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# Mutational Studies of Gene *HBB* in $\beta$ -Thalassemia Patients from Balochistan, Pakistan

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## Abstract

*Thalassemia is a hereditary blood disorder. It occurs due to two mutations in the HBB gene located on chromosome 11. This gene has 1606 base pairs and contains three exons. Moreover, HBB gene codes for  $\beta$  globin protein have been identified to possess 868 mutations, which comprise point mutation, insertion, deletion, and gene arrangement. In  $\beta$  thalassemia major, both alleles are mutated and no  $\beta$  chain is synthesized. In this study, three human families with thalassemia were selected from different areas of Balochistan. For DNA extraction and estimation, 5 ml blood samples were extracted intravenously from the affected individuals, their normal siblings, and parents in 15ml falcon tubes containing 200 $\mu$ l EDTA. Primer sequences were designed on primer 3 for the mutational analysis of the HBB gene. Since the gene has a total of three exons and two introns, three primers, namely HBBX1, HBBX2 and HBBX3, were designed. These primers were used to amplify the HBB gene responsible for  $\beta$  thalassemia in all family samples. The amplified product was sequenced through an automated 3100 ABI Prism DNA sequence. The sequencing results were analyzed by the SaqMan software. This was done to determine if any genetic variable in the selected families showed mutations. In Family 1, 1 bp substitution mutation (c.9T>C) (p.his3his) and 1bp insertion (c.111T>G) (p.Ser10 val) in exon 1 of HBB gene were identified in thalassemia locus, while in Family 2, 1*

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*bp substitution mutation (c.9T>C) (p.His3His) in exon 1 of HBB gene was identified in thalassemia locus. No mutation was observed in Family 03 after sequencing.*

**Keywords:** *HBB* gene, mutation, Sanger sequencing, thalassemia

## Introduction

Thalassemia is caused by mutations in the DNA of cells, which are also responsible for synthesizing hemoglobin. The name Thalassemia is derived from the Greek word "thalassa" meaning "the sea" since the condition was first identified in the population living near the Mediterranean Sea. The word 'thalassemia' was first introduced by noble prize-winning pathologist George Wipple and professor of Pediatrics William Bradford at the University of Rochester in 1932. It was followed by a discovery by an Italian researcher whose symptoms were similar to Cooley's anemia, it was later termed as thalassemia intermedia and characterized by disease severity ranging between the mild and severe  $\beta$  thalassemia trait [1, 2]. Different studies have shown that in families with one thalassemia carrier parent there is a 50% possibility of the children being carriers.

In contrast, in families with both  $\beta$  thalassemia carrier parents, 25% of their offspring will be normal, while 25% will be affected by Mediterranean anemia. These pursuits have been developed well-informed of American research, and individually, the two congregations reached the same results [3].

Thalassemia is a congenital blood clutter transfer through families in which the patient's body synthesizes aberrant hemoglobin. Thalassemia occurs when a globin gene fails and the production of globin protein subunits is left out of balance. The mutated gene present on chromosome 11 (beta) and chromosome 16 (alpha) causes abundant destruction of red blood cells, resulting in anemia. Clinical symptoms of this disorder are diverse, varying from lack of symptoms to intense fetal anemia if non-treated during early infancy [4].

Furthermore, several mutations can develop prenatal to mature Hb change that manifest, such as in the genetic perseverance of HbF.

Additionally, the coinheritance of alpha and gamma mutations result in the coinheritance of further hemoglobinopathies [4].

Thalassemias are a group of autosomal recessive disorders caused by the reduction or absent production of one or more of the globin chains that make up the hemoglobin (Hb) tetramers. Two main types of thalassemias, namely the  $\alpha$ - and  $\beta$ -thalassemias, can be distinguished depending upon the type of globin chain. Additionally, complex thalassemias resulting from defective production of two to four different globin chains ( $\delta\beta$ -,  $\gamma\delta\beta$ -, and  $\epsilon\gamma\delta\beta$ -thalassemia) are recognized [5]. Alpha thalassemia is more prevalent in South Asia, southern china, and Malaysia. Conversely,  $\beta$  Thalassemia was primarily observed in individuals living near the Mediterranean Sea, in South Asia, and in Africa [6].  $\beta$  thalassemia is the most familiar type of blood disorder. Individuals contain two regular copies of the  $\beta$  globin gene present on chromosomes, from which 11,200 types of mutations have been recognized to cause  $\beta$  thalassemia. The mutation occurs due to the transformation in the  $\beta$  globin gene.  $\beta$  thalassemia displays different symptoms of anemia, which can range from minor to severe, depending on the genetic changes.  $\beta$  thalassemia major is a painful anemic condition requiring constant blood transfusion. Extra iron accumulates in different organs, which results in organ failure [7].

The HBB  $\beta$  Globin gene is a small gene containing <2000 bp. It is situated on the short arm of chromosome 11. According to World Health Organization (WHO), 300,000 to 400,000 people are born with HBB globin gene mutation [8]. The HBB gene encodes beta-globin proteins that are an integral part of adult hemoglobin [9]. Previous studies have recorded 868 polymorphisms in the HBB gene, which altered globin formation. These mutations are point, insertion, deletion, and gene rearrangements [10]. Hence,  $\beta$  thalassemia is caused by quantitative abnormalities of the HBB, which either lessen or inhibit the synthesis of  $\beta$  globin [11].  $\beta$  Thalassemia is primarily caused by point mutation; however, in recent years, a deletion type mutation has also been recognized and accounted for more than 10% of  $\beta$  thalassemia mutation [11].

The main objective of this study was to identify and examine family histories containing individuals with thalassemia. The families selected for this study were inhabitants of Balochistan. For this purpose, detailed history

of each family was recorded to minimize the presence of other abnormalities and environmental causes that might result in  $\beta$  Thalassemia. The Pedigrees were drawn, and 5 mL blood samples were collected from affected and normal individuals. This was done to extract DNA and its quantity was estimated. Finally, after exon amplification and sequencing, mutational studies were performed.

### **Identification and Enrolment of Families**

Families with individuals suffering from thalassemia were screened for sequencing analysis. Participation in the study required informed consent. Each family member's medical history was recorded to rule out the possibility of other anomalies or environmental reasons for  $\beta$  Thalassemia. Three families, having a family member suffering from  $\beta$  thalassemia, were selected from BMC Quetta. The affected individuals from each family showed clinical features of  $\beta$  thalassemia major. The study was approved by the ethical board of Balochistan University of Information Technology, Engineering and Management, Quetta, Pakistan.

### **Pedigree Analysis**

The Pedigrees were drawn using Cyrillic 2.1 (Cyrillic Software, Wallingford, Oxfordshire, UK) software program based on the data and information collected from the patients and families.

### **Clinical Evaluation**

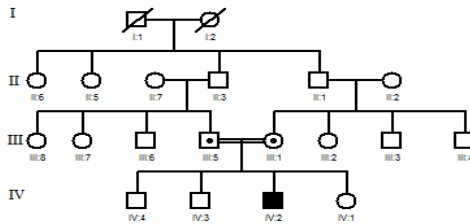
The medical history of all the affected individuals from the enrolled families was obtained. All patients were taking regular blood transfusion therapy from BMC Quetta before being enrolled for this study. Data regarding family histories, signs and symptoms, blood transfusion therapy, types, and frequency of disease was collected through a questionnaire. A hemoglobin electrophoresis test was also conducted to confirm the presence of thalassemia.

### ***Family 1***

Family 1 were inhabitants of Quetta and had one affected individual. The affected individual was a 07-year-old child, who showed growth retardation, pale skin, and enlarged abdomen. This family also had three normal individuals as shown in Figure 1a. Blood samples were collected from their parents, one normal sibling and one affected individual. The

clinical symptoms of the affected individual showed growth retardation, pale skin, enlarged abdomen, and The affected individual also required transfusion therapy every month. DNA of the affected individual was extracted and was amplified with three sets of primers for/to obtain three exons of the HBB gene. The amplified product was sequenced on a 3100 ABI Genetic Analyzer (ThermoFisher Scientific). Afterwards, the sequencing results were analyzed via SeqMan™ software.

**Figure 1a:** Pedigree of Family 01 with the Affected Individual



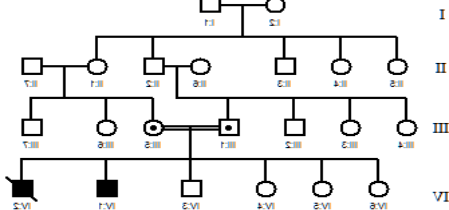
**Figure 1b.** Affected Individual



**Family 2**

Family 2 were inhabitants of Quetta (Balochistan) and had two affected individuals (Figure 2a). The clinical symptoms of the affected individual were growth retardation, pale skin, and an enlarged abdomen. They also required transfusion therapy every month (Figure 2b). The affected individual’s DNA was amplified using three sets of primers for/to obtain three exons of the HBB gene. The amplified product was sequenced through an automated 3100 ABI Prism DNA sequencer (Thermo-Fisher Scientific).

**Figure 2a:** Pedigree of Family 02 with the Affected Individual



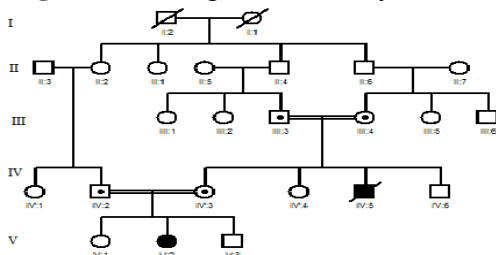
**Figure 2b:** Affected Individual



**Family 3:** Family 3 were also inhabitants of Quetta (Balochistan). They had one affected individual and two normal individuals (Figure 3a). The clinical

symptoms of the affected individual were growth retardation, pale skin, and an enlarged abdomen. The affected individual also required transfusion therapy every month (Figure 3b). Their DNA was amplified with three sets of primers and sequenced via an automated 3100 ABI Prism DNA sequencer (Thermo-Fisher Scientific)

**Figure3a:** Pedigree of Family 03



**Figure3b:** Affected Individual



### Collection of Blood Samples

Three families were identified with one or more family members affected with thalassemia. The age group of the affected individuals ranged from 2 years onwards., Their symptoms were identified to be moderate to severe.

Detailed history was taken from each family as well as to avoid the risk of misidentification of disease the prescription of doctor or diagnosed report was checked. Permission from participants was taken in the form of informed consent. 5 ml blood samples were drawn intravenously in 15ml falcon tubes containing 200 uL EDTA. The blood samples were stored at -20°C.

### DNA Extraction

Genomic DNA was extracted using the non-organic method [12]. This method involves RBCs lysis, protein digestion, and precipitation followed by DNA isolation and purification. The DNA samples were dissolved in TE buffer (pH 8.0) and stored at -20 °C for further use. DNA samples were estimated by performing gel electrophoresis on 1% agarose gel.

### Primer Designing and PCR (Polymerase Chain Reaction) Amplification

Suitable primers were designed using Primer 3 and Primer 3 plus computer program

(<http://.bioinfo.ut.ee/primer3/>)

(<http://.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>).

A total of 3 exons were processed to make the primer. Consequently, 3 forward oligonucleotide primers and 3 reverse oligonucleotide primers were designed (Table 1).

**Table1.** Sequences of Primers used for the Amplification of *HBB* Gene in Humans

EXONS	Left Primer (5' 3') →	Right Primer (5' 3') →	Product size
HBB_Exon1	ACTCCTAAGCCAGTGCCAGA	TGCCTATCAGAAACCCAAGAG	395 bp
HBB_Exon2	AGAAACTGGGCATGTGGAGA	AAAACGATCCTGAGACTTCCA	460 bp
HBB_Exon3	ATGCCTCTTTGCACCATTCT	GACCTCCCATTCCCTTTT	566 bp

PCR reactions were performed with 50 ng of template DNA in 20 µl reaction mixture for 6 samples for each exon, containing 10 µM Primers, 4µl PCR Buffer (100mM Tris-Cl, pH 8.4, 500 mM KCL, 20nM MgCL<sub>2</sub> and 1% Triton), 1.6µl of 2mM dNTPs and 5 units of Taq DNA Polymerase (ThermoFisher Scientific). The amplified products were run on 2% agarose gel in TBE buffer and visualized under UV trans-illuminator (ThermoFisher Scientific).

### DNA Sequencing

For DNA sequencing, the *HBB* gene from genomic DNA was amplified via PCR (T100, BioRad) using exon-specific primers. Subsequently, the PCR product was used as a template DNA for sequencing [13]. Direct sequencing was performed using an AB1 3100 Genetic Analyzer (ThermoFisher) and analyzed using BioEdit (version 7.0.2) software for mutation analysis.

## Results

After amplification, Sanger sequencing of the *HBB* gene was conducted on the affected individuals to identify thalassemia major patients.

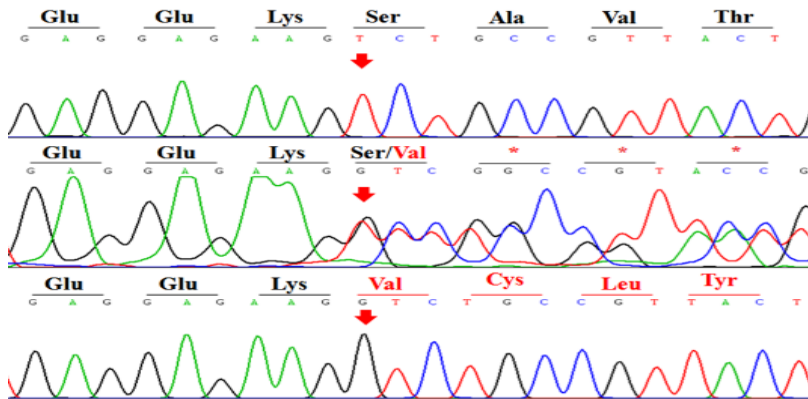
### Family 01

The sequencing results revealed the presence of two mutations: one is 1bp insertion (c.30\_31InsG) (p.Ser10valfsTer23), and the other is 1 bp silent



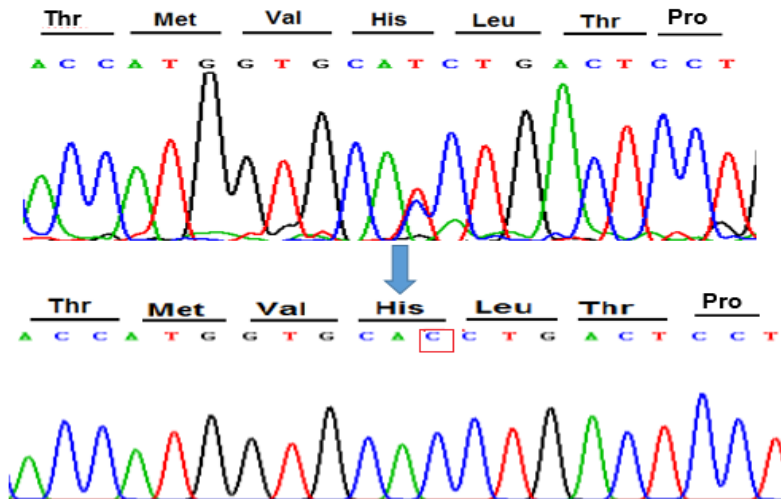
mutation (c.9T>C) (p.His3His) in exon 1 of HBB gene (Figure 4).

**Figure 4.** Electropherogram for Family 1 Showing 1 bp Insertion and a 1 bp Silent Mutation in Exon 1 of the HBB Gene



In the Second Family, 1 bp Silent Mutation (c.9T>C) (p.His3His) in Exon 1 of the HBB Gene was Identified (Figure 5)

**Figure 5.** Electropherogram for Family 2 Showing only a 1bp Silent Mutation in Exon 1 of the HBB Gene



But in the Last and Third Family, the Sequencing Detected no Mutations in any Exon of HBB Gene

## Discussion

Thalassemia is a clinically diverse disease/disorder because different genetic defects impede the globin-chain synthesis in different ways.  $\beta$ -Thalassemia, the most common type of thalassemia, is caused by more than 200 point mutations, nevertheless this is still insufficient as many other genetic variations still exist to understand the genetics of the disease [14]. The study's overall objective was to identify the molecular characterization of  $\beta$ -thalassemia disease in the selected families from Balochistan, Pakistan. A total of three families were enrolled from different areas of Quetta, Balochistan. Each family was identified with a single affected individual having thalassemia major.

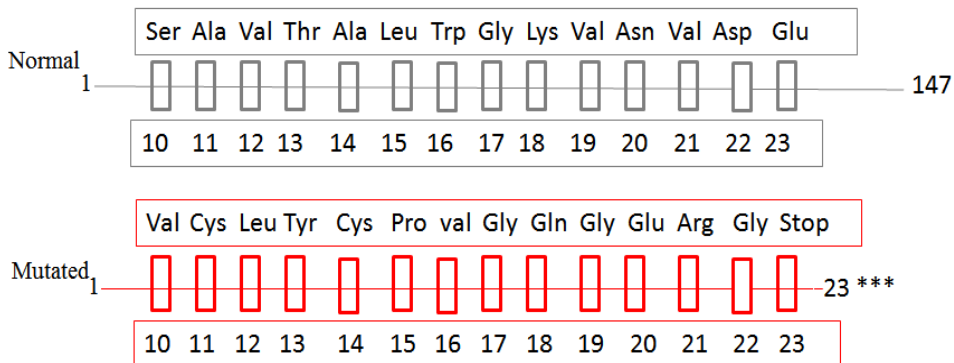
The Human *HBB* gene (OMIM, # 606352) consists of 3 exons. In humans, the *HBB* gene is located on chromosome 11 [15]. Out of the three selected families, sequencing results of three exons of the *HBB* gene from Family 1 showed a silent mutation at codon 09 CAT  $\rightarrow$  CAC on the coding strand. Both the normal codon (CAT) and the mutant codon (CAC) code for histidine. This T  $\rightarrow$  C substitution creates the sequence GTGCACC in the mutated region. The results showed two mutations in exon 1 of the *HBB* gene: one is 1 bp homozygous insertion (c.30\_31insG) (p.Ser10valfsTer23), and the other is 1bp silent mutation (c.9T>C) (p.His3His). Sequencing results of Family 02 showed 1 bp silent mutation (c.9T>C) (p.His3His) in exon 1 of the *HBB* gene. The sequencing showed a T  $\rightarrow$  C change on the coding strand. The sequencing results of Family 03 did not show any mutation.

The most common mutation identified in Indian patients was in the region c.92+5G>C. Mutation in this region was also identified in Pakistan and Sri Lankan population [16]. Previous studies have identified numerous mutations in  $\alpha$  and  $\beta$  globin genes. For example, Hb Presbyterian (*HBB*: c.327 C>G) is a naturally present variant with a reduced oxygen affinity. At codon 108 of the  $\beta$ -globin gene, the C to G conversion (AAC>AAG) causes asparagine to be replaced by a lysine. The heterozygous variant at codon 108, both in the father and daughters  $\beta$ -globin genes, was identified as Hb Presbyterian or *HBB*: c.327 C>G [17].

Previous studies have also reported a novel  $\beta$  chain mutation, the Hb gene ( $\beta 144$  Lys $\rightarrow$ Arg; *HBB*: c.434A>G), in a Chinese family. The  $\beta$ -thalassemia phenotype is minimal when the mutation is passed down in a heterozygous manner [18]. Similarly, a novel C>T substitution at codon 53 of the *HBB* gene (*HBB*: c.161C>T) was identified in a Bengali family. In this study, DNA was extracted and sequenced to identify polymorphisms in the *HBB* gene. The mutation (*HBB*: c.92+5G>C) was passed in two generations. The proband is homozygous for *HBB*: c.92+5G>C and needs monthly transfusions. On the other hand, her grandmother, mother, and sister all possessed this novel mutation cis with the heterozygous *HBB*: c.92+5G>C. They are carriers but not thalassemic. This mutation produces the substitution  $\beta 53$  Ala $\rightarrow$ Val; *HBB*: c.161C>T, new structural hemoglobin (Hb) variant [19].

Another study reported rare compound heterozygosity of two different variants, namely *HBB*c.92GNC and *HBB*c.92+5GNC, in maternal amniotic fluid sample of a Gujarati family [20]. In this study, the nucleotide sequencing method was used to detect prenatal  $\beta$ -thalassemia mutation in fetal DNA. The sequencing analysis revealed that the father was heterozygous for *HBB* c.92GNC (Codon 30 (GNC)) mutation ( $\beta 0$  type) and the mother was heterozygous for *HBB*c.92+5GNC (IVS I-5 (GNC)) mutation ( $\beta +$  type). When the amniotic fluid sample was examined for the  $\beta$  globin gene (*HBB*), they discovered a heterozygous allelic pattern for the aforementioned alterations. This fetus has compound heterozygous mutations and is classified as  $\beta +/\beta 0$  category of  $\beta$  thalassemia, which is clinically and genotypically characterized as  $\beta$ -thalassemia major.

The expression of beta-globin and surrounding globins in the  $\beta$ -globin locus is regulated by a single locus control region (LCR), which is positioned upstream of the globin genes and is the essential regulatory element in the locus. The most frequently reported allelic variant has 1600 base pairs (bp) and three exons. The order of the genes in the beta-globin cluster is 5' - epsilon - gamma-G - gamma-A - delta - beta - 3' obtained from <https://ghr.nlm.nih.gov/gene/HBB> (Figure 6). Mutations in the genetic coding sequence result in a deformed protein structure (Figure 7).

**Figure 6.** Showing the Different Domains of HBB Gene**Figure 7.** Showing Effect of Mutation on Protein Structure

## Conclusion

The study concluded with the identification of some important single nucleotide polymorphisms (SNPs) in consanguineous familial samples from Balochistan, Pakistan. These samples were identified to have homozygous insertion (c.30\_31InsG) (p.Ser10valfsTer23) and 1bp silent mutation (c.9T>C) (p.His3His) in exon 1 of the HBB gene. Future researchers should conduct a similar investigation with increased sample size and establishment of cell culture lines along with Insilico work like Homology Modeling and MD Simulations. It was noted that parental awareness regarding thalassemia, its treatment, and prevention is fair; however, the lack of health testing facilities for prenatal diagnosis in Pakistan is still a significant factor leading to the high prevalence of thalassemia. Consanguineous marriages are commonly practised in Pakistani society, which should be avoided. It is suggested that if known carriers marry, they should have prenatal screening, such as through CVS. Early screening and diagnosis may decrease the incidence of  $\beta$  thalassemia in Pakistan.

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### **Conflict of Interest**

The authors declare that they have no potential conflicts of interest.

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