of clinical history Hb Electrophoresis, and their parents Hb Electrophoresis were included. CBC was done for initial assessment. DNA extraction (Nucleic acid purification) was done with inorganic method. All specimen were tested through the modified technique of ARMS PCR as described by Newton et al, 1989 [(10)].*

1.5. RESULTS: *Mean age of the patients was 11.02+3.93 years. 68.0% of affected were males. According to this study mean of Serum Ferritin, Haemoglobin, HbA2, HbF and HbA levels were found as 2824.39±300.60ng/ml, 7.52±1.67 gm/dl, 4.05±0. 25ng/ml, 91.14±1.26ng/ml and 34.41±1.15 ng/ml respectively. Most common gene mutation found was IVS 1 - 5 (G-C) in 25% of the cases, following by the IVS 1 - 1 (G-T), Fr 8 – 9, CD 30 (G-A), Fr - 16 (-C), Fr 1 – 42, Del 619 and CD 5 (-CT).*

CONCLUSION: *We concluded that the most common gene mutation were IVS 1 - 5 (G-C), IVS 1 - 1 (G-T), Fr 8 – 9, CD 30 (G-A), Fr - 16 (-C), Fr 1 – 42, Del 619 and CD 5 (-CT).*

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INTRODUCTION:

Thalassemia is a hereditary autosomal recessive disease of blood. Thalassemia is of 2 types, α and β. The hemoglobin comprises of four chains of proteins, 2 α-globin chains and 2 β-globin chains. In thalassemia, individuals possess either disorder in alpha- or beta-globin chains. The chains of beta-globin are encoded via a mono-gene over chromosome 11; chains of alpha-globin are encoded via two narrowly allied genes in chromosome 16.[1] The thalassemia disease causes premature destruction of RBCs. Hemoglobin is a protein within RBCs which carries oxygen. Cases having thalassemia generate small quantity of hemoglobin and lesser quantity of circulating RBCs as contrasted to normal ones, which gives rise to mild/severe anemia. Thalassemia can result in prime complications, causing overload of iron, abnormalities of bone and cardiovascular disorders.[2-4] The overall annual prevalence concerning symptomatic cases with Beta thalassemia is estimated about 1/100,000 worldwide and 1/10,000 cases in European Union.[5] 3 major kinds of β- thalassemia are described as Minor, Intermediate and Major correspondingly. β-thalassemia is widespread among Mediterranean countries, Central Asia, Southern China, India, Middle East and Far East in addition to the countries alongside the African North Coast and within the South America. It is reported that carrier frequency is highest in Sardinia (10.3%), Cyprus (14%) as well as Southeast Asia.[6] Marriages among cousins, people relocating and intermarriage among various ethnic groups; established thalassemia in about each country worldwide, including Northern Europe thought safe from thalassemia in the past. It is projected that roughly 1.5% of global populations (800,000 to 900,000 individuals) are Beta-thalassaemic carriers, with around 60 thousand symptomatic cases born per annum, the majority belonging to developing world[6]. Roughly 5-9 thousand offspring is born annually with β- Thalassemia in Pakistan, because of regular control of communicable diseases and malnutrition, majority of β-thalassaemic cases who previously passed away in young age are currently living long enough to obtain medical consideration.[7]

Although in countries as Pakistan, increasing issues regarding health-care services, only low number of affected population are able to afford sufficient blood transfusions along with efficient iron chelation and transplantation of bone marrow. Thus, prevention is established to be a way forward. It may be reduced through prenatal diagnosis as well as genetic counseling to carriers. Available procedures to identify various mutations must be taken, and prenatal diagnosis may add further means for prevention. To achieve this, ARMS, PCR has been widely used for characterization of gene mutations of β-globin using allele specific PCR primers.[8]

MATERIAL AND METHODS:

This cross sectional study was held in the department of Genetics and Molecular **B**iology/Pathology department at LUMHS Jamshoro, and Diagnostic and Research Laboratory, Hyderabad, Sindh from: 06-02-2015 to 06-08-2015. All Patients of Beta thalassemia major diagnosed on the basis of clinical history Hb Electrophoresis, and their parents Hb Electrophoresis were included. All the patients with other Hemoglobinopathies or having other Genetic diseases were excluded.

- 8 ml of blood was drawn and divided into two parts.
- 3 ml in Ethylene diaminetetra acetic Acid (EDTA) bottle for Complete Blood Count (CBC).
- 5 ml blood in Tris Edta for Genomic DNA Extraction.

Complete blood counts of all samples were analyzed for basic hematological parameters; this include Hemoglobin measurement, red cell count, white cell count, platelet count, packed cell volume(PCV), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and red cell distribution width (RDW) using automated cell analyzer.

Extraction of the DNA (Nucleic acid purification) was performed through the inorganic method.[160]

- Blood is frozen at -70°c for 20-30 minutes or at -20°c for 24 hrs before the extraction of DNA
- Add 30-35ml of Tris EDTA buffer (Tris HCl 10mM, EDTA 2mM) in 5 ml blood. Mix by inverting several times. (All centrifugations in subsequent steps should be performed at 25°c.
- Centrifuge at 3000 rpm for 20 minutes
- Discard 15-20 ml supernatant. Break pellet formed at the bottom by tapping it gently. Add TE buffer upto 45 ml.
- Repeat step # 4 & 5, until pellet becomes light pink.
- Discard the supernatant leaving ~0.5ml and re-suspend pellet in 6ml Buffer TNE (Tris HCl 10mM, EDTA 2mM, NaCl 400mM) for 10 ml initial blood volume. Add 200µl 10% SDS & 50µg proteinase K (50µl of 10µg/ul concentration)
- Samples were incubated all overnight in 37°C shaker.
- Complete digestion of the pellet checked after overnight incubation. In case pellet is not

completely digested add proteinase-K, through the amount of undigested pellet. Re incubate at 37°C till the pellet is completely digested.

- Place the tubes on ice and add 1ml-saturated NaCl (6M). Shake the tube vigorously and place on ice again for 10-15min.
- Centrifuge at 3000 rpm for 15 minute to pellet down the salts and proteins.
- Decant the supernatant in a conical bottom 15 ml properly labeled falcon tube.
- Centrifuge at 3000 rpm for 15 minutes.
- Decant supernatant in a 50ml labeled falcon tube.
- Add equal volume of isopropanol and invert the tubes gently till DNA is visible.
- Leave the tubes for 10 mints at the room temperature.
- Centrifuge for 10 mints at the 3000 rpm.
- Discard supernatant carefully.
- DNA pellet wash by 10ml 70% ethanol.
- Centrifuge till 10 mints at the 3000.
- Carefully discard 70% ethanol.
- DNA pallet at 37°C air dry at room temperature in a incubator.
- Add 1.5ml low T.E (Tris HCl 10mM, EDTA 0.2mM).
- At 37°C placed the tubes in incubator shaker for dissolve the DNA all over the night. Wrap strips of Parafilm around the caps of the tubes
- Tubes placed in a shaking water bath at 70°C for inactivate nucleases till the one hour.
- Let cool tubes at the room temperature.
- Spin briefly.
- Label side and cap of 2.0 ml autoclaved tubes. Label its individual ID and the Pedigree number.
- Aliquot DNA in duplicate and store at -20°C according to pedigree number in marked & numbered Cryoboxes.
- Established the concentration of the DNA through spectrophotometery or by agarose gel estimation. Calculate the concentration of stock DNA and enter in the computer file (Annex -11)

- All samples were tested by the (ARMS) PCR as described by Newton et al, 1989

Data Analysis Procedure:

Data was analyzed on (SPSS) version 18. Frequency and percentage were computed for qualitative variables like gender and mutations. Mean and standard were calculated for the mean of serum ferritin, hemoglobin, hba2, hbf and hba levels.

RESULTS:

TABLE: 1 PATIENTS DISTRIBUTION ACCORDING TO AGE n= 80

AGE	YEARS
Mean+SD Minimum Maximum	11.02±3.93 years 3.00 years 20.0 years

Mean age of the patients was found 11.02+3.93 years, ranging from a minimum of 3 years to maximum 20 years.
TABLE: 1

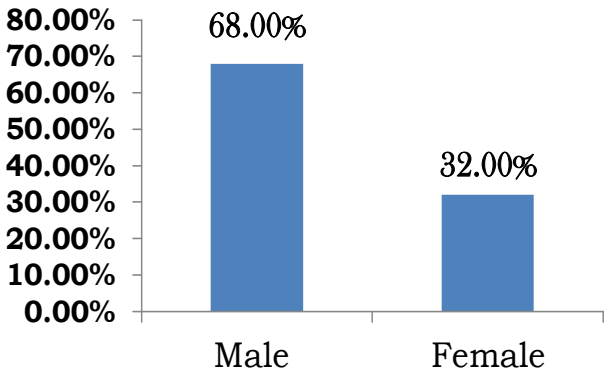

Fig 1: Patients Distribution According To Gender n= 80

According to our study % of affected males was higher than females, 68.0% compared to 32.0%. **FIGURE: 1**

Table 2: Patients Distribution According To Serum Ferritin Levels N= 80

Serum Ferritin level	**Mean±SD**
Serum Ferritin level Haemoglobin level HBA2 level HBF level HBA level	2824.39±300.60 ng/ml 7.52±1.67 gm/dl 4.05±0.25 ng/ml 91.14±1.26ng/ml 34.41±1.15 ng/m

According to this study mean of serum Ferritin level was found to be 12824.39±300.60ng/ml, y mean serum haemoglobin level was found to be 7.52±1.67 gm/d, mean of HBA2 level was found to be 4.05±0.25 ng/ml, mean of HBA2 level was found to be 4.05±0.25 ng/ml and HBA2 level was found to be 91.14±1.26ng/ml. **TABLE: 2**

Table 3: Patients Distribution According To Gene Mutation n= 80

MUTATION	**NUMBER OF PATIENTS**	**PERCENTAGE**
IVS 1 - 5 (G-C)	20	25%
IVS 1 - 1 (G-T)	16	20%
Fr 8 - 9	04	05%
CD 30 (G-A)	14	17.5%
Fr - 16 (-C)	06	7.5%
Fr 41 - 42	08	10%
Del 619	04	05%
CD 5 (-CT)	08	10%

In this study most common gene mutation found was IVS 1 - 5 (G-C) i.e. 25% of the cases, followed by IVS 1 - 1 (G-T), Fr 8 – 9, CD 30 (G-A), Fr - 16 (-C), Fr 1 – 42, Del 619 and CD 5 (-CT). **TABLE:3**

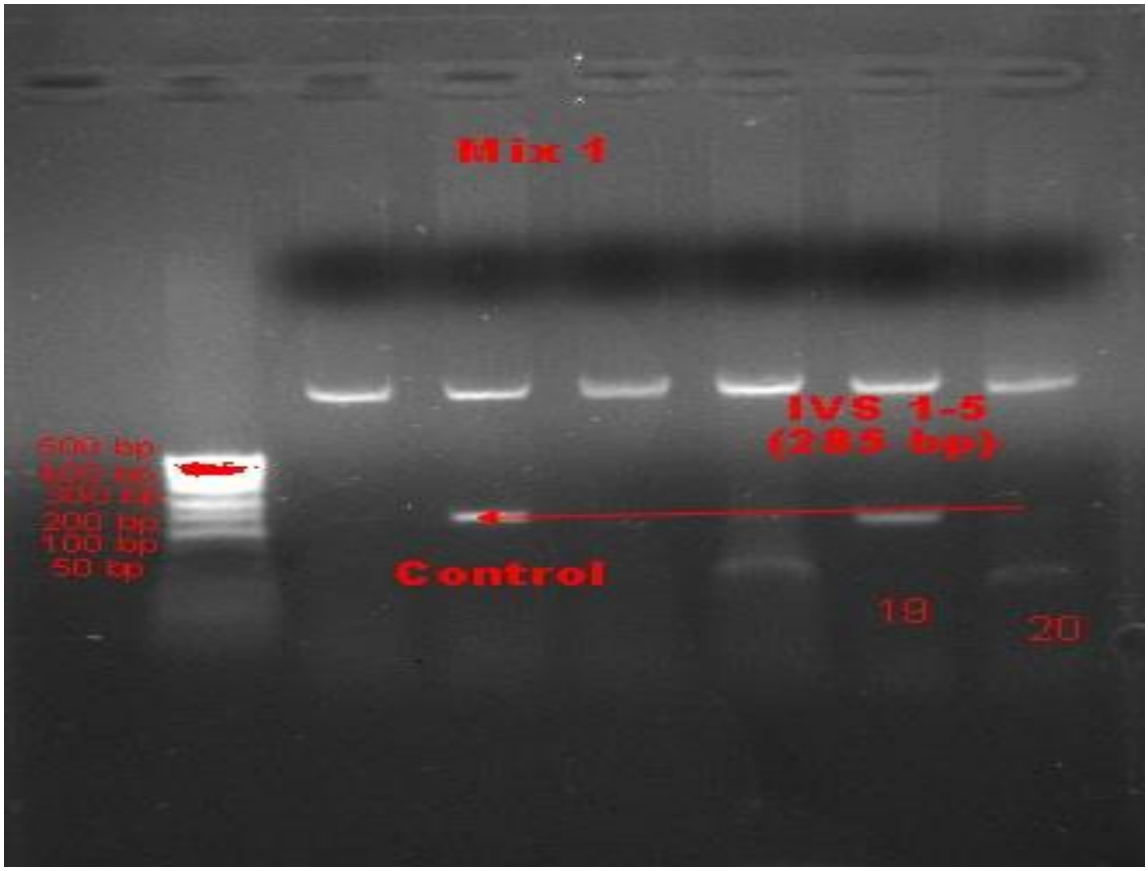

FIGURE: 2

This figure shows the positive control for Thalassemia Major Mutation (IVS 1-5).

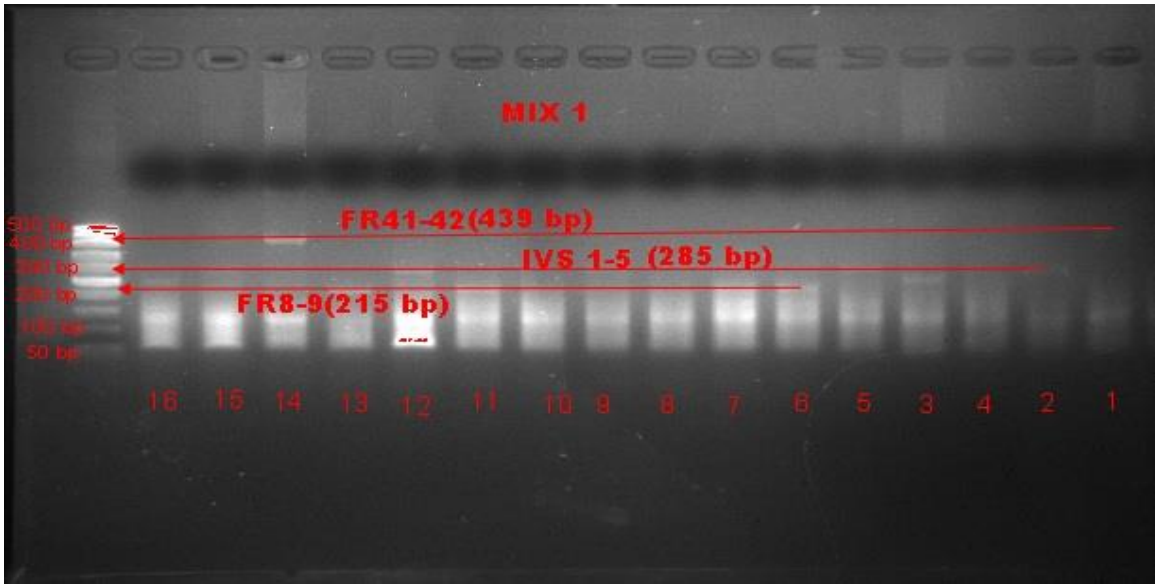

FIGURE: 3

This figure shows the amplification of the mutations which are present in MIX 1 by their fragment size in the DNA Ladder through Gel Electrophoresis.

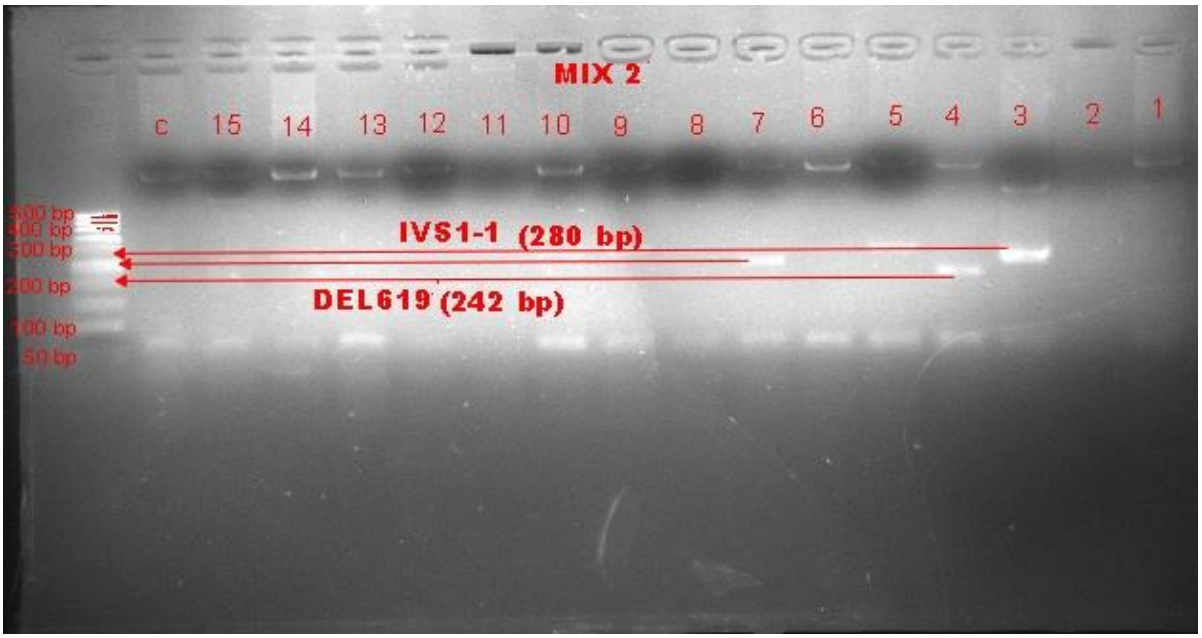

FIGURE: 4

This figure shows the amplification of the mutations which are present in MIX 2 by their fragment size in the DNA Ladder through Gel Electrophoresis.

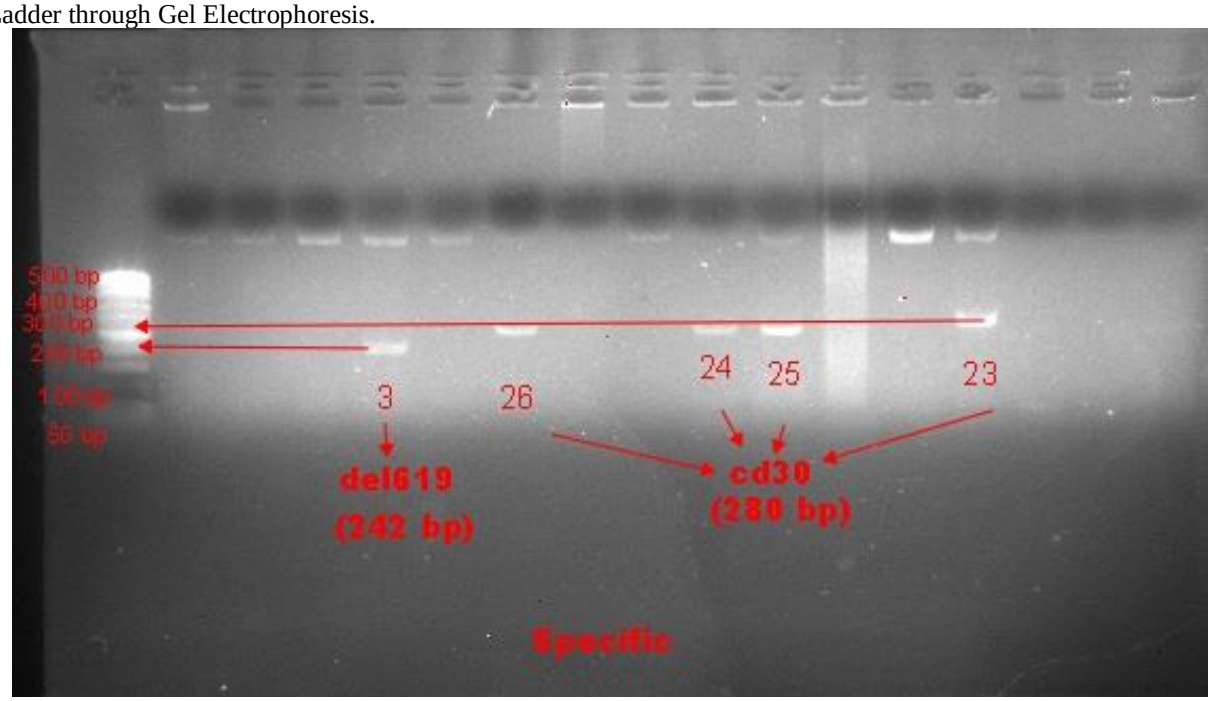

FIGURE: 5

This figure shows the amplification of the mutations by using specific primers for e.g Del 619 and Cd 30 with the help of their fragment size in the DNA Ladder through Gel Electrophoresis.

DISCUSSION:

Thalassemia is a very common (genetically determined) blood disorder prevalent worldwide. However, β thalassemia is found in about 60 nations with a carrier frequency of about 150 million. More than 300 different beta-globin gene mutations have been recognized. Most of the beta-thalassemia mutations are caused by point mutations, small

deletions or insertions within the coding regions and the exon-intron junctions. Genetic risk, such as identification of β-globin gene mutations should be integrated routinely into epidemiological studies followed by genetic counseling and prenatal diagnosis to reduce birth rate of affected infants.[1,2] Worldwide, The highest percentage of Beta thalassemia patients was between 3-5years of age group. It was observed that number of young patients was high as compared with old patients. This is due to increased disease load and shortened life expectancy.

Shahid Raza et al[9], in their study, reported age of Beta thalassemia patients was believed to be 10 years. In our study mean age of the patients was 11.02+3.93 years (range of minimum 3 years and maximum 20 years).

In our study males outnumbered females (68.0% compared to 32.0%). Shahid raza et al[9] in their study noted a ratio of 56.95% males while females were 43.05%. This finding is similar to our study also. This gender-ratio difference in thalassemia patients is significant and justifies further analysis in view of thalassemia as the single-gene disorder transmitted through the recessive mode of inheritance.

In our study mean serum Ferritin level was found to be 2824.39±300.60 ng/ml. Haris Riaz et al[10] reported levels of Ferritin 4236.5 ng/ml, which is significantly higher than normally accepted levels.[11] Choudhry VP et al in India found even higher levels of 6723 ng/ml.[12] Ferritin is the main iron-storage protein in the body. Its synthesis is regulated by quantities of iron by means of the interaction of cytoplasmic proteins bound to the messenger ribonucleic acid (mRNA), currently identified as iron regulatory proteins with specific structures of the mRNA, called iron-responsive elements.[15] It has a central role in iron homeostasis, because it binds to and sequesters intracellular iron. Serum ferritin measurement has become a routine laboratory test and high levels are a common finding in clinical practice. High serum ferritin levels are found in a large spectrum of genetic and acquired conditions, associated with or without iron overload.

High levels of serum ferritin have been observed in beta-thalassemia trait comparative studies, and even those who had never been transfused developed clinical and laboratory signs of iron overload.[15-17]

In this study most common gene mutation found was IVS 1 - 5 (G-C) i.e. 25% of the cases, followed by the IVS 1 - 1 (G-T), Fr 8 – 9, CD 30 (G-A), Fr - 16 (-C), Fr 1 – 42, Del 619 and CD 5 (-CT). Usman M et al.[13] quoted 5 general mutations IVS-1-5 (G→C), 8/9 (+G), 41/42 (-TTCT), IVS-1-1 (G→T) and 619-bp deletion (prevalence of 91.66% in Pakistani population). Usman et al.[14] reported an occurrence of these 5 mutations with 90% in population of Pakistan (N=400).

CONCLUSION:

We concluded that the most common gene mutation was IVS 1 - 5 (G-C), followed by IVS 1 - 1 (G-T), Fr 8 – 9, CD 30 (G-A), Fr - 16 (-C), Fr 1 – 42, Del 619 and CD 5 (-CT). The sample size was small for definite conclusions to be drawn. Therefore more (large) studies are required on gene mutation.

Suggestions

Preventive strategies are required all over the country to decrease the rate of thalassemia major. The important steps for prevention of thalassemia include:

1. Creating awareness in all population.
2. Family screening in thalassemia cases.
3. Genetic counseling of the cases and couples who are at risk of acquiring thalassemia.

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