Investigation of mutations in β-thalassaemic patients in the province of Khyber Pakhtunkhwa

By

Rooma Khan

NUST201361715MASAB92513F

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences and Technology,

Islamabad, Pakistan.

2015

Investigation of mutations in β-thalassaemic patients in the province of Khyber Pakhtunkhwa

A thesis submitted in partial fulfillment of the requirement for the degree of

Master of Science

In

Healthcare Biotechnology

BY

Rooma Khan

NUST201361715MASAB92513F

Supervised by: **Dr. Rumeza Hanif**

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences and Technology,

Islamabad, Pakistan.

2015

DECLARATION

I, **Rooma Khan**, declare that all work presented in this thesis is the result of my own work. Where information has been derived from other sources, I confirm that this has been mentioned in the thesis. The work herein was carried out while I was MS student at the Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan under the supervision of **Dr. Rumeza Hanif**.

TIMELINE

The following timeline was followed during this one year of research time:

September, 2014 – November, 2014	November, 2014- Februrary, 2015	March, 2015- April, 2015	May, 2015- June, 2015	July, 2015	August, 2015
Literature study and Sample Collection					
	DNA Extraction				
		PCR Optimization			
			PCR based detection of mutations		
				Data Analysis, Results, Conclusion and Thesis Writing	
					Thesis Submission

ABSTRACT

β- thalassaemia is a group of hereditary hematological disorders which are heterogeneous in nature with a number of reported mutations. These disorders have same clinical symptomatology that is loss of β- chain of haemoglobin leading to its poor or no synthesis which results in an inadequate oxygen supply causing anaemia and hypoxia. Population of KPK region shows favourable signs for β- thalassaemia prevalence. Its socio-economic system and strong cultural norms favour under-reporting of patients to healthcare units and also favour consanguineous marriages which increase the chance of β- thalassaemia prevalence. In the current study, KPK population was screened for 16 known mutations in Pakistani population. Samples were taken from Mansehra, Abbottabad and Peshawar followed by characterization by ARMS PCR. IVS-1-5 mutation was found to be the most prevalent in the sample population (53.14%) followed by FSC-8/9 (G+) and Cd 41-42 (-CTTT) which were prevalent in (17.71%) and (14.57%) of the sample population, respectively. The high percentage of β-thalassaemia was seen in Swati family (40.57%) followed by Syed (13.71%) and Tanoli (12%). High trend of consanguineous marriages were also found in the population. Complications of DFO were very common in older patients as compared to younger ones which concluded that with an increase in age risk to complications also increases.

Dedication

Fruitful efforts are dedicated first of all to Almighty ALLAH, Then to my Maternal Grandmother “Ammi Jaan”, My Parents, Mrs. And Prof. Muhammad Nawaz, to my Brother Jamal Khan and sister Afeefa Khan

To whom I am greatly thankful for their support and encouragement.

ACKNOWLEDGEMENT

All praises to Allah Almighty, the origin of all knowledge, the knower of everything, and with whom the rest all power. The favours He has bestowed upon me are uncountable, and blessed I am to have completed my work by his Will and Honour.

This effort is not mine alone- I want to share it with a lot of people because I would never have been able to finish my dissertation without them. I am truly grateful to them for providing all the small stepping stones of assistance, which brought this research up to this level.

The first person I would like to thank is my supervisor, Dr. Rumeza Hanif for continuous support of my study and research, for her patience, motivation, enthusiasm and immense knowledge. Her sage advice, insightful criticisms, and patient encouragement aided the writing of this thesis in innumerable ways. I could not have imagined having a better advisor and mentor for my study.

I think it is an opportune moment for me to thank Dr. Peter John, Principal ASAB, for providing the infrastructure for excellent research, and also Engr. Muhammad Asghar, Rector NUST, for the running of a great institution, and making the unique opportunity of research a possibility.

I would also thank my GEC members including Dr. Jawad Hassan and Dr. Aneela Javed for their help, guidance and support during this project. Whenever I was stuck in any problem, they guided me very well. I am also thankful to Dr. Aneela Javed for allowing me to work in her laboratory. I am truly grateful to Mr. Tahir Baig for help during PCR optimization when I was totally disappointed about results. At that time, his presence was light in that darkness that guided me.

I would also like to thank maternal uncles Muhammad Ayaz Khan, Prof. Muhammad Hafeez Khan and Captain Mohsin Ali for helping me obtain blood samples for this research. My humble gratitude goes to KBDO Mansehra, Thalassaemia Center Abbottabad and Fatimid Foundation Peshawar for providing the blood samples.

I would like to end acknowledging all my batch mates and friends, especially Muhammad Saeed, Muhammad Salim, Abdul Haseeb Khan, Arslan Amir and Aadil Javed who made these two years of my Masters worth living. They gave me a moral support to me in tough times. My best wishes and prayers are all with you. Also, congratulations on pulling it off successfully!

Rooma Khan

Table of Contents

Title Page …… **I**

Declaration …… **III**

Timeline …… **IV**

Abstract …… **V**

Dedication …… **VI**

Acknowledgement …… **VII**

Table of Contents …… **IX**

List of Abbreviations …… **XIII**

List of Tables …… **XIV**

List of Figures ....…… **XV**

Chapter 1: Introduction …… **1**

Chapter 2: Literature Review …… **5**

2.1. History …… 5

2.2. Classification …… 5

2.2.1. α-Thalassaemia …… 6

2.2.2. β-Thalassaemia …… 6

2.2.2.1. Thalassaemia Minor …… 7

2.2.2.2. Thalassaemia Intermedia ……..... 7

2.2.2.3. Thalassaemia Major …… 8

2.3. Expression of α and β-Globin Genes …… 8

2.4. Molecular basis of β-thalassaemia ……... 9

2.5. Mechanisms Altering the Expression of β-Globin Gene …… 10

2.5.1. Transcriptional Promoter Mutations ……... 10

2.5.2. Chain Termination Mutations …… 11

2.5.3. Deletions as a Cause of β-Thalassaemia …… 11

2.5.4. Dominant β-Thalassaemia Mutations …… 12

2.5.5. β-Thalassaemia Due To Unknown Mutations …… 12

2.6. Pathophysiology of β-thalassaemia …… 13

2.7. Epidemiology of β-Thalassaemia …… 14

2.8. Epidemiology of β-Thalassaemia in Pakistan…… 14

2.9. Diagnosis …… 15

2.9.1. Clinical diagnosis …… 15

2.9.2. Haematological diagnosis …… 15

2.9.3. Molecular diagnosis …… 16

2.9.4. Prenatal diagnosis …… 16

2.10. Genetic counseling …… 17

2.11. Prognosis …… 17

2.12. Clinical management for βThalassaemia …… 18

2.12.1. Transfusions …… 18

2.12.2. Iron chelation therapy …… 19

2.12.3. Bone marrow transplantation …… 20

2.12.4. Lifestyle and Diet in β-Thalassaemia …… 21

Research Objectives …… 24

Chapter 3: Materials and Methods …… 25

3.1. Study Subjects …… 25

3.2. Blood Sample Collection …… 25

3.3. Sample Storage and Transport ……..……. 25

3.4. Solutions Used In DNA Extraction ……..... 26

3.4.1. RBC Lysis Buffer …… 26

3.4.2. Proteinase K …… 26

3.4.3. 20% SDS solution …… 26

3.4.4. 6 M NaCl Solution …… 26

3.4.5. Tris-EDTA Buffer solution (TE Buffer) …… 27

3.4.6. 1X Tris Acetate Ethylenediamine tetraacetic acid (TAE)…… 27

3.5. Genomic DNA extraction by salt extraction method …… 27

3.6. DNA Dilution …… 28

3.7. Primer Designing …… 28

3.8. Detection of Mutations …… 29

3.8.1. Amplification Refractory Mutations System –
Polymerase Chain Reaction (ARMS-PCR) ……..... 29

3.8.2. PCR Process …… 29

3.8.3. Validation of ARMS PCR …… 32

3.9. Pedigree Analysis …… 33

Chapter 4: Results …… 34

4.1. Genotyping of β-Globin Gene Mutations in β-Thalassaemia Patients 34

4.2. Age-wise Distribution of Study Samples …… 37

4.3. Gender-wise Distribution of Study Samples …… 38

4.4. Parental Relationship of Study Samples …… 39

4.5. Economic Status-wise Distribution of Study Samples ……..... 40

4.6. Caste-wise Distribution of Study Samples …… 41

4.7. Other complications in β-Thalassaemia Major Patients …… 42

4.8. Complications of DFO Therapy in β-Thalassaemia Major Patients… 43

4.9. Pedigree Analysis …… 44

Chapter 5: Discussion …… 46

Conclusion …… 50

Future Prospects …… 51

References …… 53

Appendix …… 66

List of Abbreviations

β	Beta
α	Alpha
γ	Gamma
Δ	Epsilon
HBB	Haemoglobin Gene
KPK	Khyber Pakhtunkhuwa
NaCl	Sodium Chloride
$MgCl_2$	Magnesium Chloride
TAE	Tris Acetate EDTA
EDTA	Ethylenediaminetetraacetic Acid
SDS	Sodium Dodecyl Sulphate
RBCs	Red Blood Cells
WBCs	White Blood Cells
N.F.	Nuclease Free Water
PCR	Polymerase Chain Reaction
ARMS	Amplification Refractory Mutations System
µl	Micro-liter
dNTPS	Deoxyribonucleotide Triphosphates
%	Percentage
Ml	Millimeter

List of Tables

Table 3.1. Primer Sequences and Amplicon Sizes forDetection of Mutations 30

Table 4.1. Distribution of β – Globin Gene Mutations across β – Thalassaemia Major Patients …… 36

Table 4.2. Other Complications in β-Thalassaemia Major Patients …… 42

Table 4.3. Complications associated with DFO Therapy ……..... 43

List of Figures

Figure 4.1: Representative gel image of IVS 1-5, FSC 8/9 and CD 41/42…. 35

Figure 4.2: Age wise Distribution of Study Samples ……..... 37

Figure 4.3: Gender Wise Distribution of Study Subjects …… 38

Figure 4.4: Parental Relationship of β- Thalassaemia Patients …….…… 39

Figure 4.5: Economic Status-wise Distribution of Study Samples..……... 40

Figure 4.6. Caste-wise Distribution of β-thalassaemia in Study Subjects…. 41

Figure 4.7. Pedigree of a Family having β-thalassaemia major Patient …… 45

CHAPTER 1

INTRODUCTION

β-thalassaemia is an autosomal recessive genetic disorder that results mainly from mutations, decreasing (b+) or eliminating (b0) the expression of β-globin gene (Weatherall *et al*., 1981). The disease is heterogeneous at the molecular level, with about 200 different molecular defects and is associated with variable degrees of anaemia, bone marrow hyperplasia, splenomegaly, and other related complications. The most severe form of this disease is β-thalassemia major, which becomes manifest several months after birth, when the foetal to adult haemoglobin (Hb) switching is completed (Weatherall, 1998).

In 1925, β-thalassaemia was first characterized by Thomas Cooley as a disease characterized by severe anaemia, peculiar bone deformities and splenomegaly among the children of Italian and Greek decent currently known as β-thalassaemia major which was named as Cooley's anaemia. From year 1925 to 1935, several other researchers also explained the condition of mild anaemia and augmented osmotic resistance of erythrocytes. Symptoms of mild anaemia were found to be less dangerous to Cooley's anaemia and the condition was named as 'La Malattia di Rietti-Greppi-Micheli and nowadays is known by the name thalassemia intermedia (Pignatti *et al*., 2011).

The thalassemias (named from the Greek word for sea, thalassa) (Whipple, 1936) are a group of inherited autosomal recessive hematologic disorders that cause haemolytic anaemia because of the decreased or absent synthesis of a globin chain and this makes

thalassaemia different from haemoglobinopathies. Imbalances of globin chains cause haemolysis and impair erythropoiesis as well (Muncie *et al*., 2009).

Thalassaemias are commonly found in the populations of Asia, Africa, Mediterranean and spread worldwide mainly because of migrating populations (Model, 2003). There are different types of this disease including α-thalassaemia, β-thalassemia and HbE-thalassemia. The rate of their occurrence depends on ethnicity as α-thalassaemia and HbE- thalassaemia occur more frequently in South East Asia while β-thalassaemia occurs most frequently in Africa, Mediterranean region, Indian Subcontinent and South East Asia (Cao *et al*., 1997). Prevalence of thalassaemia is more in Mediterranean Countries, Central Asia, the Middle East, India, Southern China and Far East, also in the countries along the North coast of Africa and in South America (Flint *et al*., 1998). Reasons behind its wide spread in almost every country of the world are population migration and intermarriages between different ethnic groups, including Northern Europe where thalassaemia was previously not found (Vichinsky, 2005).

In Pakistan, β-thalassemia is the most common disease among genetic disorders related to Hb (Rafique, 1990). The population in Pakistan is divided into four regions, with Pakhtuns and Punjabis in the north whereas Balochs and Sindhis in the south of the country. Besides this, there are other divisions which are based on ethnicity such as Urdu speaking, Gujratis and Memons who migrated to Pakistan at the time of partition in 1947. Due to cultural and ethnic set-up, consanguineous marriages are quite common in Pakistan. Consanguineous marriages, high gene frequency, high birth rate, lack of awareness about premarital screening and counselling of those people with family history are common reasons that nurture this fatal disease among population of Pakistan

(Arif *et al*., 2008). The disease can only be prevented by proper genetic counselling, genetic screening and prenatal diagnosis (Cao *et al*., 1991). The life expectancy and quality of life of patients is low due to lack of availability of fresh and screened blood at low cost. Moreover, bone marrow transplant is not in practice in Pakistan due to high cost. The average cost of transplant for thalassemia is 2 Million Pakistani Rupees approximately (Shamsi *et al*., 2008).

β-thalassaemia may be classified as thalassaemia major, intermedia and minor. Clinical manifestations of thalassemia major start to occur between the age of 6 and 24 months. Infants affected with this disease succumb to grow normally and become progressively pale. Individuals with thalassemia intermedia develop symptoms later than thalassemia major, such patients have milder anaemia and by definition do not require transfusion. The clinical symptoms of severe form of β-thalassemia intermedia occur between the ages of 2 to 6 years. On the other hand, individuals with thalassemia minor remain completely asymptomatic with little or no anaemia (Danjou *et al*., 2011).

Different populations and ethnic groups in Pakistan have specific set of mutations in β-globin genes. The study of β-thalassaemia at its molecular level in different ethnic groups is very important. A handful of studies have been conducted in the past but sill it is an uncluttered field for molecular researchers (Ansari *et al*., 2011). Considering the literature, the KP population is not studied as a whole KP province population rather all the data available about this population is studied by taking samples of Pakhtuns living in provinces other than KP. So, in this study, samples are gathered from the KP and β-thalassemia molecular epidemiological study is done highlighting the pattern of common mutations for β-thalassemia major in KP province in comparison to common

mutations residing in rest of the Pakistan. The major aim of this study was to study the prevalence of this fatal disease, its pattern and detection of mutations in different ethnic groups of KP. This is the most untouched and unstudied area so far in Pakistan regarding genetic diseases and specially β-thalassemia.

CHAPTER 2

LITERATURE REVIEW

2.1 History:

In 1925, Cooley and Lee first identified anaemia characterized by splenomegaly and bone deformities among children of Italian and Greek descents. The disease was named as Cooley's anaemia which is now known by the name thalassaemia major (Whipple and Bradford 1933). Later on another group of researchers found that this disease is homozygous for a recessive autosomal gene (Pauling *et al*., 1949).

In early 1950's, several studies were executed on haemoglobin of patients with diverse clinical forms of thalassaemia which led to a conclusion that it is a hereditary heterogeneous blood disorder which is passed from parents to off-springs through defective genes (Pauling *et al*., 1949). The study of haemoglobin patterns of patients with different forms of thalassaemia and structural haemoglobin variations led scientists to the point that there might be different types of thalassaemia (Ingram and Stretton, 1959).

In 1970s, the existence of thalassaemia in many forms and significant heterogeneity was proved. Hence, thalassaemia was defined as heterogeneous group of inherited haemoglobin disorders which affect the synthesis of haemoglobin, characterized by deficiency or reduced output of one or more of the globin chains of the haemoglobin (Weatherall and Clegg, 2001).

2.2. Classification:

Genetically, thalassaemia is described on the basis of the presence or absence of globin chains which in turn show the structure of globin genes that are involved in

their synthesis. On this basis, they are classified into two major types: α-thalassaemia and β-thalassaemia (Flint *et al*., 1993).

2.2.1. α-Thalassaemia:

α-thalassaemia is caused by less production of the alpha globin chain of haemoglobin molecule. Haemoglobin is a tetrameric molecule, comprised of two α like and two β like globin chains. Excess in β like globin chains occurs due to under production of α globin chains. Patients with mutations on one of the chromosomes affecting the α globin genes, linked with less anaemia have α-thalassaemic traits(Chui *et al*., 1998; Liang *et al*., 1985; Nakayama *et al*., 1986).

2.2.2. β-Thalassaemia:

β-thalassaemia is a group of hereditary blood disorders characterized by anomalies in the synthesis of the β chains of haemoglobin resulting in variable phenotypes ranging from severe anaemia to clinically asymptomatic individuals (Galanello and Origa, 2010). Besides classical recessive forms of β-thalassaemia, there are some very rare mutations which lead to synthesis of highly unstable variants of β globin chains which during the process of erythropoiesis precipitate in erythroid precursors resulting in an inadequate erythrocytes formation. These mutations are associated with a clinically detectable thalassaemia phenotype in the heterozygote and are therefore referred to as dominant β-thalassaemia (Croteau *et al*., 2013). β-thalassaemia has been shown to be very heterogeneous in clinical as well as molecular levels. About 217 causative molecular mutations have been reported in the β-globin gene resulting in β-thalassaemia, most of them are point mutations in functionally active regions of the β globin gene (Hardison *et al*., 2002). There are different molecular mechanisms by which point mutations in β-globin gene occur. Most of them are base substitutions or

small deletions or insertions of one or two nucleotides in the β-globin gene (Clark *et al*., 2004). Moreover, it has also been observed that these mutations are relatively specific to particular population or ethnic group, i.e. certain population or ethnic group has its own unique set of common mutations in β-globin gene (Xu *et al*., 2004). For example, haemoglobin E occurs as a result of a point mutation at position 26 of the β-globin chain (glu->Iys). As a consequence of this mutation, the abnormal gene leads to the production of decreased levels of β-mRNA as well as β-globin chains (Thein, 2005). Clinically, it exist in 3 main forms on the basis of the severity and worsening of the disease namely thalassaemia minor, thalassaemia intermedia and thalassaemia major (Flint *et al*., 1993).

2.2.2.1. Thalassaemia Minor:

Individuals with β-thalassaemia minor are the carriers of the disease. They usually do not show clinical symptoms but they may have mild anaemia during their life span. If both parents are thalassaemia minor carriers, they have 25% chance of having children with homozygous β-thalassaemia at each pregnancy (Weatherall, 2004). As far as thalassaemia minor is concerned, it is clinically asymptomatic, however, in some individuals moderate anaemia has been reported. Individuals having a thalassemic trait in one gene are known as carriers or have thalassaemia minor.

2.2.2.2. Thalassaemia Intermedia:

Moderate anaemia have been reported in patients with thalassaemia intermedia and required no habitual or regular blood transfusions. In the patients of thalassaemia intermedia, several clinical features have been reported such as hypertrophy of erythroid marrow with medullary and extramedullary haematopoiesis (Galanello *et*

al., 2001; Taher *et al*., 2008; Sanctis *et al*., 1998; Nassar *et al*., 2008; Farmakis *et al*., 2005, 2002).

2.2.2.3. Thalassaemia Major:

Severe anaemia and need of red blood cells transfusions have been reported to be presented in patients in the first two years of life in case of thalassaemia major. Jaundice, growth retardation, poor musculature, enlargement of spleen and liver and some bone marrow abnormalities which may lead to modifications in skeleton and development of masses from extrame dullary haematopoiesis have been seen in patients which are untreated and not transfused properly in many developing countries. Transfusion therapy, if given regularly may cause iron overload related problems like endocrine complication (Borgna *et al*., 2004, 2005).

2.3. Expression of α and β-Globin Genes:

The expression of α and β-globin genes start to activate by the end of third week of gestation. During foetal development, an alteration in the expressions of globin genes have been reported (Stamatoyannopoulos, 2005; Weatherall, 1986). Initially, there is production of embryonic haemoglobin, then foetal haemoglobin is formed leading to the production of adult haemoglobin. The expressions of α -globin genes stay even throughout all life. The final protein product of both α 1 and α 2 genes are almost same, however α 2 mRNA levels are seemed to be in majority as compare to α 1-mRNA. While on the other hand, expressions of more related genes such as ε-, and γ-globin gene are down regulated alongside the increased expression of β-globin gene with the transition from foetal to adult haematopoiesis (McGrath *et al*., 2011). By the six month of time after birth, the process of switching γ to β-globin usually reaches to an end (Kingsley *et al*., 2006).

For controlled expressions of ε, γ and β-globin, trans-acting factors like GATA binding proteins play an important role. These GATA binding proteins are synthesized in yolk sac, foetal liver and bone marrow and bind to DNA sequence motif (T/A)GATA(A/G) present in the ε, γ and β-globin promoters. The two important promoter binding proteins GATA- 1 and GATA-2 have been shown to be very crucial for the controlled expression of erythroid specific genes (Moritz *et al*., 1997).

The process of transcription for globin genes begins at the transcription initiation site corresponding to the Cap site which is located 50 bp upstream from the starting codon (AUG) and it also labels the 5' end of the mature cytoplasmic mRNA. The transcription starts on reading all the exons introns and all the sequences beyond the highly conserved 3' "AATAAA" polyadenylation site (Barber, 2011).

2.4. Molecular basis of β-thalassaemia:

The β-globin gene is present on short arm of chromosome 11p15.4-11p15.5 almost of 45 Kb and consists of 5 genes (Das & Talukder, 2002). Mutations in different regions of β-globin gene either result in the reduction or the absence of the globin chain synthesis while in some cases highly unstable β-globin product is formed is known as β-thalassaemia major while the type in which complete absence of β-globin chain occurs is known as β-thalassaemia (Higgs *et al*., 2001). The common cause of β-thalassaemia is mainly of point due to point mutations, small deletions and insertions in the β-globin gene. Point mutations in the β-thalassaemia cause defective synthesis of the β chain. Mutations arising in promotor regions affect transcription process. Mutations in the coding regions or splicing sites or termination codons affect RNA

functioning, processing and translation where a few small deletions or insertions leading to frameshifts (Mansoor *et al*., 1998).

2.5. Mechanisms Altering the Expression of β-Globin Gene:

Expression of β-globin gene is kept in harmony by locus control region. On chromosome 11, there is a cluster of gene present out there and β globin gene is a part of that cluster (FU *et al*., 2002). Due to the presence of different types of mutations, several defects arise in the process of transcription (termination and initiation), often causes unusual RNA splicing and substitutions. As a result of these abnormalities, either there will be less production of β-globin or total absence of it which leads to the development of β- thalassaemia. There are different kinds of mutations including β zero mutations characterized by the complete non-existence of β globin chain production. These type of mutations are usually nonsense, splicing mutations or frameshifts. On the other hand, β-plus mutations are the ones having β-globin production in splicing defects and promoter regions unlike β zero mutations (Weatherall, 1994).

A number of mechanisms have been reported by which following mutations alter the expression of β-globin genes. Few are explained below.

2.5.1. Transcriptional Promoter Mutations:

One of the most significant mutations that cause β-thalassaemia are the mutations in the promoter sequences of the β-globin genes. The mutations lead to diminished promoter function. Most of the mutations are found to be concerted in the "TATA" box and in the proximal and distal "CACACCC" sequences at -90 and -105 nucleotides upstream of the gene (Rahimi, 2010). Such promoter mutations have the tendency to lower the rate of transcription by 20-30% as compare to normal one by

reducing the binding of RNA polymerase to β-globin gene sequences. For example, one of common mutations in the promoter site of β-globin gene i.e. C-T substitution at position -88 relative to Cap site results into a mild phenotype. Another mutation at the Cap site of the β-globin gene also influences the transcription as well as generates secondary effects on the process of translation. There are several other mutations in the promoter region of β-globingene which are known to cause β-thalassaemia (Patrinos*et al*., 2005).

2.5.2. Chain Termination Mutations:

Nonsense mutations may lead to the premature termination of transcription. A single nucleotide substitution in a certain codon which actually codes for a functional amino acid could turn into a stop codon. One of the common examples of nonsense codon is CAG-TAG substitution at codon 39. Apart from nonsense mutations, frame shift mutation either by an insertion or deletion of nucleotide(s) may also lead to premature chain termination. For example, four nucleotide deletion (-TTCT) in codons 41 and 42 results in introduction of a stop codon in another codon 59. All of the chain termination mutants have been shown to produce β°-thalassaemia phenotype. A number of nonsense as well as frame-shift mutations have been reported to cause of β°-thalassaemia (Weatherall, 2001).

2.5.3. Deletions as a Cause of β-Thalassaemia:

Deletions that cause β-thalassaemia are very common. 13 different deletions of variable lengths and sizes have been documented so far (Harteveld and Higgs, 2010). One of the most common deletions is the removal of 619 bp of IVS-H, exon-3 and sequences 3' of the gene. Some deletions also engross a large portion of the 5' untranslated region of β-globin gene and seemed to be linked with an abnormally high

level of Haemoglobin-A2 (Pagon *et al*., 2013). In a previous study, a 10.3kb deletion comprising the 5' untranslated region of β-globin gene in an Asian Indian is found to be linked with high Haemoglobin-A2 level has been reported. Such mutations presumably reduce the chances of competition among β-globin gene promoters (Weatherall, 2004).

2.5.4. Dominant β-Thalassaemia Mutations:

These mutations are responsible for dominant form of β-thalassaemia, in which heterozygote shows severe anaemia, have been identified (Galanello *et al*., 2011).A total of 27 different mutations, generally in the 3rd exon, are shown to be associated with this unusual phenotype. These mutations result in altering different and extremely significant mechanisms at molecular level which lead to production of highly unstable β-variant. These mechanisms are single base substitution or deletion of functionally intact codons, premature termination or truncation of β-globin chain, and an elongated β-globin chain with an modified carboxyl terminal end due to the of frame-shift mutation . Moreover, these dominant thalassaemia mutations in a heterozygous state also form highly unstable haemoglobin variant that has a tendency to precipitate in erythrocytes and leads to clinical manifestations of thalassaemia intermedia (Galanello *et al*., 2011).

2.5.5. β-Thalassaemia Due To Unknown Mutations:

In some cases of classic β-thalassaemia, not even a single detectable mutation has been identified in the β-globin gene or its immediate flanking regions. It is thought that in such cases β-thalassaemia occurs due to the mutations in some other genes located elsewhere in the genome or in the vicinity of β-globin genes and have a central role in β-globin gene expression (Serjeant *et al*., 2011).

2.6. Pathophysiology of β-thalassaemia:

In β-thalassaemia, unproductive erythropoiesis is the fundamental pathophysiology. After regular conversion of haemoglobin of the foetus which is a tetramer with 2 α and 2 γ globin chains to haemoglobin of adult which has 2 α and two β-globin chains, usually then clinical manifestations are seen owing to imperfection in β globin gene. Due to the deficiency or absence in the formation of β-globin chains, disproportion among α and β-chain occurs which causes excess α chains to precipitate off in red cells which are getting mature and erythroid precursors which leads to damage of membrane and destruction of cells. These destructed cells cannot survive leading to unproductive erythropoiesis which in turn causes anaemia as well as erythroid hyperplasia. Erythroid hyperplasia causes change in the bones of skull. Enlargement of liver and spleen also occurs due to later one because of extramedullary haematopoiesis in these organs. Severe anaemia occurs which is usually transfusion dependent in case of β thalassaemia major as in this there is either low production of β globin chain or either incomplete production. In case of moderate reduction, less anaemia has been reported in some individuals (β-thalassaemia intermedia) and in case of β thalassemic trait the heterozygous individual have mild anaemia. Intermediate phenotypes may develop due to mutations in different globin genes which can change the severity. From the intestine, increased absorption of iron as well as hepcidin's downregulated expression has been reported which may be due to inappropriate erythropoiesis and to some extent anaemia as well (Gardenghi, 2010). In case of β thalassaemia intermedia this is more distinct (Galanello, 2012).

2.7. Epidemiology of β-Thalassaemia:

Population of Mediterranean countries, North Africa, Middle East region and many areas of SouthAsia have been reported with mutations in β-globin chain with a very high frequency of greater than 1 percent (Livingstone, 1985). Immigration in areas of northern Europe and North America has led to the worldwide distribution of this disease, though mutations in β cluster gene is low in these region's natives. One of the other reason is intermarriages within different ethnic group of immigrants in the countries of Northern Europe where thalassaemia was totally absent. Approximately, 1.5% of global population is a carrier of β-thalassaemia, along with 60,000 symptomatic individuals born annually. However, the correct data about thalassemic carriers is unknown in the populations, especially in the areas of the world known or expected to be heavily affected (Vichinsky,2005). β-thalassaemia intermedia and βthalassaemia major have phenotypic similarity to complex heterozygosity in the company of E mutations and frequency has been reported to be higher in some parts of Southern China, Cambodia and Thailand too. According to an estimate made in 1998, 2001 and 2010, among the total children born with thalassaemia, 95% were from Middle East and Asia with mostly haemoglobin E mutations (Weatherall *et al*., 2001; Angastiniotis *et al*., 1998; Colah *et al*., 2010). In Pakistan, it has been reported that an estimated 5000-9000 children with β-thalassaemia are born per year, although no documentary registry is available at government level. The estimated carrier rate is 5-7%, with approximately 9.8 million carriers in total population (Lodhi, 2003).

2.8. Epidemiology of β-Thalassaemia in Pakistan:

In Pakistan, several molecular and epidemiological studies have been conducted mostly for larger cities and 20 different mutations have been identified (Ahmed *et al*.,

1996; Khateeb *et al*., 2000; El- Kalla and Mathews, 1997; Baig *et al*., 2005, 2006 a, b). Out of them, three most commonlyfound mutations are IVS-1-5 (G>C), FSC 8/9 (+G) and CD 41/42 (-CTTT). FSC 8-9 (+G) is the most prevalent mutation with a percentage of 38.59%, IVS-1-5 (G->C) being the second most prevalent with a percentage of 37.89% and third most common mutation is counted as Cd41/42 (-CTTT) with a percentage of 9.12 % (Baig *et al*., 2005). In Sindh and Baluchistan, IVS 1-5 (G->C) mutation was found prevalent while in Punjab and Khyber Pakhtunkhwa FSC8/9 (+G) is found more prevalent (Baig *et al*., 2005). IVS-1-5 (G-C) mutation was considered the most common mutation identified in Pakistani population and was found in 53% of the test subjects and was represented almost equally in all the ethnic groups except Pathans (Khateeb *et al*., 2000). FSC 8-9 is found to be the most common mutation followed by IVS-1-5 then followed by Cd 41-42 but still this study stated that FSC-8/9 was prevalent in Punjabis and Pathans (Khattak *et al*., 2012).

2.9. Diagnosis:

2.9.1. Clinical diagnosis:

Thalassaemia major is severe form of thalassaemia and usually seen in infants less than 2 years characterized by microcytic anaemia, mild jaundice and hepatosplenomegaly. Thalassaemia intermedia expresses itself in a later age with milder and less aggressive clinical findings as compared to major one. Individuals with thalassaemia minor are mostly asymptomatic but in some cases show signs of mild anaemia(Galanello *et al*., 1979).

2.9.2. Haematological diagnosis:

Thalassaemia can be diagnosed by Complete Blood Count (CBC) and Peripheral Blood Smear in which different morphological changes are examined which shows

signs of anaemiai.e. either hypochromic or hypocytic cells etc. Other quantitative and qualitative techniques are available for example HPLC and electrophoresis to identify the type of haemoglobin (Galanello *et al*., 1979).

2.9.3. Molecular diagnosis:

Commonly occurring mutations of the β-globin gene are normally detected by a number of polymerase chain reaction (PCR)-based procedures. The most frequently used methods are reverse dot blot analysis or primer-specific amplification by means of a set of probes or primers complementary to the most common mutations in the population from which the affected individual originated (Old *et al*., 2005). Additional methods based on real-time PCR or microarray technology due to their reproducibility, speediness, and easy handling are potentially suitable for the routine clinical laboratory (Vrettou *et al*., 2003;Ye B *et al*., 2007). If targeted mutation analysis fails to detect the mutation, scanning or sequence analysis can be used. Sensitivity of both mutation scanning and sequence analysis is 99%. In the meantime, the presence of an extended deletion should be investigated by using multiplex ligation-dependent probe amplification (MPLA) (Vrettou *et al*., 2003)

2.9.4. Prenatal diagnosis:

Prenatal diagnosis of β- thalassaemia was performed for the first time in the 1970s by using globin chain synthesis analysis of foetal blood attained by foetoscopy or placental aspiration (Kan *et al*., 1975). The molecular characterization of the thalassaemias, the improvement of methods for their detection by DNA analysis and the introduction of chorionic villus sampling in the last span have led to a dramatic development in the practices to be used in the prenatal detection of these disorders. For a brief period, the diagnosis of thalassaemia was achievedeither by indirect

method i.e. polymorphism analysis (Kan *et al*., 1980) or via direct method i.e.oligonucleotide hybridization on electrophoretically separated DNA fragments (Pirastu *et al*., 1983). Prenatal diagnosis is by getting DNA from foetal cells of amniocentesis, which is usually performed at 15-18 weeks of gestation period or chorionic villi sampling at 11 weeks gestation. Preimplantation genetic diagnosis may be available for families in which the disease causing mutation have been identified (Mavrou *et al*., 2007).

2.10. Genetic counseling:

Preconception genetic counseling is strongly recommended forall thalassemic patients (Sanctis, 2001).Thalassaemia can only be prevented by the identification of carriers and genetic counselling (Cao *et al*., 1998).If two parents have βthalassaemia trait, they will have one in four chance of having a child with βthalassaemia major and remaining three will be either normal or will have thalassaemia trait. On the other hand, individuals with αthalassaemia trait usually have a very complex pattern of inheritance. Chorionic villus sampling using polymerase chain reaction technology to detect point mutations or deletions can identify infants affected with βthalassaemia (Rappaport *et al*., 2004).

2.11. Prognosis:

Life expectancy of α-thalassemic patients is normal. Individuals with thalassemic major live an average of 17 years. Most of the patients die early and deaths are mostly due to iron overload that cause cardiac complications and liver cirrhosis (Modell *et al*., 2000).Severe β thalassaemia major (also called Cooley's anaemia) has traditionally had a poor prognosis with 80% dying from complications of the disease in the first five years of life (Peters *et al*., 2012).

2.12. Clinical management for βThalassaemia:

Since there is not any standard cure for this appalling disease, so one of the best ways to deal with this genetic disorder is to take preventive measures as well as accentuates the already existing thalassaemia management. Regular blood transfusions, iron chelation therapy and prerequisite for other facilities to support patient's life (Origa, & Galanello, 2010).

2.12.1. Transfusions:

The main goal in case of long term transfusion is to keep haemoglobin of patient at 9-10 g/dL, therefore improving patient's condition whereas at the same time suppressing increasing erythropoiesis which helps not only in treating anaemia.Hepatitis B vaccination shots is required before starting transfusion therapy as treatment. The transfusion is planned in such a manner that the pre-transfusionHaemoglobin concentration remains 95-100g/L and is normally planned after 2-3 weeks. There is increased possibility of developing increased iron amounts from gastrointestinal absorption of iron due to low hepcidin production in such patients. If serum ferritin concentration is increased more than 300µg/L, in this case chelation therapy is started (Origa *et al*., 2007, Borgna- Pignatti., 2007). The process of transfusion helps the patient to have normal development of body and allows performing physical activities like a healthy individual. It also decreases the chances of marrow hyperplasia and related bone deformities and prevents splenomegaly and hyperspienism (Birgens & Ljung., 2007).Though, there is a drawback of hyper-transfusion which is accumulation of excess iron present in the body which has huge tendency to harm many vital organs in the body such as liver and endocrine glands. Human body has not any definite mechanism to remove the excessive iron which gets

accumulated due to frequent blood transfusions over a period of time. To eliminate the iron overloads due to increased intestinal absorption, iron chelation therapy is given to the patient (Musallam *et al*., 2012).

2.12.2. Iron chelation therapy:

Chelation therapy is usually initiated after 10-12 transfusions have been given. Desferoxamine (DFO) is the most commonly used iron chelator which is administered generally 5-7 days a week for 12 hours of subcutaneous infusion. Dosage must be calculated on the basis of patient's age and concentration of serum ferritin. It may be started with 20-30mg/kg/day increasing to 40mg/kg/day (5-6 years of age) and reaching maximum of 50mg/kg/day after the growth is completed. Dosage can be reduced depending on the low serum ferritin concentration. This DFO therapy lessens the secondary effects of iron overload and hence, causes a fall in morbidity and mortality (Borga-Pignatti *et al*., 2004).

DFO chelation therapy has its own side effects which are more obvious in less iron burden conditions and can cause growth retardations in some individuals, ocular and auditory toxicity or rarely renal impairment and interstitial pneumonitis. Its administration also makes the patient vulnerable to Yersinia infections (bacteria which causes intestinal infections in human). Low compliance has been seen in this therapy because of several complications in its administration process which is found to be the major drawback in its administration. Effective response of this therapy can only be monitored by regular determination of serum ferritin concentration. Though, it is not a single parameter, other factors are there too which can influence that is liver damage, so MRI are used in recent years to detect iron load in liver and heart (Clark *et al*., 2003).

Response of Deferiprone and Defersirox is also seen recently. Deferiprone is considered aprogressing useful iron chelator substituteto DFO because of fewer myocardial side effects (Maggio, 2007).

Alternatively, Deferasirox has shown good results in patients with a particular safety profile that is clinically manageable along with appropriate check. This therapy also comes up with certain side effects like any other therapy are gastrointestinal problems, skin rashes and increase in non-progressive serum creatinine concentration (Neufeld, 2006).

When iron levels are very high in patients then combination therapy of DFO and Deferiprone can give promising results and iron toxicity can also be decreased. The decrease in frequency needed for subcutaneous infusions of DFX, when it is combined with deferiprone therapy, may significantly improve patient compliance with DFX as well as potentially reducing the costs of DFX therapy (Wonke *et al*., 1998).

2.12.3. Bone marrow transplantation:

As thalassaemia major is an inherited disease requiring chronic long-life transfusions to treat the anaemia triggered by enhanced red blood cell destruction. Still this regimen cause iron overload and following organ deterioration. Iron chelation regularly by deferoxamine slows down this process however the iron overload gradually increases leading to portal fibrosis which may be enhanced by the concomitant presence of blood-borne infections such as Hepatitis C Virus (HCV) infection. The only fundamental cure for thalassaemia today, is to correct the genetic defect by haemopoietic stem cell transplantation (Lucarelli *et al*., 2002). Stem cell transplant if taken from a matched related donor is emerging therapy for

βthalassaemia major and it can produce promising results (Perumbeti *et al*., 2010; Bank, 2010).

The success of this treatment strategy is mostly dependent on pre-transplantation clinical condition of patient that is the presence of hepatomegaly, amount of iron accumulation in the liver and chance of liver fibrosis. If these risk factors are not present in a patient then the percentage disease free survival becomes 90 % in case of children. But in the presence of these risk factors, survival rate becomes 60 % in all age groups (Gaziev & Lucarelli, 2003).But unfortunately it difficult to find related donor for at least 70 % of the needy. Animal models have been experimented using vectors like lenti viruses which have shown the effectiveness of transfer of globin gene in mice with β globin mutations (May *et al*., 2000. 2002; Malik *et al*., 2005). These experiments showed good results like their expression was stable still when low dose of vector was used. On the other hand they also came up with some problems like instability of vector, probability for insertional mutagenesis as well as variable expression (Persons. 2010; Yannaki *et al*., 2010).

2.12.4. Lifestyle and Diet in β-Thalassaemia:

If the disease is completely compensated by treatment, a patient of thalassaemia major can delight in a near-normal way of life and experience normal physical and emotional development since childhood to adulthood, including parenthood. Thalassaemic patients do not have specific dietary requirements, except they have been advised by the physician. Patients already take a heavy treatment besides it is counterproductive to supplement further restrictions without the likelihood of flawless benefit. For the duration of growth, a normal energy intake including normal fat and sugar content is highly recommended. In adolescence and adult life, a diet having low highly refined carbohydrates is useful in inhibiting or delaying the commencement of

impaired glucose tolerance or diabetes. No clear evidence is there that specific diet is advantageous in preventing or managing liver disease, except at late stages. The major characteristic of thalassaemia is increased iron absorption. The amount is influenced by degree of erythropoiesis, the Haemoglobin level and several potential independent factors. Iron absorption from food can be reduced by having black tea with food particularly in patient suffering from thalassaemia intermedia (Borgna-Pignatti, 2007). There is no proof that iron-poor diets are beneficial in thalassaemia major (Galanello and Origa, 2010). The foods very rich in iron (such as liver and multivitamin preparations contain added iron, along with other vitamin supplements) should not be used. Since many factors in thalassaemia stimulate calcium depletion, a diet having adequate calcium (e.g. milk, cheese, dairy products) is recommended at all times. However, nephrolithiasis is seen in some patients with thalassaemia major, in those individuals calcium supplements must not be given lest there is a clear indication; as an alternative a low oxalate diet should be considered. Thalassaemia patients who remain untransfused or the ones on low transfusion regimens have greater folate consumption and might develop folate deficiency. Supplements (1 mg/day) may be given to these type of patients. Patients who are transfused very frequently rarely develop this condition, and generally have no requirement of supplements. Vitamin C deficiency also occurs in some patients as iron overload causes vitamin C to be oxidized at an amplified rate. About 50 mg of vitamin C in children 10 years along with DFO infusion may increase the 'chelatable iron' present in the body, therefore increasing the efficacy of chelation therapy. Still, yet there is no confirmation supporting the consumption of vitamin C supplements in patients on DFP, DFX or combination treatment. Iron absorption is increased from the gut with vitamin C, labile iron and hence iron toxicity and may consequently be particularly harmful to

patients who are not receiving DFO, as iron mobilized by the vitamin C will stay unbound, triggering tissue damage. The efficacy and safety of vitamin E supplementation in thalassaemia major has not been formally assessed and it is not possible to give recommendations about its use at this time. Patients with thalassaemia should be not use alcohol, as it can facilitate the oxidative destruction of iron and worsens the influence of HBV and HCV on liver tissue. Physical activity should always be encouraged lest there is a precise secondary medical condition. Some conditions that demand special attention include splenomegaly, osteoporosis and severe heart disease.

Standard recommended vaccinations should not be skipped or delayed. Proper immunization with Haemophilus Influenza and meningococcal vaccines should be done before two weeks of splenectomy and after surgery to prevent and decrease the risk of infection. Like other chronic diseases, certain psychological complications are also seen in this disease.

The strategy using which the family and the patient cope up with the disease and its treatment will have a life-threatening effect on the patient's survival and quality of life, and an overall acceptance by the patient of his/her own condition creates the key to normal development from childhood to adulthood. A significant role of physicians and other health care professionals in treatment is to support patients and families to face up to the challenging demands of treatment, whereas preserving a positive role (Galanello and Origa, 2010).

Research Objectives:

This is the first such pilot study in Khyber Pakhtunkhwa, no such study has been reported yet in this region of Pakistan. In this study, it is hypothesized that the mutations present in the β- thalassemic patients of the KPK population are the same as in the rest of the country. The major objectives of the study are:

- To investigate the prevalence of β-thalassaemia in the province of KPK.
- To conduct a preliminary study to correlate clinical pathological profile and taxonomy of molecular subtypes of thalassaemia.
- To investigate age related gender specific prevalence of βthalassaemia.
- To investigate population wise distribution of the disease.
- To conduct a pedigree analysis of a family affected with this disorder.

CHAPTER 3

MATERIALS AND METHODS

3.1. Study Subjects:

The designed study was a case study. The sample size for the study was 350 thalassaemic patients which were taken from various thalassemia centers of Mansehra, Abbottabad and Peshawar and 30 control samples. All the patients considered under the study were clinically diagnosed patients of β-thalassaemia major. The patient's data including age, gender, current treatment and family history was recorded.

Thirty normal individuals from the same region were taken as healthy control, falling in the corresponding age and sex group as made for the case group. Before conducting this study, written consent was taken from the patients after inferring them about the study.

3.2. Blood Sample Collection:

Sterilized syringes of 5ml (BD 0.6mm X 25 mm, 23 G X 1 TW) and 10 ml (BD 0.6 mm X 38 mm, 21 G X 1 TW) were used to draw, venous blood from the subjects under study. All the samples were collected in 2.5 ml (BD vacutainer TM, Frankin Lakes, New Jersey, USA) and 3 ml ethylenediaminetetraacetic acid (EDTA) tubes (BD vacutainer TM, Frankin Lakes, New Jersey, USA). EDTA tubes were labelled properly by giving identification number (ID num.) to each sample, date and time on which the sample was collected along with name and age of the individuals.

3.3. Sample Storage and Transport:

The blood samples were then dispatched to Thalassemia Laboratory Atta-ur-Rehman School of Applied Biosciences (ASAB), National University of Sciences and

Technology (NUST), Islamabad, Pakistan. Samples were stored at 4 ^{o}C for further processing.

3.4. Solutions Used In DNA Extraction:

For DNA extraction, four different types of solutions were made i.e. RBC lysis buffer solution, Proteinase K buffer solution, 20% SDS solution and 6 M NaCl solution.

3.4.1. RBC Lysis Buffer:

For the lysis of red blood cells, lysis buffer was made by adding 10mM Tris (PH 7.5), 5mM magnesium chloride ($MgCl_2$) (Tris Merck, Gemany) and 0.32 M sucrose. Distilled water was then added to make up the volume and solution was autoclaved at 121^{o} C temperature and pressure 100 kPa. After that Triton X-100 (1% Vol/vol) was added to the autoclaved solution.

3.4.2. Proteinase K:

Proteinase K is an enzyme that digests proteins. In order to ensure better performance of this enzyme, Proteinase K buffer was made by adding 10 mM Tris (Tris Merck, Gemany) (PH7.5), 2 mM EDTA (Affymetrix, USA) (pH 8.0) and 400 mM sodium chloride (Tris Merck, Gemany) to distilled water.

3.4.3. 20% SDS solution:

20% SDS solution was made for the degradation of cell membrane and lysis of WBCs (White Blood Cells).

3.4.4. 6 M NaCl Solution:

6 M NaCl solution was used to salt out all the proteins from the mixture.

3.4.5. Tris-EDTA Buffer solution (TE Buffer):

TE Buffer was prepared by adding 10mM Tris HCL in 1 mM EDTA (pH 8.0) (Affymetrix, USA). 10 ml of TE buffer was dissolved to get 1X buffer in 990 ml distilled water. pH of the buffer was maintained at 8.0 and it was stored in room temperature.

3.4.6. 1X Tris Acetate Ethylenediamine tetraacetic acid (TAE):

50X stock solution of Tris Acetate (EDTA) was prepared. By adding 890 mM of Tris base (Tris Merck, Gemany), 20 mM of EDTA (Affymetrix, USA) (pH 8.0) and 890 mM of acetic acid in distilled water. In order to make up the desired volume, distilled water was added. 1X TAE was prepared from 50X stock solution. As final concentration, 1X TAE had 89 mM Tris base (Tris Merck, Gemany), 89 mM Acetic acid and 2 mM of EDTA (Affymetrix, USA).

3.5. Genomic DNA extraction by salt extraction method:

Genomic DNA was extracted by proteinase K/ Sodium dodecyl-sulphate (SDS) digestion and proteins were separated from the blood (Miller M *et al*, 1988). Salt extraction method is cost effective and reliable method for DNA extraction. This method is less time consuming and DNA obtained at the end is of high yield. The blood samples stored in EDTA tubes were stored in incubator at room temperature half an hour before processing. Approximately 750 µl of blood was taken in 1.5ml eppendorf tube. 750 µl of RBC Lysis Buffer was then added in it. The tube was tightly closed and placed at room temperature till it became clear red. This mixture was then centrifuged at 13,000 rpm (revolution per minute) for 1 minute in the centrifuge machine (spectrafuge 24D Labnet, Edison, New Jersey, USA). After centrifugation, supernatant was discarded carefully and nuclear pallet was left in it for further processing. Pallet

was re-suspended in 400 µl of RBC Lysis Buffer, centrifuged again at 13,000 rpm for 1 min and supernatant was again discarded. Nuclear pallet obtained after this step was re-suspended in 400 µl of Proteinase K Buffer, 14 µl of 20% sodium dodecyl sulphate (SDS) and 5µl of proteinase K (PK). After this step, overnight shaking incubation was done at 37^{o} C. The next day 500µl of 6M NaCl solution was added in the tube and mixed properly using vortex. The tube was then centrifuged for 20 minutes at 4,500 rpm. After centrifugation, the upper aqueous phase was collected in a clean and appropriately labeled tube. Then, 500µl of isopropyl alcohol was added to precipitate the DNA by inverting it several times. Then again centrifugation was done at 13,000 rpm for 10 minutes and the supernatant was discarded to get the DNA pellet. In DNA pallet, 200 µl of 70% ethanol was added in order to wash the DNA and for about 7 minutes it was centrifuged again at 13,000 rpm. Then upper layer having ethanol was discarded leaving DNA at the bottom. DNA pallet was then dried by keeping tubes in incubator at 37^{o} C to evaporate all the excess ethanol. To the DNA pallet, 100µl of TE buffer was added and left for 4-5 hours for the DNA to dissolve.

3.6. DNA Dilution:

For checking the purity and quantification of DNA, nanodrop (Eppendorf, USA) was used. Stock DNA with high concentration and purity was diluted to get the concentration of 40-50 ng/µl for PCR amplification. All the dilutions were stored at -20^{0} C for further use in PCR amplification. In PCR optimization different concentrations of DNA was used to get an optimum and specific band of amplicon.

3.7. Primer Designing

Primer sequences were adopted from literature that amplified a specific region in both diseased and healthy controls (Baig *et al*., 2005; Baig *et al*., 2006). Primers were

checked for complementarity with the desired region of interest using NCBI primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LOC=BlastHome). Other properties were evaluated using Oligocalc, an online oligonuceotide properties calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). The primer sequences and product sizes of their amplicons are given in Table 3.1. All the primers were amplified against a single common reverse primer, the sequence on which was

5' CGG CAG ACT TCT CCT CAG GAG TCA GGC GCA 3'

3.8. Detection of Mutations:

The DNA stocks were screened for 16 known β-Thalassaemia mutations in Pakistani population.

3.8.1. Amplification Refractory Mutations System – Polymerase Chain Reaction (ARMS-PCR)

ARMS PCR is a very simple and economical technique for the detection of point mutations or small insertions or deletions. It works on the principle of sequence specific priming (SSP). For screening of a specific mutations, PCR reaction encompasses four primers in one reaction mixture.

3.8.2. PCR Process:

The PCR was performed in 0.2 ml PCR tubes (Axygen, California, USA). A 20µl of reaction mixture having 10µl of PCR water (Invitrogen, USA) , 2 µl of $MgCl_2$ (Invitrogen, USA) , 2.5 µl of dNTP's (Invitrogen, USA) , 1 µl of forward primer (Eurofins, Germany), 1 µl of reverse primers (Eurofins, Germany), 2 µl of Taq Buffer (Invitrogen,USA) and 1 µl of extracted genomic DNA. PCR tubes were swirled and 0.5 µl of Taq polymerase (Invitrogen, USA) was added to PCR tubes which are sited on

ice. They were gently mixed in order to avoid bubble formation. In case, bubbles were found, they were removed by tapping gently. Then, tubes were taken for amplification process to PCR.

Table 3.1: Primer Sequences and Amplicon Sizes for Detection of Mutations

Mutation	Primer Sequences (5' – 3")	Amplicon Size (bp)
IVS-I-5	**Mutant:** *CTCCTTAAACCTGTCTTGTAACCTTGATAG* **Normal:** *CTCCTTAAACCTGTCTTGTAACCTTGATAC*	**293**
FSC 8-9	**Mutant:** *CCTTGCCCCACAGGGCAGTAACGGCACACC* **Normal:** *CCTTGCCCCACAGGGCAGTAACGGCACACT*	**222**
CD 41/42	**Mutant:** *GAGTGGACAGATCCCCAAAGGACCAACCT* **Normal:** *GAGTGGACAGATCCCCAAAGGACTCAAAG*	**451**
IVS-II-I	**Mutant:** *AAGAAAACATCAAGGGTCCCATAGACACAT* **Normal:** *AAGAAAACATCAAGGGTCCCATAGACACAC*	**642**
IVS-II-848	Mutant: *CACACAGACCAGCACGTTGCCCAGGAGCTT* Normal: CACACAGACCAGCACGTTGCCCAGGAGCTG	**1489**
IVS-I-I (G to T)	**Mutant:** *TTAAACCTGTCTTGTAACCTTGATACGAAA* **Normal:** *TTAAACCTGTCTTGTAACCTTGATACGAAC*	**289**

Mutation	Primer Sequences (5' – 3")	Amplicon Size (bp)
IVS-I-I (G to A)	**Mutant:** *TTAAACCTGTCTTGTAACCTTGATACCAAT* **Normal:** *TTAAACCTGTCTTGTAACCTTGATACGAAC*	**289**
Cd 15	**Mutant:** *CACCAACTTCATCCACGTTCACCTTGGCCT* **Normal:** *CACCAACTTCATCCACGTTCACCTTGGCCC*	**243**
Cd 16	**Mutant:** *TCACCACCAACTTCATCCACGTTCAGCTTC* **Normal:** *TCACCACCAACTTCATCCACGTTCAGCTTG*	**247**
Cd 26 (G-A)	**Mutant:** *TAACCTTGATACCAACCTGCCCAGGGGCTT* **Normal:** *TAACCTTGATACCAACCTGCCCAGGGGCTC*	**275**
Cd 30 (G-C)	**Mutant:** *TAAACCTGTCTTGTAACCTTGATACCTACG* **Normal:** *TAAACCTGTCTTGTAACCTTGATACCTACC*	**288**
Cd 30 (G-A)	**Mutant:** *TAAACCTGTCTTGTAACCTTGATACCTACT* **Normal:** *TAAACCTGTCTTGTAACCTTGATACCTACC*	**288**
Cd 39 (C-T)	**Mutant:** *CAGATCCCCAAAGGACTCAAAGAACCACTA* **Normal:** *CAGATCCCCAAAGGACTCAAAGAACCACTG*	**444**

Mutation	Primer Sequences (5' – 3")	Amplicon Size (bp)
-88	**Mutant:** *TCACTTAGACCTCACCCTGTGGAGCCTCAT* **Normal:** *TCACTTAGACCTCACCCTGTGGAGCCTCAC*	**288**
Cap +1 (A-C)	**Mutant:** *AAAAGTCAGGGCAGAGCCATCTATTGGTTC* **Normal:** *AAAAGTCAGGGCAGAGCCATCTATTGGTTT*	**350**
Initiation Cd (T-C)	**Mutant:** *CGGCAGACTTCTCCTCAGGAGTCAGGCGCG* **Normal:** *CGGCAGACTTCTCCTCAGGAGTCAGGCGCA*	**320**
Internal Control	**Forward:** *CAATGTATCATGCCTCTTTGCACC* **Reverse:** *GAGTCAAGGCTGAGAAGATGCAGGATA*	**862**

A Thermo-cycler (Applied Biosystem, Foster City, USA) was used in order to perform cycling process. It was first optimized following this protocol : 5 minutes for initial denaturation at 95^{O} C, trailed by 35 cycles at 95^{O} C for 45 seconds (denaturation), 60 ^{O} for 45 seconds (annealing) and 72 ^{O} C for 45 seconds (elongation) and final allowance at 72 ^{O} C for 10 minutes for elongation of any incomplete PCR products. PCR products were then kept at 4 ^{O} C.

3.8.3. Validation of ARMS PCR:

For analysis of PCR products, agarose gel (2% w/w) was made using 100ml 1 X Tris acetate EDTA (TAE) buffer and 1.6g agarose and then simply heating it for 120 seconds in microwave oven. It was then allowed to cool till that point when no fumes were

coming out from the container. After that, 8 µl of Ethidium Bromide (EtBr) was also added to the conical flask to stain the gel. That aqueous solution was poured in gel casting tray and allowed to solidify at room temperature. The gel was placed in buffer tank of electrophoresis apparatus (Cleaver Scientific Ltd.). For loading the samples, 15 µl of PCR product was mixed with 3 µl of loading dye (bromophenol blue). All that mixture was loaded into wells carefully. Electrophoresis was done at 100 volts for 25 minutes in 1X TAE. Gel was visualized in UV Trans-illuminator (Biometra, Goettingen, Germany). At last, it was photographed by using Gel Documentation System (Wealtec Dolphin Doc, Sparks, USA).

3.9. Pedigree Analysis:

A family was selected from Mansehra, KPK, Pakistan for pedigree analysis. Pedigree was constructed by using a standard method (Bennett *et al*., 1995). The exact genetic relationships of different individuals were obtained through interview of index patient. All the information taken from the proband was used for the pedigree analysis. Males were symbolized by squares while females were symbolized by circles. Unfilled circles and squares were used for the representation of normal individuals while filled circles and squares were used to represent abnormal / affected individuals. Carrier individuals were represented by using squares and circles having a dot in the centre. Half-filled circles and squares were used to represent thalassaemia minor. For the deceased ones, a line was drawn across circles and squares. Proband was represented by an arrow indicating towards it. Each generation was indicated by Roman numbers while each individual in a generation was named using Arabic numbers.

CHAPTER 4

RESULTS

4.1. Genotyping of β-Globin Gene Mutations in β-Thalassaemia Patients:

The present study was carried out on β-thalassaemia major patients (350 patients) from different cities of Khyber Pakhtunkhuwa (KPK). Mutations were detected through amplification refractory mutation system-polymerase chain (ARMS-PCR). The sample DNA was screened for 16 different mutations that has been reported in Sub-Continent. Internal control primers were used to confirm amplification and in order to avoid mis-priming, a negative control of a normal subject was added that produced no band other than the control band.

From overall 350 diseased subjects, 299 showed three most prevalent mutations; IVS 1-5, FSC 8/9 and CD 41 /42, which are reported in Sub-continent while the remaining 51 subjects showed other uncommon mutations. Our study showed high prevalence of these three mutations in KP population which is almost similar to rest of the Pakistani population. Out of these three, IVS-1-5 was found to be the most prevalent which was found in 186 patients that is almost 53 percent of the subjects under observation, followed by FSC 8/9 present in 62 patients (17.71 %) while CD 41/42 was reported in 51 subjects (14.57 %). The representative gel image can be seen in Figure 4.1. IVS-1-5 normal and mutated can be seen in well labelled as 2A and 1B, respectively, i.e. amplicon of 293 bp. Similarly CD 41/42 normal and mutated amplicons can be observed in well labelled as 3A and 4B respectively giving an amplification product of 451 bp. FSC 8/9 normal amplicon can be seen in well labelled as 5A while mutated amplicons in well 6B and 7B. Negative and positive controls were also run and can be

seen in the gel image. Amplicon sizes were calculated by comparing the band sizes with the respective bands on the DNA ladder.

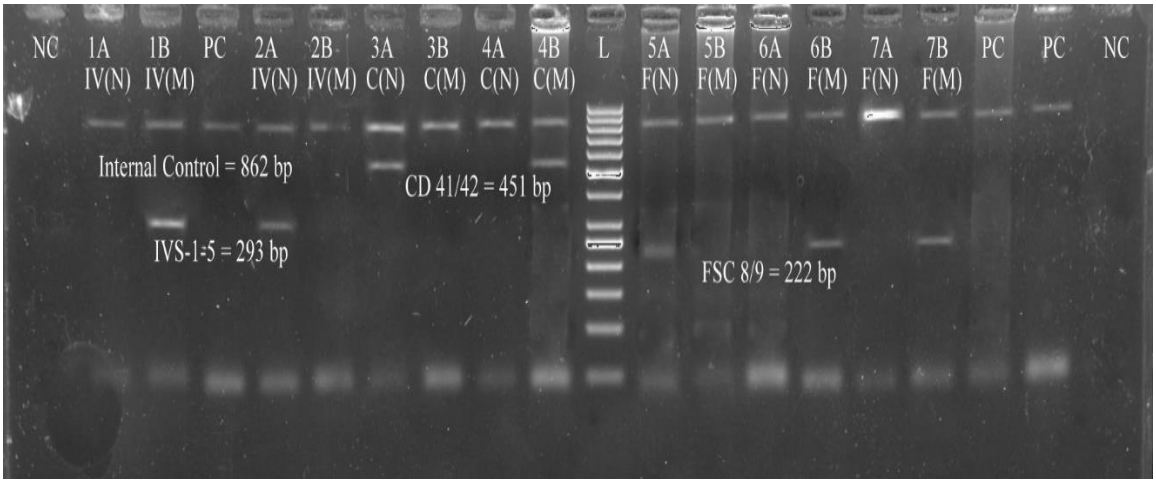


Figure 4.1: Representative gel image of IVS 1-5, FSC 8/9 and CD 41/42

(NC = Negative control; IV (N) = IVS 1-5 Normal Primer in Lane 1A and 2A; IV (M) = IVS 1-5 Mutant Primer in Lane 1B and 2B; F (N) = FSC 8/9 Normal Primer in 5A, 6A and 7A; F (M) = FSC 8/9 Mutant Primer in 5B, 6B and 7B; C (N) = CD 41/42 Normal Primer in 3A and 3B; C (M) = CD 41/42 Mutant Primer in 3A and 4A; PC = Positive controls; L = 50 bp Ladder; CD = Codon IVS= Intervening region FSC= Frame Shift Codon)

Other Mutations present in the population were: IVS-I-I (G>T), CD 15, IVS-I-I (G>A), CD 30 (G>C), CD 26, CD 39, CD 16 and IVS-II-I. Random samples were screened for other mutations including IVS-II-848, CD 30 (G>A), Cap +1 (A-C) and Initiation Cd (T-C) but no mutation was detected in the population under study. The incidence of mutation along with their percentage distribution is shown in Table 4.1.

Table 4.1: Distribution of β – Globin Gene Mutations across β – Thalassaemia Major Patients

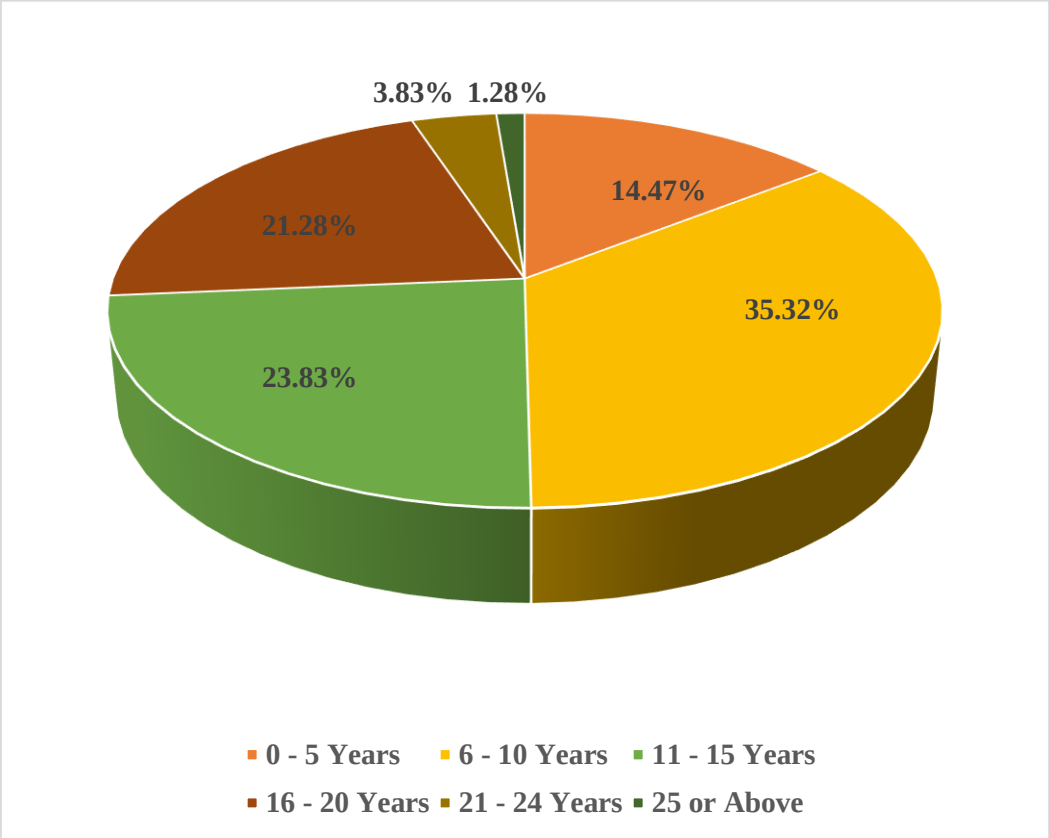
S. No.	Mutation	No. of Patients (Percentage) (n=350)
1	IVS 1-5	186 (53.14)
2	FSC 8/9	62 (17.71)
3	CD 41 / 42	51 (14.57)
4	IVS-I-I (G>T)	13 (3.71)
5	CD 15	10 (2.86)
6	IVS-I-I (G>A)	9 (2.57)
7	CD 30 (G>C)	6 (1.71)
8	CD 26	6 (1.71)
9	CD 39	3 (0.86)
10	CD 16	2 (0.57)
11	IVS-II-I	2 (0.57)

CD= codon IVS=intervening sequence FSC= Frame Shift Codon

4.2. Age-wise Distribution of Study Samples:

The age wise distribution of sample was also checked. For this purpose sample was divided into six groups i.e. 0-5 years, 6-10 years, 11-15 years, 16-20 years, 20-24 years and 25 and above. The data is represented graphically in Figure 4.2. Less than 5% of the patients were above the age of 21 years.

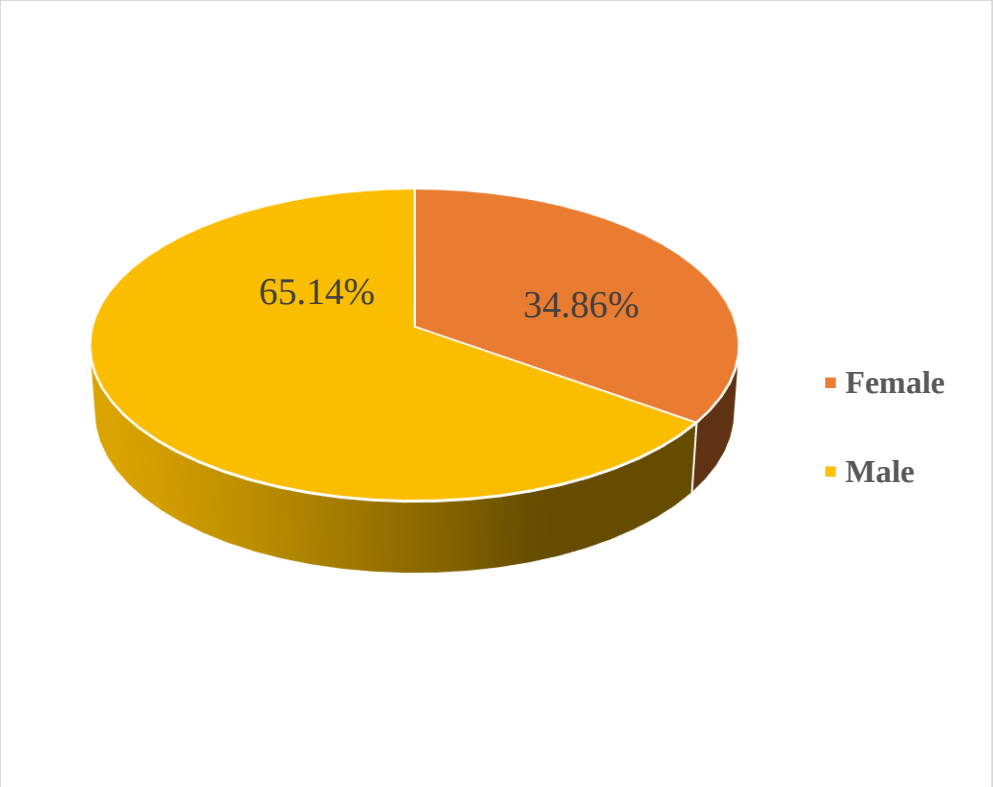
Figure 4.2: Age wise Distribution of Study Samples



4.3. Gender-wise Distribution of Study Samples:

The prevalence of β-thalassaemia is not dependent on the gender of the individual because it is an autosomal recessive disorder, even though, the sample population was found to be biased where male subjects were dominated statistically. The results are depicted in the Figure 4.3. Among all patients, 228 were male while 122 were female, presenting imbalance of gender in the sample population.

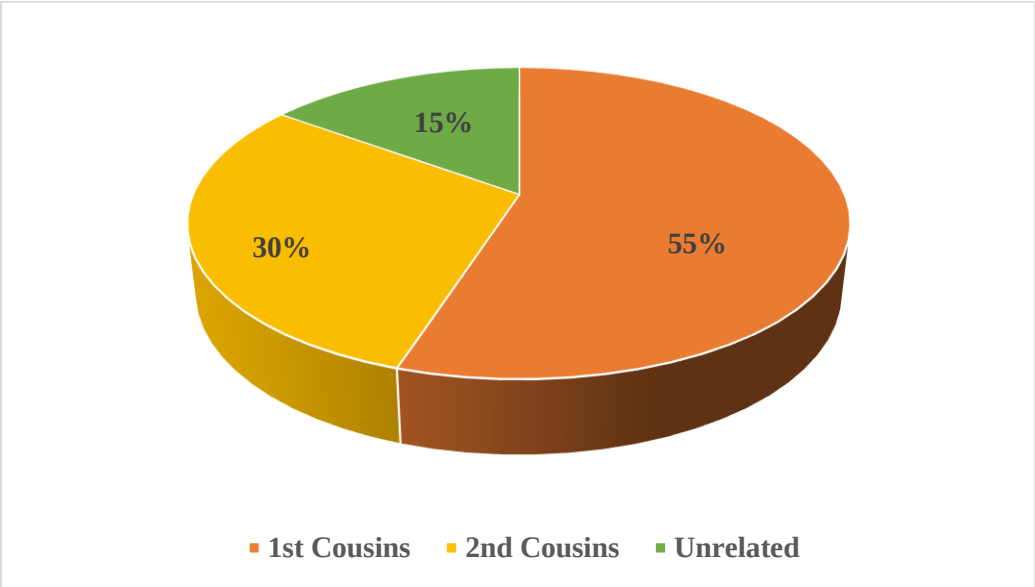
Figure 4.3: Gender Wise Distribution of Study Subjects



4.4. Parental Relationship of Study Samples:

The trend of consanguineous marriages was also checked because it is a genetic disorder and was found to be very high. The parental distribution of β-thalassaemia patients according to parental genetic relationships is given in Figure 4.4. It was found that parents of about 55% of the sample population are related as 1st cousins whereas 30% were related as 2nd cousins and remaining 15% were found to be unrelated.

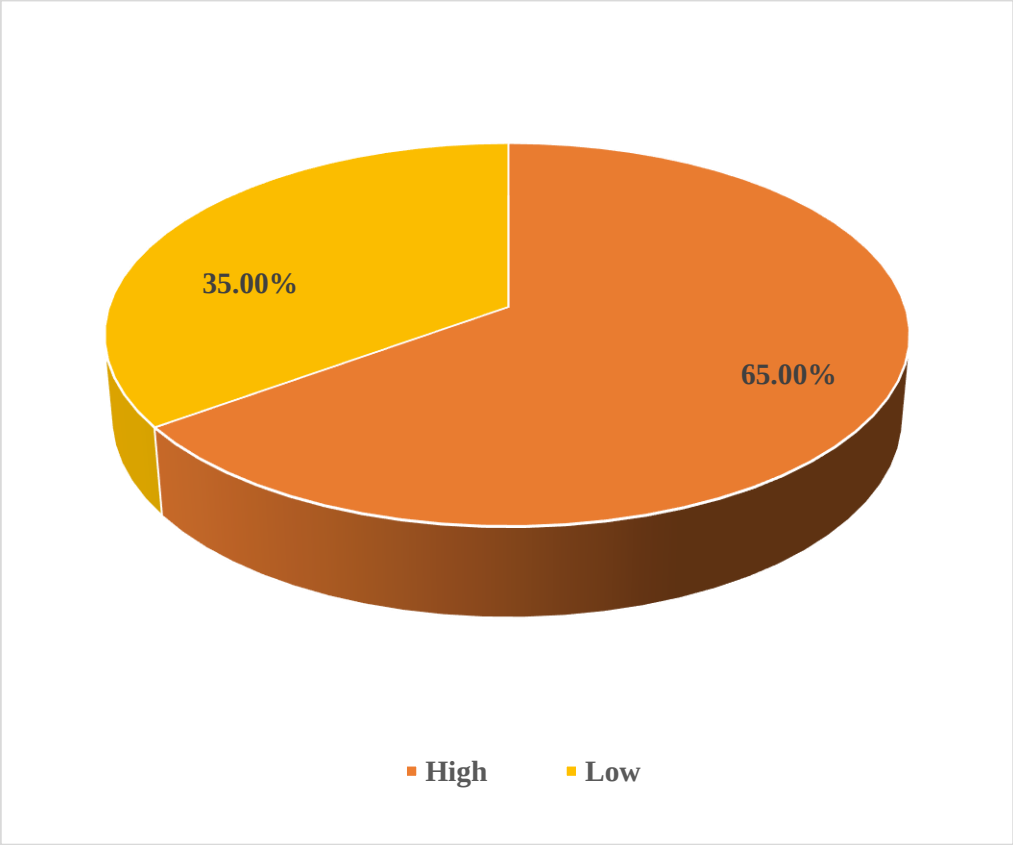
Figure 4.4: Parental Relationship of β- Thalassaemia Patients



4.5. Economic Status-wise Distribution of Study Samples:

The Economic status of the subjects was also checked in this study because this disease needs a lot of resources to manage the treatment and to decrease the rate of morbidity and mortality at early age. The results are shown in Figure 4.5. It was found that out of all patients, 51.9 % of subjects were from low income family and 49.1 % had high economic status.

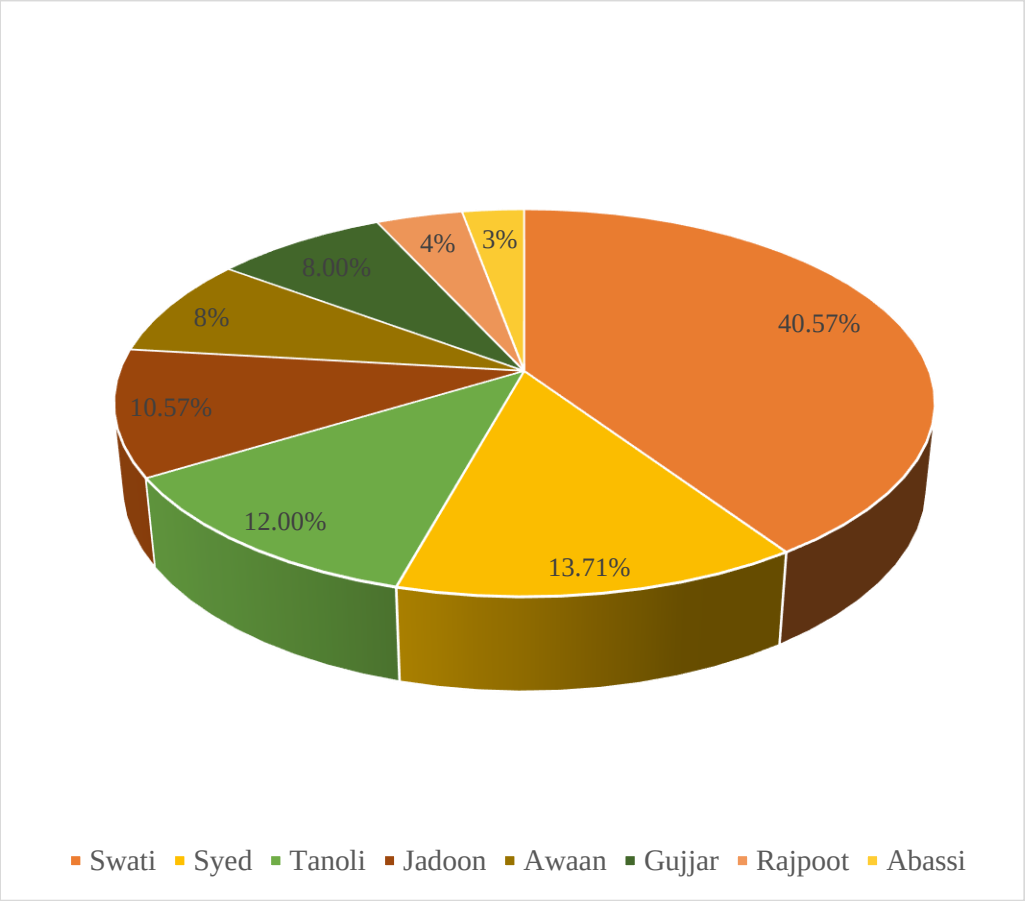
Figure 4.5: Economic Status-wise Distribution of Study Samples



4.6. Caste-wise Distribution of Study Samples:

The caste wise distribution of this disease was also checked. The high percentage of β-thalassaemia was seen in Swati family (40.57%) followed by Syed (13.71%) and Tanoli (12%). The distribution of β-thalassemic patients in different caste groups is shown in Figure 4.. The high percentage of this fatal disease in patients was associated to first cousin relations compared to unrelated ones.

Figure 4.6. Caste-wise Distribution of β-thalassaemia in Study Subjects



4.7. Other complications in β-Thalassaemia Major Patients:

β-thalassaemic patients getting more than 8 transfusions annually were taken into consideration. Patients requiring fewer than 8 transfusions per year are considered to have thalassaemia intermedia and were excluded from this study. Out of the 250 patients examined for this study, none of them reported to be Hepatitis (HCV) or HIV positive. Almost 89% of the patients that were in the age group 11 – 24 years have reported heart complications while in overall sample pool 52% showed heart complications. 24% of patients have diabetes mellitus in overall sample pool. These complications are summarized in Table 4.2.

Table 4.2. Other Complications in β-Thalassaemia Major Patients

Complications	0 – 10 Y, % (No. Positive / No. Tested)	11 – 20 Y, % (No. Positive / No. Tested)	21 Y and above, % (No. Positive / No. Tested)	Overall, % (No. Positive / No. Tested)
Infectious				
Hepatitis	**0 (0 / 60)**	**0 (0 / 90)**	**0 (0 / 100)**	**0 (0 / 250)**
HIV	**0 (0 / 60)**	**0 (0 / 90)**	**0 (0 / 100)**	**0 (0 / 250)**
Heart Disease	**0 (0 / 60)**	**89 (80 / 90)**	**50 (50 /100)**	**52 (130 / 250)**
Liver Failure	**0 (0 / 60)**	**0 (0 / 90)**	**20 (20 / 100)**	**8 (20 / 250)**
Endocrine disorders				
Thyroid Disease	**0 (0 / 60)**	**33 (30 / 90)**	**40 (40 / 100)**	**28 (70 / 250)**
Diabetes Mellitus	**0 (0 / 60)**	**33 (30 / 90)**	**30 (30 / 100)**	**24 (60 / 250)**
Osteoporosis	**50 (30 / 60)**	**56 (50 / 90)**	**40 (40 / 100)**	**48 (120 / 250)**
Splenomegaly	**12.5 (10 / 60)**	**44 (40 / 90)**	**30 (30 / 100)**	**32 (80 / 250)**
Endocrinopathies	**0 (0 / 60)**	**33 (30 / 90)**	**20 (20 / 100)**	**20 (50 / 250)**

4.8. Complications of DFO Therapy in β-Thalassaemia Major Patients:

Among 250 patients who had taken chelation therapy with Deferroximine (DFO), majority of patients have shown complications which required the adjustment of the dose and modification of the dose of the patient. Complications of DFO were very common in older patients as compared to younger ones which included hearing loss, vision problems and different types of allergic reactions. Table 4.3 shows specific complications related with DFO therapy and their frequencies in patients.

Table 4.3. Complications associated with DFO Therapy

Complications	0 – 10 Y, % (No. Positive / No. Tested)	11 – 20 Y, % (No. Positive / No. Tested)	21 Y and above, % (No. Positive / No. Tested)	Overall, % (No. Positive / No. Tested)
Hearing Loss	**0 (0 / 60)**	**33 (30 / 90)**	**60 (60/100)**	**36 (90 / 250)**
Vision Disturbances	**0 (0 / 60)**	**44 (40 / 90)**	**20 (20/100)**	**24 (60 / 250)**
Allergy	**50 (30 / 60)**	**22 (20 / 90)**	**40 (40/100)**	**36 (90 / 250)**

4.9. Pedigree Analysis:

A family was selected for pedigree analysis having one β-thalassaemia major patient. All the information gathered through an interview with the proband was used to create this pedigree. The proband belong to 3rd generation in the pedigree named as III-6. Proband was suffering from β-thalassaemia major. She had two brothers, one normal while other had β-thalassaemia. She had a sister suffering from β-thalassaemia major who died almost 29 years ago. The pedigree is graphically represented in Figure 4.7. The parents of the proband (II-5 and II-6) were not related to each other. No member of parental and maternal family was diagnosed with thalassaemia minor or major but pedigree analysis revealed them to be carrier of the disease. The pedigree is graphically represented in Figure 4.7.

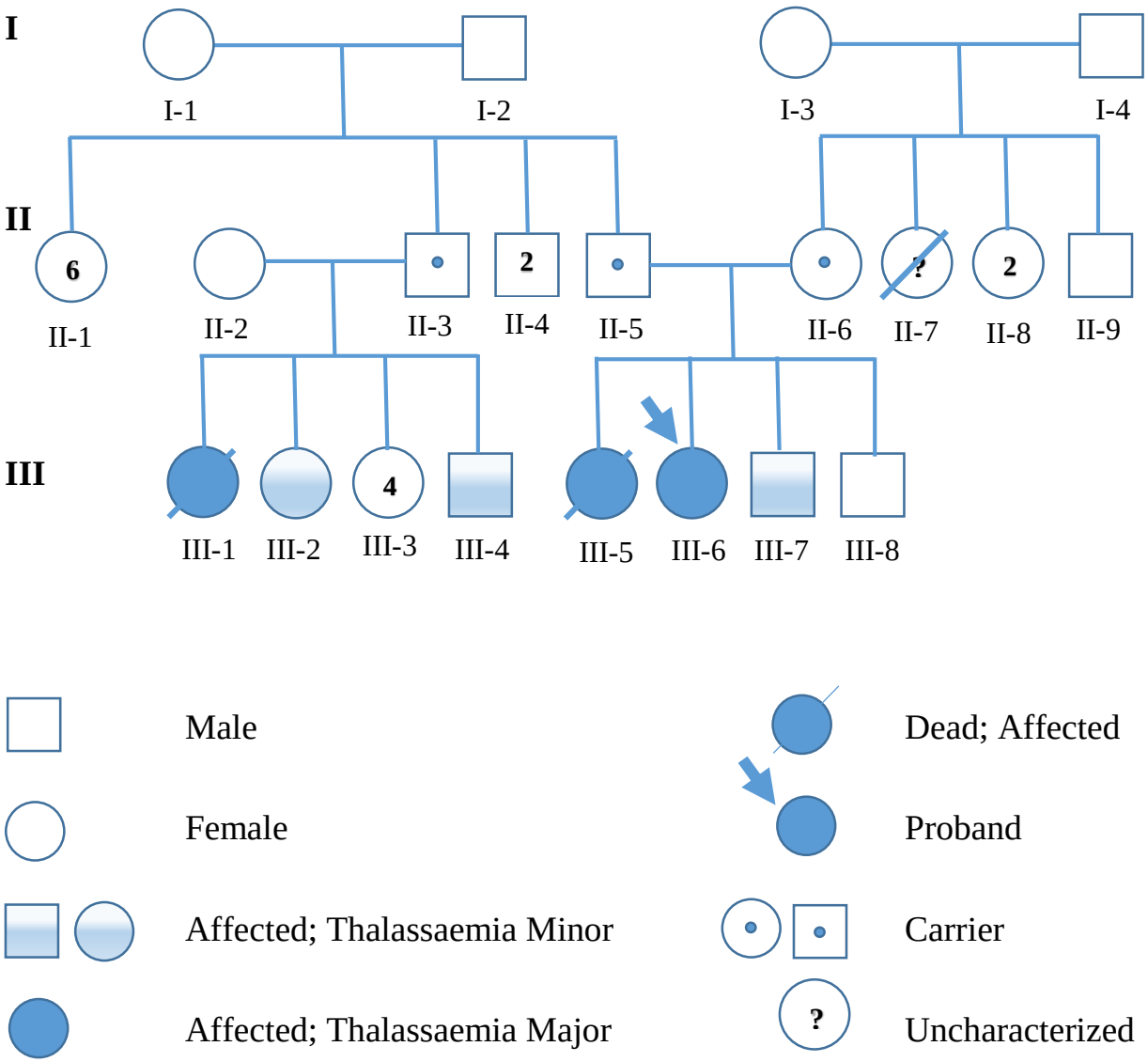


Figure 4.7: Pedigree of a Family having β-thalassaemia major Patient

CHAPTER 5

DISCUSSION

Pakistan has a significant genetic diversity and racial variety due to its long history beginning from Indus civilization which was further richened culturally by the invasion of Persian and Arab invaders. Pakistani ethnic culture depicts a blend of Greek, Persian, Indian and Middle Eastern descendants. Secondly, Arabic and Persian cultural norms strongly support consanguineous marriages which has made Pakistan a home of different genetic diseases including β-thalassaemia.

β-thalassaemia has no permanent therapy, so the patient is sentenced to life of misery with repeated blood transfusions for the rest of the life. Pakistan shows all the physiognomies of the developing countries including uncontrollable growth rates, poor education standards, inadequate health care system and escalating poverty levels. All these factors not only contribute to prevalence of genetic disorders like β-thalassaemia but also increase its complexities and hinder its proper treatment. In such environment where treatment is very expensive, the prevention is the only cure of thalassaemia.

For its prevention, many effective measures should be taken into consideration like phylogenetic analysis and characterization of carrier families, genetic counseling of prospective parents and prenatal diagnosis if the child has been conceived. These preventive measures can reduce the number of β-thalassaemia patients thus reducing its economic burden and hence improving health care system for the existing patients. But in this poverty stricken country, even prevention is not economical as it includes prenatal diagnosis which utilizes the process of ARMS- PCR.

Without prior knowledge of the most prevalent mutations, characterization of these mutations can only be done by using the primers of all existing mutations. This results

in an increased cost of diagnostic procedure. On the other hand, if the prevalent mutation of a particular ethnic group residing in a specific location is known, then it might cut this resource wastage which can prove valuable for a limited resource health care unit, which majorly constitute the health care systems of Pakistan. Our research aims to bridge this gap of knowledge.

Many researchers have done work in this area (Qurat-ul-ain *et al*., 2011; Ahmed *et al*., 1996; Khateeb *et al*., 2000; El- Kalla and Mathews, 1997; Baig *et al*., 2005; 2006 a,b ; Khattak *et al*., 2012) but while reviewing the literature it was revealed that none of them have work specifically on the population residing in Khyber Pakhtunkhuwa. Previous work was done on Pakhtuns living in other parts of the country. This province has been neglected as a territory and due to its Afghan origin; consanguinity is very common, hence increasing the chances of β-thalassaemia. Due to its tribal socio-economic system, many of such cases go unreported which makes this area more considerable for this research.

By molecular characterization of β-thalassaemia mutations, we aimed to find the most prevalent mutation occurring in this region which will help in its prenatal diagnosis and will make people more aware and psychologically equipped for prevention of β-thalassaemia. We have found that the most prevalent mutation according to our data is IVS-1-5 (G→C) which matches results obtained from the study on population of other provinces of Pakistan (Khateb *et al*., 2000). FSC-8/9 (+G) was found to be the second most prevalent mutation in our study followed by Cd 41/42 (-CTTT). FSC-8/9 (+G) was in the highest percentage according to other studies (Baig *et al*., 2005; Khattak *et al*., 2012). The knowledge of this trend may prove useful for optimizing the prenatal diagnosis and PCR based screening methods to increase their efficiency at the lower

cost. Furthermore, genetic counseling of the subjects was also done so that public awareness about this disease would increase thus helping in its prevention.

Age wise distribution of β-thalassaemia was also checked that revealed that death rate of patients was increasing as the age increases and after 20 years of age life expectancy decreases drastically. Male patients were found to outnumber female ones in this study which were somehow similar to the results of Qurat-ul-ain *et al*., 2011 and this difference in patients (more males and less females) is noteworthy and deserves further investigation. However, as observed during sample collection, the regional and cultural norms tend to be over-protected for the female sex preventing their interaction not only with the sample collectors but also with health care staff and their information is also kept confidential which explains low registered female patients in hospitals in that particular region.

Furthermore, our study revealed that consanguineous marriages were very common and the occurrence of β-thalassaemia was more in case of consanguineous marriages including first cousins and second cousins marriages and these results were almost in agreement with those observed by Qurat-ul-ain *et al*., 2011 and Baig *et al*., 2005. Cultural norms and tribal social system were found to be the major cause of this trend hence converging the diversity of gene pool of this population. Not only the consanguineous marriages but the intra-tribal and distant cousin marriages produce homozygous patients but heterozygous carriers thus keeping the high chance of manifestation of this disease in generations to come.

We also found that our sample size was almost belonging to families having higher economic status. From which we concluded that due to expensive therapy only a certain part of the population can afford it that is why these patients are a large part of our sample data. Families having low economic status have cultural norms and customs

including cousin marriages and poor awareness about the disease. According to our expectations, incidence of β-thalassaemia in these families may be much higher than actually reported.

In this study, it was revealed that Swati and Syed caste has higher incidence of this disease. It is arguable that usual practice of cousin marriages may be a cause of higher incidence of the disease in these caste. This is the most untouched area by researchers so no data is available in this regard.

With an increase in age, other complications in β-thalassaemia patients also increases. Similar results were obtained by Cunningham *et al*., 2004. β-thalassaemia patients tend to have different complications, for example heart diseases, diabetes mellitus, splenomegaly and different endocrinopathies with the increase in age. We also observed that the complications associated with DFO therapy including hear loss, visual disturbances and allergies are also getting severe with increase in age of overall population.

CONCLUSION

In the light of our findings, we conclude that:

- Most prevalent mutation in our sample population was IVS-1-5 (G→C) followed by FSC 8/9 (G+) and Cd 41-42 (-CTTT) respectively.
- Other mutations were also found in our sample population but they were less common. This pattern followed the mutation pattern existing in rest of Pakistani and South East Asian Population.
- Cultural norms were found to be pro-cousin marriages thus contributing to the prevalence of β-thalassaemia.
- Low number of female subjects may indicate over-protected cultural norms in KPK region. Moreover, the number of registered female patients was also very low. This might indicate that the females are constrained from availing such health care facilities.
- The patients with higher economic status were registered more as compared to patients with low economic status.
- Several other complications develop with an increase in age which may lead to death of the patient.
- DFO therapy also comes up with complications which includes hearing loss, visual disturbances and allergies. The incidence of complications of DFO therapy was reported more in older patients which shows that complications increase after continuous consumption of the therapy.

FUTURE PROSPECTS

Sample size of the study was small because of time constraints. In order to yield a significant conclusion about the prevalence of mutations in this population, sample size should be in the range of 2000 to 3000. So, we encourage other research groups to take this study further in order to reach a meaningful conclusion.

Studies like this can be very valuable and should be done more often to optimize prenatal diagnosis in a way that it can become more efficient and less costly to the public. If prevalence of all the mutations is checked and screened in KPK population according to their ethnic background and locality, we can develop a β-thalassaemia genetic map which can help us in predicting the causative mutation in any individual with β-thalassaemia disorder by his/her basic information such as location and ethnic background. This will help not only in prenatal diagnosis but launching such a map will also give awareness to the public.

By studying mutation prevalence's in different ethnic groups, we can relate them phylogenetically to different populations which can give us insight to their phylogenetic ancestry and can help us in relating them to their neighboring cultures.

This type of studies can lead to the revealing of very rare mutations which are unreported in these areas and owing to complex socio-economic system and low research there may be a possibility that a novel mutation may be loitering in this population undetected.

There is a need to increase the number of centers in this area able to perform prenatal diagnosis, and provide this facility at a subsidized cost, or free for the poor, and introduce quality control programs. An important challenge is to develop pre-implantation genetic diagnosis as many couples are distressed by having affected

children in consecutive pregnancies. Investment in non-invasive techniques for prenatal diagnosis would be worthwhile, as this would help to provide prenatal diagnosis.

The need of the hour is to introduce β-thalassaemia control programs in the high risk areas and they should be monitored by a competent authority. Even without a control program in place we must motivate the obstetricians to screen every woman at first visit for carrier status of thalassaemia, haemoglobin E and sickle cell disease. As far as feasible, husband should be screened at the same time.

REFERENCES:

Aessopos A, Farmakis D, Deftereos S, Tsironi M, Tassiopoulos S, Moyssakis I, Karagiorga M (2005). Thalassaemia heart disease: a comparative evaluation of thalassaemia major and thalassaemia intermedia. Chest, 127:1523-1530.

Aessopos A, Farmakis D, Loukopoulos D (2002). Elastic tissue abnormalities resembling pseudoxanthoma elasticum in β thalassaemia and the sickling syndromes. Blood, 99:30-35.

Ahmad, S., Petrou, M., & Saleem, M. (1996). molecular genetics of B Thalassaemia in Pakistan. Br J Hematol, 94, 1-7.

Alpha and Beta Thalassemia HERBERT L. MUNCIE, JR., MD, and JAMES S. CAMPBELL, MD Louisiana State University Health Sciences Center, New Orleans, Louisiana

Angastiniotis, M., & Modell, B. (1998). Global epidemiology of hemoglobin disorders. Annals of the New York Academy of Sciences, 850(1), 251-269.

Ansari, S. H., Shamsi, T. S., Ashraf, M., Bohray, M., Farzana, T., Khan, M. T., ... & Raza, F. (2011). Molecular epidemiology of β-thalassemia in Pakistan: far reaching implications. International journal of molecular epidemiology and genetics, 2(4), 403.

Ansari, S. H., Shamsi, T. S., Ashraf, M., Bohray, M., Farzana, T., Khan, M. T., ... & Raza, F. (2011). Molecular epidemiology of β-thalassaemia in Pakistan: far reaching implications. International journal of molecular epidemiology and genetics, 2(4), 403.

Arif, F., Fayyaz, J., & Hamid, A. (2008). Awareness among parents of children with thalassemia major. J Pak Med Assoc, 58(11), 621-624.

Baig, S. M., Azhar, A., Hassan, H., Baig, J. M., Kiyani, A., Hameed, U., ... & Zaman, T. (2006). Spectrum of β-thalassaemia mutations in various regions of Punjab and Islamabad, Pakistan: establishment of prenatal diagnosis.haematologica, 91(3), ELT02-ELT02.

Baig, S. M., Rabbi, F., Hameed, U., Qureshi, J. A., Mahmood, Z., Bokhari, S. H., ... & Zaman, T. (2005). Molecular characterization of mutations causing β-thalassaemia in Faisalabad Pakistan using the amplification refractory mutation system (ARMS-PCR). Indian Journal of Human Genetics, 11(2), 80.

Bank, A. (2010). Hemoglobin gene therapy for β-thalassaemia.Hematology/oncology clinics of North America, 24(6), 1187-1201.

Barber, E. K. (2011). U.S. Patent No. 8,080,376. Washington, DC: U.S. Patent and Trademark Office.

Bennett, R. L., Steinhaus, K. A., Uhrich, S. B., O'Sullivan, C. K., Resta, R. G., Lochner-Doyle, D., ... & Hamanishi, J. (1995). Recommendations for standardized human pedigree nomenclature. Journal of genetic counseling, 4(4), 267-279.

Birgens, H., & Ljung, R. (2007). The thalassaemia syndromes. Scandinavian journal of clinical & laboratory investigation, 67(1), 11-26.

Borgna-Pignatti C, Cappellini MD, De Stefano P, Del Vecchio GC, Forni GL, Gamberini MR, Ghilardi R, Origa R, Piga A, Romeo MA, Zhao H, Cnaan A (2005). Survival and complications in thalassaemia.Ann N Y Acad Sci, 1054:40-47.

Borgna-Pignatti C, Galanello R (2004). Thalassaemias and related disorders: quantitative disorders of hemoglobin synthesis. In Wintrobe's Clinical Hematology. Volume 42. 11th edition. Lippincott Williams & Wilkins. Philadelphia; 1319-1365.

Borgna-Pignatti C, Vergine G, Lombardo T, Cappellini MD, Cianciulli P, Maggio A, Renda D, Lai ME, Mandas A, Forni G, Piga A, Bisconte MG (2004). Hepatocellular carcinoma in the thalassaemia syndromes.Br J Haematol, 124:114-117.

Borgna-Pignatti, C. (2007). Modern treatment of thalassaemia intermedia.British journal of haematology, 138(3), 291-304.

Borgna-Pignatti, Rugolotto, De Stefano, P., Zhao, Cappellini, M. D., Del Vecchio, G. C., ... & Cnaan, A. (2004). Survival and complications in patients with thalassaemia major treated with transfusion and deferoxamine. haematologica, 89(10), 1187-1193.

Cao, A., Galanello, R., & Rosatelli, M. C. (1994). Genotype-phenotype correlations in β-thalassaemias. Blood reviews, 8(1), 1-12.

Cao, A., Galanello, R., & Rosatelli, M. C. (1998). 8 Prenatal diagnosis and screening of the haemoglobinopathies. Baillière's clinical haematology, 11(1), 215-238.

Cao, A., Rosatelli, M. C., & Galanello, R. (1991). Population-based genetic screening. Current opinion in genetics & development, 1(1), 48-53.

Cao, A., Saba, L., Galanello, R., & Rosatelli, M. C. (1997). Molecular diagnosis and carrier screening for β thalassemia. Jama, 278(15), 1273-1277.

Chui, D. H., & Waye, J. S. (1998). Hydrops fetalis caused by α-thalassaemia: an emerging health care problem. Blood, 91(7), 2213-2222.

Clark, B. E., & Thein, S. L. (2004). Molecular diagnosis of haemoglobin disorders. Clinical & Laboratory Haematology, 26(3), 159-176.

Colah, R., Gorakshakar, A., & Nadkarni, A. (2010). Global burden, distribution and prevention of β-thalassaemias and hemoglobin E disorders. Expert review of hematology, 3(1), 103-117.

Croteau, S. E., Luo, H. Y., Lehmann, L. E., Chui, D. H., & Neufeld, E. J. (2013). Novel dominant β-thalassaemia: Hb Boston-Kuwait [Codon 139/140 (+ T)]. Pediatric blood & cancer, 60(10), E131-E134.

Cunningham, M. J., Macklin, E. A., Neufeld, E. J., Cohen, A. R., & Thalassemia Clinical Research Network. (2004). Complications of β-thalassemia major in North America. Blood, 104(1), 34-39.

Danjou, F., Anni, F., & Galanello, R. (2011). Beta-thalassemia: from genotype to phenotype. Haematologica, 96(11), 1573-1575.

Das, S. K., & Talukder, G. (2002). B globin gene and related diseases: A review. Int J Hum Genet, 2(3), 139-152.

De Sanctis V, Tangerini A, Testa MR, Lauriola AL, Gamberini MR, Cavallini AR, Rigolin F:Final height and endocrine function in Thalassaemia Intermedia. J Pediatr Endocrinol Metab 1998, 11:965-971

El-Kalla, S., & Mathews, A. R. (1997). A significant β-thalassaemia heterogeneity in the United Arab Emirates. Hemoglobin, 21(3), 237-247.

Flint, J., Harding, R. M., Boyce, A. J., & Clegg, J. B. (1993). 8 The population genetics of the haemoglobinopathies. Baillière's clinical haematology, 6(1), 215-262.

Flint, J., Harding, R. M., Boyce, A. J., & Clegg, J. B. (1998). 1 The population genetics of the haemoglobinopathies. Baillière's clinical haematology, 11(1), 1-51.

Fu, X. H., Liu, D. P., & Liang, C. C. (2002). Chromatin structure and transcriptional regulation of the β-globin locus. Experimental cell research,278(1), 1-11.

Galanello R, Piras S, Barella S, Leoni GB, Cipollina MD, Perseu L, Cao A (2001). Cholelithiasis and Gilbert's syndrome in homozygous β-thalassaemia. Br J Haematol, 115:926-928.

Galanello, R. (2007). Deferiprone in the treatment of transfusion-dependent thalassaemia: a review and perspective. Therapeutics and clinical risk management, 3(5), 795.

Galanello, R. (2012). Recent advances in the molecular understanding of non-transfusion-dependent thalassaemia. Blood reviews, 26, S7-S11.

Galanello, R., & Origa, R. (2010). Review: β-thalassaemia. Orphanet J Rare Dis, 5(11).

Galanello, R., Melis, M. A., Ruggeri, R., Addis, N., Scalas, M. T., Maccioni, L., ... & Cao, A. (1979). β° Thalassaemia Trait in Sardinia. Hemoglobin, 3(1), 33-46.

Galanello, R., Perseu, L., Satta, S., Demartis, F. R., & Campus, S. (2011). Phenotype-genotype correlation in β-thalassaemia. Thalassaemia Reports, 1(1), e6.

Gardenghi, S., Grady, R. W., & Rivella, S. (2010). Anemia, ineffective erythropoiesis and hepcidin: Interacting factors in abnormal iron metabolism leading to iron overload in β-thalassaemia. Hematology/oncology clinics of North America, 24(6), 1089.

Gaziev, J., & Lucarelli, G. (2003). Stem cell transplantation for hemoglobinopathies. Current opinion in pediatrics, 15(1), 24-31.

Giardine, B., van Baal, S., Kaimakis, P., Riemer, C., Miller, W., Samara, M., ... & Patrinos, G. P. (2007). HbVar database of human hemoglobin variants and thalassaemia mutations: 2007 update. Human Mutation, 28(2), 206-206.

Hardison, R. C., Chui, D. H., Giardine, B., Riemer, C., Patrinos, G. P., Anagnou, N., ... & Wajcman, H. (2002). HbVar: a relational database of human hemoglobin variants and thalassaemia mutations at the globin gene server.Human mutation, 19(3), 225-233.

Harteveld, C. L., & Higgs, D. R. (2010). Review α-thalassaemia. Orphanet J Rare Dis, 5, 13.

Higgs, D. R., Thein, S. L., & Wood, W. G. (2001). Distribution and population genetics of the thalassaemias. The thalassaemia syndromes, 4, 237-84. http://emedicine.medscape.com/article/206490-treatment

Ingram, V. M. & Stretton, A. O. W. (1959). Genetic basis of thalassaemia diseases.

Kan YW, Gotbus MS, Klein P & Dozy AM (1975). Successful application of prenatal diagnosis in a pregnancy at risk for homozygous ~-thalassaemia. New England Journal of Medicine 292: 1096-1099.

Kan YW, Lee KY, Furbetta M et al (1980). Polymorphism of DNA sequence in the [3-globin gene region: application to prenatal diagnosis of ~-thalassaemia in Sardinia. New England Journal ~" Medicine 302: 185-188.

Kazazian, H. H., Dowling, C. E., Boehm, C. D., Warren, T. C., Economou, E. P., Katz, J., & Antonarakis, S. E. (1990). Gene Defects in β-Thalassemia and Their Prenatal Diagnosis. Annals of the New York Academy of Sciences,612(1), 1-14.

Khateeb, B., Moatter, T., Shaghil, A. M., Haroon, S., & Kakepoto, G. N. (2000). Genetic diversity of β-thalassaemia mutations in Pakistani population. JPMA. The Journal of the Pakistan Medical Association, 50(9), 293-296.

Kingsley, P. D., Malik, J., Emerson, R. L., Bushnell, T. P., McGrath, K. E., Bloedorn, L. A., ... & Palis, J. (2006). "Maturational" globin switching in primary primitive erythroid cells. Blood, 107(4), 1665-1672.

Liang, S. T., Wong, V. C. W., So, W. W. K., Ma, H. K., Chan, V., & Todd, D. (1985). Homozygous α-thalassaemia: clinical presentation, diagnosis and management. A review of 46 cases. BJOG: An International Journal of Obstetrics & Gynaecology, 92(7), 680-684.

Livingstone, F. B. (1985). Frequencies of hemoglobin variants: thalassaemia, the glucose-6-phosphate dehydrogenase deficiency, G6PD variants, and ovalocytosis in human populations (p. 67). New York:: Oxford University Press

Lodhi, Y. (2003). Economics of thalassaemia management in Pakistan. Thalassaemia awareness week.

Lucarelli, G., Andreani, M., & Angelucci, E. (2002). The cure of thalassaemia by bone marrow transplantation. Blood reviews, 16(2), 81-85.

Maggio, A. (2007). Light and shadows in the iron chelation treatment of haematological diseases. British journal of haematology, 138(4), 407-421.

Malik, P., Arumugam, P. I., Yee, J. K., & Puthenveetil, G. (2005). Successful Correction of the Human Cooley's Anemia β-Thalassaemia Major Phenotype Using a Lentiviral Vector Flanked by the Chicken Hypersensitive Site 4 Chromatin Insulator. Annals of the New York Academy of Sciences,1054(1), 238-249.

Marsella, M., Borgna-Pignatti, C., Meloni, A., Caldarelli, V., Dell'Amico, M. C., Spasiano, A., ... & Pepe, A. (2011). Cardiac iron and cardiac disease in males and females with transfusion-dependent thalassemia major: a T2* magnetic resonance imaging study. haematologica, 96(4), 515-520.

Mavrou, A., Kouvidi, E., Antsaklis, A., Souka, A., Kitsiou Tzeli, S., & Kolialexi, A. (2007). Identification of nucleated red blood cells in maternal circulation: a second step in screening for fetal aneuploidies and pregnancy complications.Prenatal diagnosis, 27(2), 150-153.

May, C., Rivella, S., Callegari, J., Heller, G., Gaensler, K. M., Luzzatto, L., & Sadelain, M. (2000). Therapeutic haemoglobin synthesis in β-thalassaemic mice expressing lentivirus-encoded human β-globin. Nature, 406(6791), 82-86.

May, C., Rivella, S., Chadburn, A., & Sadelain, M. (2002). Successful treatment of murine β-thalassaemia intermedia by transfer of the human β-globin gene.Blood, 99(6), 1902-1908.

McGrath, K. E., Frame, J. M., Fromm, G. J., Koniski, A. D., Kingsley, P. D., Little, J., ... & Palis, J. (2011). A transient definitive erythroid lineage with unique regulation of the β-globin locus in the mammalian embryo. Blood, 117(17), 4600-4608.

Model B, Berdoukas V. The clinical approach to thalassemia. London: Grune and Stratton; 1981. 35 p.

Modell, B. (2003). Recommendations for introducing genetics services in developing countries. Nature Reviews Genetics, 4(1), 61-68.

Modell, B., Khan, M., & Darlison, M. (2000). Survival in β-thalassaemia major in the UK: data from the UK Thalassaemia Register. The Lancet, 355(9220), 2051-2052.

Moritz, K. M., Lim, G. B., & Wintour, E. M. (1997). Developmental regulation of erythropoietin and erythropoiesis. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 273(6), R1829-R1844.

Muncie Jr, H. L., & Campbell, J. (2009). Alpha and beta thalassemia. American family physician, 80(4), 339-344.

Nakayama, R., Yamada, D., Steinmiller, V., Hsia, E., & Hale, R. W. (1986). Hydrops fetalis secondary to Bart hemoglobinopathy. Obstetrics & Gynecology, 67(2), 176-180.

Nassar AH, Naja M, Cesaretti C, Eprassi B, Cappellini MD, Taher A: Pregnancy outcome in patients with β-thalassaemia intermedia at two tertiary care centers, in Beirut and Milan.Haematologica 2008, 93:1586-1587.

Neufeld, E. J. (2006). Oral chelators deferasirox and deferiprone for transfusional iron overload in thalassaemia major: new data, new questions.Blood, 107(9), 3436-3441.

Old, J., Traeger-Synodinos, J., Galanello, R., Petrou, M., & Angastiniotis, M. (2005). Prevention of thalassaemias and other haemoglobin disorders.Thalassaemia International Federation Publications, 2, 113-116.

Origa, R., Galanello, R., Ganz, T., Giagu, N., Maccioni, L., Faa, G., & Nemeth, E. (2007). Liver iron concentrations and urinary hepcidin in β-thalassaemia.Haematologica, 92(5), 583-588.

Pagon, R. A., Adam, M. P., Ardinger, H. H., Bird, T. D., Dolan, C. R., Fong, C. T., ... & Origa, R. (2013). Beta-Thalassemia.

Patrinos, G. P., Kollia, P., & Papadakis, M. N. (2005). Molecular diagnosis of inherited disorders: lessons from hemoglobinopathies. Human mutation, 26(5), 399-412.

Pauling, L., Itano, H. A., Singer, S. J., & Wells, I. C. (1949). Sickle Cell Anemia, a Molecular Disease1. Landmarks in Medical Genetics: Classic Papers with Commentaries, 51, 200.

Persons, D. A. (2010). The challenge of obtaining therapeutic levels of genetically modified hematopoietic stem cells in β-thalassaemia patients. Annals of the New York Academy of Sciences, 1202(1), 69-74.

Perumbeti, A., & Malik, P. (2010). Therapy for β-globinopathies: a brief review and determinants for successful and safe correction. Annals of the New York Academy of Sciences, 1202(1), 36-44.

Peters, M., Heijboer, H., Smiers, F., & Giordano, P. C. (2012). Diagnosis and management of thalassaemia. BMJ, 344.

Pirastu M, Kan YW, Can A et al (1983) Prenatal diagnosis of I]-thalassaemia. Detection of a single nucleotide mutation in DNA. New England Journal ~'Medicine 309: 284-287.

Qurat-ul-Ain, L. A., Hassan, M., Rana, S. M., & Jabeen, F. (2011). Prevalence of β-thalassemic patients associated with consanguinity and anti-HCV-antibody positivity–a cross sectional study. Pak J Zool, 43(1), 29-36.

Rafique, B. (1990). Haemoglobinopathies (thalassaemia). Review and analysis of 1510 cases. Pak. Paediatr. J, 14, 85-95.

Rahimi, Z., Muniz, A., & Parsian, A. (2010). Detection of responsible mutations for β thalassaemia in the Kermanshah Province of Iran using PCR-based techniques. Molecular biology reports, 37(1), 149-154.

Rappaport, V. J., Velazquez, M., & Williams, K. (2004). Hemoglobinopathies in pregnancy. Obstetrics and gynecology clinics of North America, 31(2), 287-317.

Sabath, D. E., & Greisman, H. A. (2011). A multiplex approach to the molecular diagnosis of β-thalassaemia. The Journal of Molecular Diagnostics, 13(4), 369-370.

Sabath, D. E., & Greisman, H. A. (2011). A multiplex approach to the molecular diagnosis of β-thalassaemia. The Journal of Molecular Diagnostics, 13(4), 369-370.

Schrier, S. L. (2002). Pathophysiology of thalassaemia. Current opinion in hematology, 9(2), 123-126.

Serjeant, G. R., Serjeant, B. E., Fraser, R. A., Hambleton, I. R., Higgs, D. R., Kulozik, A. E., & Donaldson, A. (2011). Hb S-β-thalassaemia: molecular, hematological and clinical comparisons. Hemoglobin, 35(1), 1-12.

Shamsi, T. S., Hashmi, K., Adil, S., Ahmad, P., Irfan, M., Raza, S., ... & Ansari, S. (2008). The stem cell transplant program in Pakistan—the first decade. Bone marrow transplantation, 42, S114-S117.

Stamatoyannopoulos, G. (2005). Control of globin gene expression during development and erythroid differentiation. Experimental hematology, 33(3), 259-271.

Taher AT, Otrock ZK, Uthman I, Cappellini MD: Thalassaemia and hypercoagulability.Blood Rev 2008, 22:283-292.

Thein, S. L. (1992). Dominant β thalassaemia: molecular basis and pathophysiology. British journal of haematology, 80(3), 273-277.

Thein, S. L. (2005). Genetic modifiers of β-thalassaemia. haematologica,90(5), 649-660.

Thein, S. L. (2005). Pathophysiology of β thalassaemia—a guide to molecular therapies. ASH Education Program Book, 2005(1), 31-37.

Vichinsky, E. P. (2005). Changing patterns of thalassaemia worldwide. Annals of the New York Academy of Sciences, 1054(1), 18-24.

Vichinsky, E. P. (2005). Changing patterns of thalassemia worldwide. Annals of the New York Academy of Sciences, 1054(1), 18-24.

Vrettou, C., Traeger-Synodinos, J., Tzetis, M., Malamis, G., & Kanavakis, E. (2003). Rapid screening of multiple β-globin gene mutations by real-time PCR on the LightCycler: application to carrier screening and prenatal diagnosis of thalassaemia syndromes. Clinical chemistry, 49(5), 769-776.

Weatherall DJ, Clegg JB. The thalassemia syndromes. Oxford: Blackwell Scientific Publications; 1981. 223 p.

Weatherall DJ. The thalassaemias. In: Stamatoyannopoulos G, et al. (1994). The molecular basis of blood diseases. Philadelphia, PA: WB Saunders Company; 157-205

Weatherall, D. (2004). The thalassaemias: the role of molecular genetics in an evolving global health problem. The American Journal of Human Genetics,74(3), 385-392.

Weatherall, D. J. (1986). The regulation of the differential expression of the human globin genes during development. Journal of Cell Science, 1986(Supplement 4), 319-336.

Weatherall, D. J. (1998). 4 Pathophysiology of thalassaemia. Baillière's clinical haematology, 11(1), 127-146.

Weatherall, D. J. (2001). Phenotype—genotype relationships in monogenic disease: lessons from the thalassaemias. Nature Reviews Genetics, 2(4), 245-255.

Weatherall, D. J. (2004). Thalassaemia: the long road from bedside to genome.Nature Reviews Genetics, 5(8), 625-631.

Weatherall, D. J. (2012). The definition and epidemiology of non-transfusion-dependent thalassaemia. Blood reviews, 26, S3-S6.

Weatherall, D. J., & Clegg, J. B. (2001). Inherited haemoglobin disorders: an increasing global health problem. Bulletin of the World Health Organization,79(8), 704-712.

Weatherall, D. J., & Clegg, J. B. (2008). The thalassaemia syndromes. John Wiley & Sons.

Weatherall,D.J., & Clegg, J.B (2001). The molecular pathology of the thalassaemias. The Thalassaemia Syndromes, Fourth Edition, 133-191.

Whipple GH, Bradford WL (1936). Mediterranean disease: thalassemia (erythroblastic anemia of Cooley). J Pediatr.; 9:279-311.

Whipple, G.H. & Bradford, W. L. (1936). Meditterranean disease thalassaemia (erthythroblastic anaemia of Cooley): associated pigment abnormalities simulating hemochromatosis. The Journal of pediatrics, 9 (3), 279-311.

Wonke, B., Wright, C., & Hoffbrand, A. V. (1998). Combined therapy with deferiprone and desferrioxamine. British journal of haematology, 103, 361-364.

Xu, X. M., Zhou, Y. Q., Luo, G. X., Liao, C., Zhou, M., Chen, P. Y., & Zhong, M. (2004). The prevalence and spectrum of α and β thalassaemia in Guangdong Province: implications for the future health burden and population screening. Journal of clinical pathology, 57(5), 517-522.

Yannaki, E., Emery, D. W., & Stamatoyannopoulos, G. (2010). Gene therapy for β-thalassaemia: the continuing challenge. Expert Rev Mol Med, 12, e31.

Ye, B. C., Zhang, Z., & Lei, Z. (2007). Molecular Analysis of α/β-Thalassaemia in A Southern Chinese Population. Genetic testing, 11(1), 75-83.

Zuhur-ur-Rehman, M. S., Alvi, A. A., Anwar, M., Ahmed, P. A., & Ahmad, M. (1991). α-Thalassaemia Prevalence And Pattern In Northern Pakistan. J Pak Med Assoc, 41, 246.

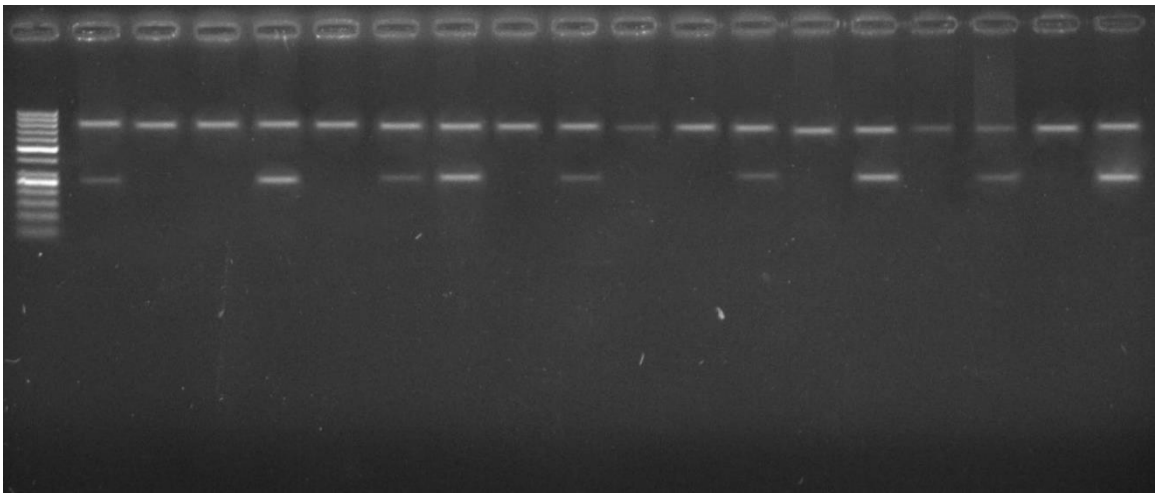


Figure 1: Gel Image of IVS 1 – 5

Lane 1 = Ladder; IVS Normal Primers in Lane 2, 4, 6, 8, 10, 12, 14, 16 and 18. IVS Mutant Primers in lane 3, 5, 7, 9, 11, 13, 15, 17 and 19 (Product Size = 293 bp). Control Primers were added in each reaction (Product size = 862 bp).

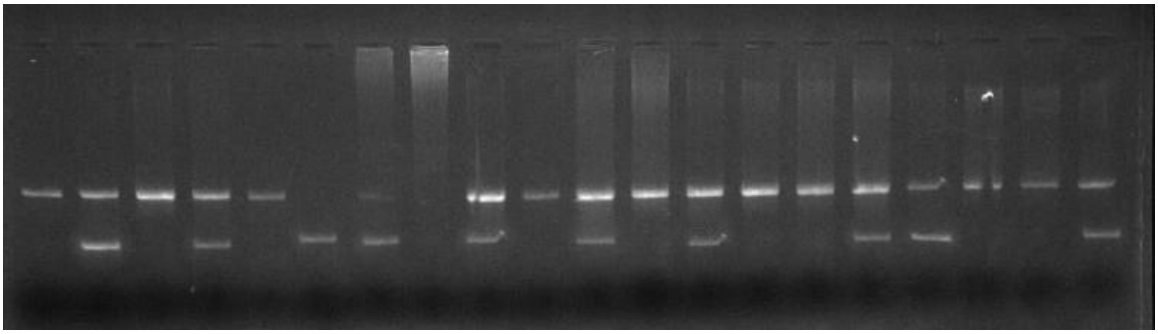


Figure 2: Gel Image of IVS-I-I

IVS-I-I Normal Primers = Lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 (Product Size = 289). IVS-I-I Mutant Primers in Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 (Product Size = 289). Control primers were added in each reaction (Product size = 862 bp)

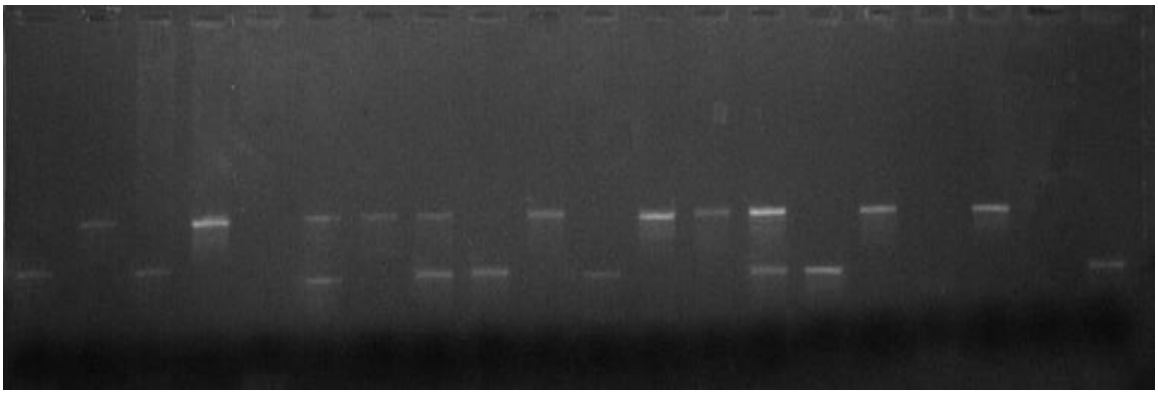


Figure 3: Gel Image of CD 15

CD 15 Normal Primers in Lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 (Product size = 243 bp). CD 15 Mutant Primers in Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 (product size = 243). Control Primers were added in each reaction (Product size = 862 bp)

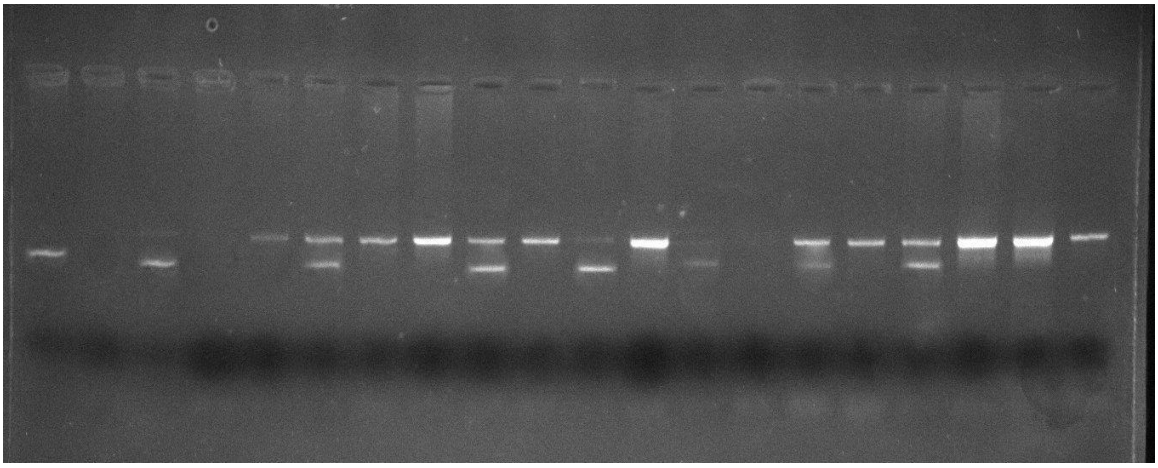


Figure 4: Gel Image of CD 41 / 42

CD 41 / 42 Normal Primers in Lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 (Product size = 451 bp). CD 41 / 42 Mutant Primers in Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 (Product size = 451 bp). Control Primers were added in each reaction (Product size = 862 bp)

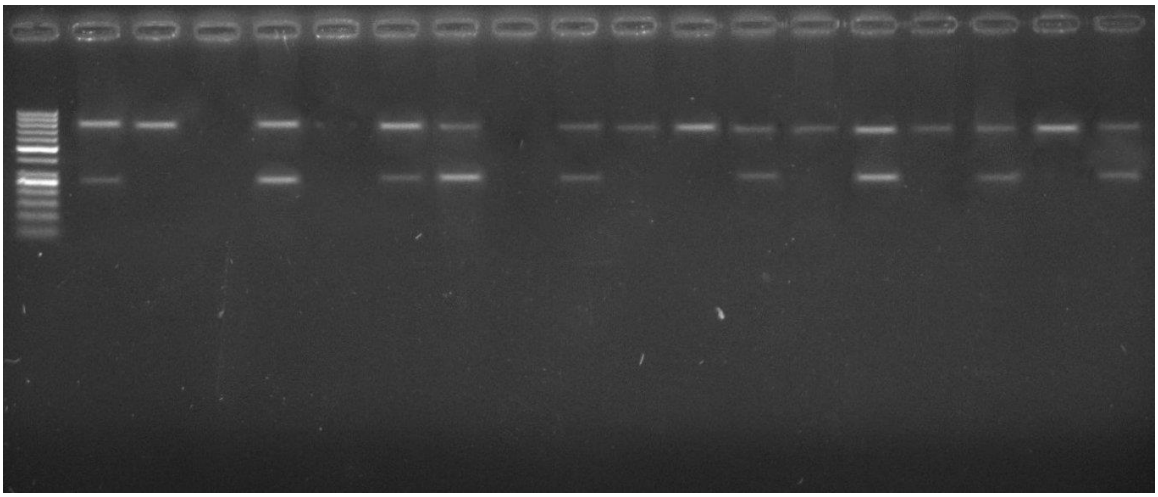


Figure 5: Gel Image of CD 26

CD 26 Normal Primers in Lane 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 (Product size = 451 bp). CD 26 Mutant Primers in Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 (Product size = 451 bp). Control Primers were added in each reaction (Product size = 862 bp)

Rooma Khan - Thesis

ORIGINALITY REPORT

17%	11%	12%	6%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.ncbi.nlm.nih.gov Internet Source	6%
2	Submitted to University of Warwick Student Paper	1%
3	prr.hec.gov.pk Internet Source	1%
4	Submitted to Higher Education Commission Pakistan Student Paper	1%
5	icmr.nic.in Internet Source	1%
6	Cao, A.. "9 Screening and prenatal diagnosis of the haemoglobinopathies", Bailliere's Clinical Haematology, 199303 Publication	1%
7	Antonio Cao. "Beta-thalassemia :", Genetics in Medicine, 02/2010 Publication	1%
	M.F. Sadiq. "Spectrum of β-thalassemia in	