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ORIGINAL ARTICLE

Molecular Analysis and Prenatal Diagnosis of Segregating B-Thalassemia

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ABSTRACT

Aims: This study aimed to comprehensively investigate the status of β-thalassemia mutational screening and prenatal diagnosis techniques in Pakistan, with a specific focus on understanding their implications for disease diagnosis and management.

Methods: Blood and fetal samples were meticulously collected from three families affected by β-thalassemia. Employing the advanced Amplification Refractory Mutation System (ARMS) PCR technique, DNA isolation and amplification were carried out, leading to the identification of the three most prevalent β-thalassemia mutations—IVS-I-5, -619bp, and FSC 8-9—within these familial contexts.

Results: The study unveiled the presence of the three predominant β-thalassemia mutations IVS-I-5, -619bp, and FSC 8-9 in the collected samples. These findings hold significant implications for the improvement of β-thalassemia diagnosis and management.

Practical Implications: The identification of these common mutations has the potential to streamline genetic testing and prenatal diagnosis, potentially reducing β-thalassemia cases. Additionally, mutation analysis provides valuable insights into the disorder's pathophysiology, enabling informed decisions and targeted genetic counseling for affected families to minimize transmission risk.

Conclusion: In conclusion, this study constitutes a pivotal advancement in the ongoing efforts to mitigate the profound impact of β-thalassemia, both within Pakistan and on a broader global scale. By not only pinpointing the prevalent mutations responsible for the disorder but also delving into the techniques for diagnosis, the research lays a robust foundation for the enhancement of disease management strategies and preventive measures.

Keywords: β-thalassemia; Chorionic villus sampling; ARMS PCR; Prenatal diagnosis; Mutation analysis; Disease prevention; Genetic counseling.

INTRODUCTION

β-thalassemia is a complex and heterogeneous genetic blood disorder that affects millions of people worldwide, with the highest concentration of carriers in the "thalassemia belt" stretching from the Mediterranean to Southeast Asia (1). The condition is caused by mutations in the HBB gene, leading to reduced or absent production of β-globin chains, a crucial component of adult hemoglobin (2). This imbalance in the α/β-globin chain ratio results in the formation of insoluble globin aggregates and precipitates that damage red blood cells, leading to anemia (3).

The clinical presentation of β-thalassemia is highly variable (4) ranging from the severe thalassemia major, which requires regular blood transfusions and iron chelation therapy, to the milder thalassemia intermedia and thalassemia minor (5). The severity and phenotypic expression of β-thalassemia are determined by the type of mutation in the HBB gene, the presence of co-inherited modifying factors, and the interaction between α- and β-globin chain production(6).

Various therapeutic approaches are employed to manage β-thalassemia, including blood transfusions, iron chelation therapy, hematopoietic stem cell transplantation (HSCT), and gene therapy. However, each approach carries risks and limitations, highlighting the need for continued research and development in the field(7).

The global prevalence of β-thalassemia remains alarmingly high, with millions of carriers and affected individuals worldwide (8). The carrier rate varies from 0.1% to 22% in selected Southeast Asia, Middle East, countries, Mediterranean Countries, America and in Europe with an estimated number of carriers ranging from 330,000 to 10,000,000. The diversity of β-thalassemia mutations among various countries and regions emphasizes the need for tailored prevention strategies and precise diagnostic tools (9).

To combat the spread of β-thalassemia, it is crucial to raise awareness about the disease and its genetic basis, implement effective prevention and management strategies, and prioritize research into the molecular basis of β-thalassemia and the development of new diagnostic and treatment options. International cooperation and information sharing among healthcare professionals, researchers, policymakers, and affected communities are essential to tackling β-thalassemia on a global scale (10)

In conclusion, β-thalassemia remains a significant health challenge worldwide, requiring a coordinated global response to address this genetic disorder effectively. With a combination of public health measures, research and innovation, and international cooperation, it is possible to reduce the disease burden and improve health outcomes for affected individuals and communities around the world (11).

Revolutionizing β-Thalassemia Management: The Power of Mutational Screening and Prenatal Diagnosis (PND): Early detection is key in managing β-thalassemia, and mutational screening and prenatal diagnosis are game-changers in achieving this (12). These essential tools not only enable the identification of β-thalassemia carriers in at-risk communities but also inform reproductive decision-making and significantly reduce the disease's incidence, ultimately improving the quality of life for affected individuals and their families (13).

In regions with high disease prevalence, mutational screening is particularly crucial, as it facilitates prompt detection and raises awareness. Healthcare professionals can provide personalized genetic counseling to couples, informing them about their reproductive options and risks of having a child with β-thalassemia(14). Prenatal diagnosis, on the other hand, examines fetal samples to detect β-globin gene mutations, allowing at-risk couples to make informed decisions about their pregnancies (15).

They can choose to continue with the pregnancy, consider adoption, or opt for in-vitro fertilization (IVF) with preimplantation genetic testing (PGT) for future pregnancies (16). The implementation of mutational screening and prenatal diagnosis programs has led to remarkable success stories. Cyprus, for example, saw a staggering 95% reduction in affected births after introducing a national screening program(17)

Similar triumphs have occurred in Italy, Greece, and Iran, with comprehensive prevention programs leading to significant declines in β-thalassemia births. In conclusion, mutational

screening and prenatal diagnosis are indispensable tools in the fight against β-thalassemia(18). By investing in these groundbreaking programs, healthcare systems and policymakers can transform health outcomes and provide hope for affected individuals and families worldwide, especially in regions with high disease prevalence.

This research aims to transform β-thalassemia prevention and management in developing countries, specifically targeting Pakistan. The study's primary goal is to critically assess current mutational screening approaches and pinpoint opportunities for enhancement, ultimately crafting more effective, locally adapted screening programs.

The research will also examine the efficacy and precision of prenatal diagnosis methods, such as CVS and amniocentesis, within Pakistani populations(19). This ensures accurate genetic information for informed reproductive decision-making, leading to improved population health. Furthermore, the study intends to uncover the challenges and barriers to implementing mutational screening and prenatal diagnosis in Pakistan, including limited funding, insufficient healthcare infrastructure, and cultural factors.

Finally, this research will offer evidence-based recommendations for future research and implementation strategies. These include innovative screening technologies, capacity building, and public health interventions to elevate β-thalassemia awareness and encourage genetic testing services. Together, these efforts will significantly improve β-thalassemia prevention and management in Pakistan and beyond.

MATERIALS AND METHODS

Prior to commencing the study, formal approval was granted by the Institutional Review Board (IRB) of Health biotechnology department (HBD) of the National Institute for Biotechnology and Genetic Engineering (NIBGE) in Faisalabad, Pakistan.

Participant Selection and Clinical Evaluation: This study involved the examination of three families with β-thalassemia. The Multan Institute of Nuclear Medicine and Radiotherapy (MINAR) identified these families, and medical histories were recorded after conducting appropriate tests before molecular investigation. These families exhibited strong evidence of autosomal recessive inheritance and consanguinity.

Pedigree Analysis: After obtaining local ethical committee approval and written consent from all participating families, pedigrees were constructed using the standard method introduced by Bennett et al. (20). Cyrillic software version 2.1.3 was employed to create extensive pedigrees, using distinct symbols to represent family structure. Males and females are represented by squares and circles, respectively, with numbers enclosed denoting individual siblings.

Family Profiles: The families were identified from southern Punjab, with 1-3 affected members from each family undergoing genotypic examination. All three families' parents were consanguineous and clinically healthy. Genetic screening for known mutations was performed on thalassemic families before prenatal diagnosis. Chorionic villus sampling (CVS) was collected during the 12th week of pregnancy.

BLOOD SAMPLE COLLECTION

Family 1: After collecting blood samples from both parents and an affected child (III:1, III:2, and IV:1) in EDTA-coated tubes at MINAR, they were sent to the National Institute of Biotechnology and Genetic Engineering (NIBGE), Human Molecular Genetics and Metabolic Disorders (HMG laboratory) Faisalabad, Pakistan. Subsequently, a CVS sample (IV:1) was obtained during the 12th week of pregnancy and stored in normal saline. ARMS PCR was performed for the detection of the -619bp mutation, along with mutation-specific positive and negative controls, for the most prevalent mutations IVS-I-5 and FSC 8-9.

Family 2: To consider the second family, we obtained blood samples from both parents (III:1 and III:2) and their affected child (IV:2) at a designated location. The samples were sent to NIBGE for further processing. Moreover, during the 12th week of pregnancy, a CVS sample was collected, stored in normal saline, and sent to the HMG laboratory. Prior to the study, the parental mutations were unknown. We performed ARMS PCR to detect one of the most common β-thalassemia mutations, IVS-I-5. To confirm the results and compare them with control samples, we analyzed homozygous mutant (affected), heterozygous (carrier), and homozygous normal (negative control) samples alongside the collected samples. The PCR product was then analyzed using 1.8% agarose gel electrophoresis.

Family 3: In Thalassemia-3 family, a couple who were related to each other had one healthy child. During the 12th week of pregnancy, a chorionic villus sample (CVS) was obtained. Prior to their blood samples being sent to the following destination, the parents were screened for thalassemia minor through complete blood count (CBC) and hemoglobin electrophoresis. Parental mutations were identified before the prenatal diagnosis. The mother was found to carry the IVS-I-5 mutation, while the father carried the FSC 8-9 mutation. The fetus could only be affected if both mutations were inherited in a compound heterozygous state, the CVS was tested for both parental mutations. Following analysis of the ARMS PCR results, the fetus was found to only carry the maternal mutation (IVS-I-5) and tested negative for the paternal mutation.

Blood Sample Acquisition: Peripheral venous blood was also collected at MINAR, Multan, after obtaining consent from individuals or their legal guardians following proper diagnosis. Non-infectious 5cc syringes were used to draw blood, which was then transferred into anticoagulant EDTA vacutainers for storage. After completing all investigations, blood samples were sent to the Human Molecular Genetics Laboratory (HMGL), NIBGE, Faisalabad**.**

DNA Extraction and Purification Genomic DNA: First, we extracted genomic DNA from blood samples using the phenol chloroform method, adhering to the standard proteinase K digestion and phenol chloroform extraction protocol established by Cortes et al. (21). A 1.5mL microcenter refuge tube containing 0.75mL blood was mixed with an equal volume of solution A and incubated for 10 minutes at room temperature. The mixture was then centrifuged, and the supernatant was discarded. The nuclear pellet was resuspended in solution B, SDS, and proteinase K, and the tubes were incubated at the appropriate temperatures. After incubation, a freshly prepared mixture of solutions C and D was added to the tube, followed by centrifugation and the collection of the upper aqueous phase. This process was repeated with solution D alone. DNA was then precipitated using chilled isopropanol and sodium acetate, washed with ethanol, and dissolved in a suitable amount of DNA dissolving buffer or double distilled PCR water.

DNA Extraction from CVS: Blood drawn during the second step for DNA extraction from CVS samples following (22), the CVS sample was separated from normal saline and lysed with CVS lysis buffer. It was then incubated with proteinase K and SDS before being mixed with a solution of C and D, vortexed, and centrifuged. This step was repeated three times for highly purified DNA. The aqueous layer was then separated and mixed with solution D, chloroform, sodium acetate, and isopropanol to precipitate DNA. The DNA pellet was then washed with ethanol and dissolved in TE buffer or double distilled PCR water.

DNA Quantification: After DNA extraction, genomic DNA concentration was determined using the Thermo Scientific Nanodrop 8000 UV-VIS Spectrophotometer (23). We assessed the integrity of the extracted DNA samples by visualizing them on a 1% agarose gel under UV light. Genetic screening of thalassemia families was conducted using Amplification Refractory Mutation System (ARMS) PCR (24), which is a modified PCR technique that utilizes two oligonucleotide primers with differing 3' terminal nucleotides. The resulting PCR products were then visualized on a 1.8% agarose gel (25).

Agarose Gel Electrophoresis (Horizontal): After quantifying the extracted DNA, we checked its integrity by using 1% (w/v) agarose gel electrophoresis to separate the amplified PCR products (25).

First, we prepared the agarose gel by melting agarose in TBE buffer, adding ethidium bromide solution, and then casting the agarose solution into a tray, allowing it to solidify. Next, we placed the solidified gel in an electrophoresis tank with TBE buffer and loaded and ran the samples. We then used a UV Gel (Uvitec, UK) Documentation system to visualize and capture the resolved PCR products.

Amplified Refractory Mutation System (ARMS) PCR: Once the extraction and electrophoresis were completed, we proceeded to the crucial step of ARMS PCR (26), a highly effective genetic screening method employed for detecting genetic mutations in thalassemia families. This technique is also referred to as allele-specific PCR or PCR amplification of specific alleles (PASA). Below is the entire procedure:

Procedure: ARMS PCR is a modified version of traditional PCR and requires two oligonucleotide primers that are identical in sequence, apart from their terminal 3' nucleotides. One primer has its 3' terminal nucleotide complementary to the mutated sequence (Mt ARMS primer), while the other corresponds to the normal DNA sequence (N ARMS primer). Both primers act as reverse primers in the reaction. A common forward primer, C, was used to amplify both mutant and normal templates for specific mutation screening. In addition to the primary pair of primers, a second pair was always included in the reaction mixture to simultaneously amplify an unrelated DNA sequence, serving as an internal control to ensure optimal performance of the reaction mixture and thermal cycler. The ARMS analysis was carried out in a 20 µl reaction mixture, with the PCR product then visualized on 1.8% agarose gel.

Agarose Gel Electrophoresis: Separating Amplified DNA Products, the amplified PCR products were separated using 1.8-2% agarose gel electrophoresis(27). Samples mixed with bromophenol blue were loaded into the gel and run at 90 volts (55mA) for 45 minutes in a 0.5X TBE-filled (running buffer) electrophoresis tank.

Creating the Gel: To prepare the gel, we melted 1.8-2 grams of agarose in 100-200ml of 0.5 X TBE buffer in a microwave for 1-2 minutes. After cooling the agarose solution to below 50°C, we added 4.5 µl of ethidium bromide solution (10mg/ml) to stain the DNA. Then, we poured the agarose solution into a tray with positioned combs and allowed it to solidify at room temperature, which took approximately 30-35 minutes. Once the gel had polymerized, we gently removed the combs to create slots or wells for loading samples. We placed the tray containing the gel into an electrophoresis tank filled with 0.5X TBE buffer, as described in Appendix B. Finally, we visualized and captured the separated PCR products using a UV Gel Documentation system (Uvitec, UK).

RESULTS

Mutational analysis: Our study comprehensively investigates the management of β-thalassemia in Pakistan, identifying effective proposing solutions to improve diagnosis and management. We analyzed prevailing mutational screening and prenatal diagnosis methods and utilized ARMS PCR to screen for mutations in three affected families. Additionally, we performed first trimester prenatal diagnosis using chorionic villus sample DNA to detect high-risk pregnancies.

Family 1: The quality of the extracted genomic DNA samples was excellent, as demonstrated by the Nanodrop values shown in Table 1. ARMS PCR results showed that the parents were negative for the most common mutations, IVS 1-5 and FSC 8-9. However, further screening revealed the presence of a rare -619 bp mutation in both parents (Figure 1). The affected child (IV: 1) was found to be homozygous for this mutation, while the CVS analysis indicated that the fetus was negative for the parental mutation (Figure 2).

Table 1: DNA Quantification of Family 1 Samples Using Nanodrop

#	DNA Source	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)
1	Mother	1403.8	ng/µl	28.075	14.980
2	Father	1425.6	ng/µl	28.513	15.162
3	Child	195.7	ng/µl	3.915	2.092
4	CVS	167.9	ng/µl	3.358	1.854

Note: This table presents the DNA quantification results of five samples from Family 1, as measured using a Nanodrop spectrophotometer.

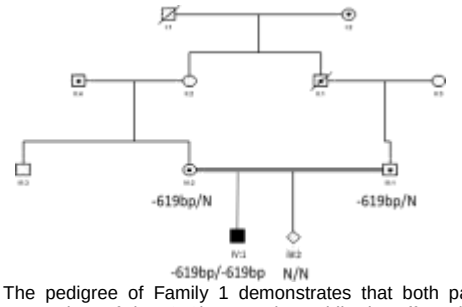

Figure 1: The pedigree of Family 1 demonstrates that both parents are heterozygous carriers of the -619bp mutation, while the affected child is a homozygous mutant. In contrast, the fetus exhibits a wild-type (standard) genotype.

Genotyping Results:

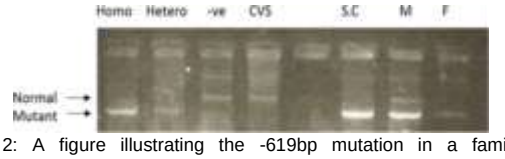

Figure 2: A figure illustrating the -619bp mutation in a family with thalassemia-1 is presented. The figure displays control samples in lanes 1-3, including homozygous, heterozygous, and negative control DNA leaders, followed by blood samples in lanes 4-7, representing the carrier child, effected child (SC), mother, father, and chorionic villus sampling (CVS). The mutant allele is indicated by the lower band, while the upper band represents the normal allele.

Family 2: Identifying the Most Prevalent Mutation: Table 2 presents the remarkable Nanodrop values that demonstrate the exceptional quality of the extracted genomic DNA samples. In Family 2, the ARMS PCR analysis revealed that both parents carried the most common mutation, IVS-I-5. The affected child (IV: 2) and the fetus (IV: 1) shown in Figure 3 were identified to be homozygous for the mutated allele depicted in Figure 4, indicating a heightened risk for β-thalassemia. These findings underscore the crucial significance of early detection and precise prenatal diagnosis in managing β-thalassemia in Pakistan.

Table 2: DNA Quantification of Samples Using Nanodrop

#	DNA Source	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)
1	Mother	167.9	ng/µl	3.358	1.854
2	Father	563.2	ng/µl	11.264	6.126
3	Child	617.8	ng/µl	12.358	6.742
4	CVS	914.0	ng/µl	18.280	9.775
5	Sick child (proband)	703.0	ng/µl	14.060	7.534

Note: This table 2 presents the DNA quantification results of five samples from Family 2, as measured using a Nanodrop spectrophotometer.

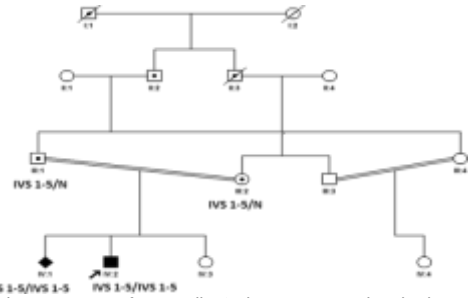

Figure 3: The genogram for Family 2 demonstrates that both parents are heterozygous carriers of the IVS-I-5 mutation. The affected child and fetus both exhibit a homozygous genotype for this mutation.

Genotyping Results:

Figure 4: The figure presents the findings of the IVS-I-5 mutation in family 2 with thalassemia. Control samples in lanes 1-7 consist of homozygous, heterozygous, and negative control DNA leaders, followed by blood samples in lanes 8-11. These samples represent the effected chorionic villus sampling (CVS), effected Sick child (SC), mother, father, and the top row of bands represents mutant alleles, while the bottom row indicates normal alleles. The affected child and CVS both exhibit homozygous IVS-I-5 mutation, whereas the parents carry heterozygous alleles as carriers.

Family 3: High-Quality Genomic DNA Samples Unveil Key Insights. The extracted genomic DNA samples displayed exceptional quality, as demonstrated by the outstanding Nanodrop values presented in Table 3. We genotyped three blood samples (III:1, III:2, and IV:1) (Figure 5) and one chorionic villus sample (IV:2) from the family β-thalassemia-3 (Figure 6) for β-thalassemia mutations. Notably, this family does not have a proband.

Table 3: DNA Quantification of Family 3 Samples Using Nanodrop

#	DNA Source	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)
1	Mother	824.1	ng/µl	16.482	8.925
2	Father	678.4	ng/µl	13.568	7.395
3	Sick child (SC)	321.9	ng/µl	6.438	3.498
4	CVS	234.7	ng/µl	4.694	2.552

Note: This table presents the DNA quantification results of five samples from Family 3, as measured using a Nanodrop spectrophotometer

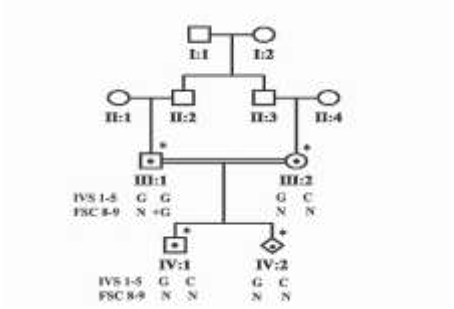

Figure 5: Genogram illustrating the autosomal recessive inheritance pattern of β-thalassemia-3 is presented. The symbol for chorionic villus sampling (CVS) is represented by a diamond shape in the pedigree. Both children in the pedigree are carriers or have the trait for the IVS-I-5 mutation.

Genotyping Results: A

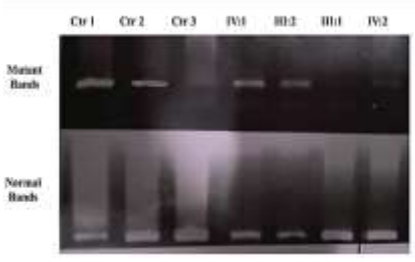

Figure 6: displays Electropherogram "A", demonstrating the IVS-I-5 mutation in the thalassemia-3 family. Control samples are shown in lanes 1-3, with two heterozygous samples and one negative control. Lanes 4-7 display samples for the normal child (sibling), mother, father, and chorionic villus sampling (CVS), respectively.

Genotyping Results: B

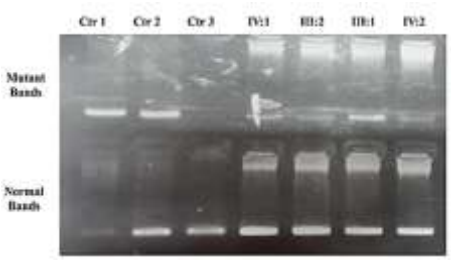

Figure 7: depicts Electropherogram "B" demonstrating the FSC 8-9 mutation in the same family. Homozygous, heterozygous, and negative control samples are displayed in lanes 1-3, respectively. Lanes 4-7 exhibit samples for the normal child (sibling), mother, father, and chorionic villus sampling (CVS), respectively.

Table 4: Genotyping Results Summary for three families

Family ID	Diagnostic Approach	Paternal Genotype (M: F)	Fetal Phenotype	Pregnancy Outcomes
Family 1	ARMS PCR	M-619 bp/N: F -619 bp/N	-619 bp/N	Carrier
Family 2	ARMS PCR	M IVS-I-5/N: F IVS-I-5/N	IVS-I-5/IVS-I-5	Affected
Family 3	ARMS PCR	M IVS-I-5/N: F FSC 8-9/N	IVS-I-5/N	Carrier

Note: This table provides a summary of the genotyping results for three families, including diagnostic approaches, parental genotypes, fetal phenotypes, and pregnancy outcomes.

DISCUSSION

At the beginning of our discussion as we embark on this in-depth exploration of Table 4, we find a detailed summary of genotyping results for three unique families, offering valuable insights into diagnostic approaches, parental genotypes (M: F), fetal phenotypes, and pregnancy outcomes.

The first family underwent diagnostic testing using the ARMS PCR method. The paternal genotypes were found to be M-619 bp/N and F-619 bp/N, indicating that both parents carried a specific genetic variation. The fetal phenotype also revealed the presence of the -619 bp/N genotype. As a result, the pregnancy outcome for Family 1 was classified as a carrier, meaning the child would carry the genetic variation but would not display symptoms of the condition.

In the case of Family 2, the diagnostic approach employed was also ARMS PCR. The paternal genotypes were identified as M IVS-I-5/N and F IVS-I-5/N, signifying that both parents carried a different genetic variation compared to Family 1. The fetal phenotype displayed the genotype IVS-I-5/IVS-I-5, which indicates that the child inherited the genetic variation from both parents. Consequently, the pregnancy outcome for Family 2 was categorized as affected, meaning the child would most likely exhibit symptoms of the genetic condition.

Finally, Family 3 was also examined using the ARMS PCR diagnostic approach. The paternal genotypes for this family were M IVS-I-5/N and F FSC 8-9/N, with each parent carrying a distinct genetic variation. The fetal phenotype of IVS-I-5/N implied that the child inherited the genetic variation from one parent only.

Hereditary genetic disorders, particularly autosomal recessive diseases, contribute to numerous premature deaths and chronic health problems (28). β-thalassemia, a prime example, is the most prevalent autosomal recessive monogenic disorder globally and in Pakistan (29). The high incidence in Pakistan is primarily due to widespread consanguineous marriages (~60%), influenced by a rigid caste system and concerns over property distribution among offspring (30).

To address the significant health issue of thalassemia in Pakistan, carrier screening and prenatal testing have been established. The screening process involves analyzing the five most commonly reported mutations(31), namely IVS-I-5 (c. 92 + 5

G > C), FSC 8-9 (c. 27_28insG), FSC 41-42 (c.124_127delTTCT), IVS-I-1 (c. 92 + 1 G > T), and -619 bp. Notably, IVS-I-5 (c. 92 + 5 G > C) and FSC 8-9 (c. 27_28insG) are the most prevalent mutations in southern Pakistan (52.2%) and northern Pakistan (41.3%), respectively(32).

Today we discuss a vital study on thalassemia in Pakistan, a genetic disorder that affects countless families. Our research focused on carrier screening and prenatal testing in three affected families, identifying prevalent mutations like IVS-I-5, FSC 8-9 and -619 bp. By using Chorionic Villus Sampling (CVS), we further highlighted the importance of accessible prenatal diagnosis to reduce affected births and the need for genetic linkage analysis, mutational analysis, and prenatal screening(33).

With a staggering consanguinity rate of 70%, Pakistan faces increased risks for thalassemia and other genetic disorders. Our study, which concentrated on β-thalassemia families in Minar Multan city of Pakistan, is part of a broader effort across the country to better understand and manage these genetic diseases. We used Chorionic Villus Sampling (CVS) to identify heterozygosity for thalassemia mutations, showing homozygosity for both normal and mutant alleles. Although CVS and amniotic fluid sampling are invasive, they pose a low risk of fetal injury or death (34). Researchers are working on less invasive techniques to detect and genotype cell-free nucleic acids in maternal circulation (35), (36). These methods, however, face technical and ethical challenges and are not yet commercially available.

In Pakistan, parental screening is limited, but the National Institute for Biotechnology and Genetic Engineering (NIBGE) offers this service to at-risk couples. Regrettably, many thalassemia patients don't survive past adolescence due to limited access to chelation and transfusions, as well as unsafe transfusion practices (37). Developing innovative, minimally invasive methods for detecting cell-free DNA in maternal blood is a crucial research area.

Our research aims to reduce β-thalassemia incidence in Pakistan by enhancing our understanding of the disorder and promoting improved management practices (38). We focused on families with recessively inherited β-thalassemia and used the rapid and reliable Amplification Refractory Mutation System (ARMS) PCR diagnostic method (33) to identify common mutations in the Pakistani population.

Despite the importance of prenatal diagnosis and carrier screening, challenges persist in raising public awareness and providing basic healthcare facilities. To reduce affected births, accessible prenatal diagnosis is crucial (39). Hospitals and clinics should offer free carrier screening and prenatal diagnosis, and public awareness campaigns should stress the risks of consanguineous unions (40)

Our findings emphasize the importance of genetic linkage analysis, mutational analysis, and prenatal screening. Expanding the mutational database for carrier screening within Pakistani families will help identify at-risk individuals and pave the way for DNA-based treatment strategies, prenatal screening, and genetic counseling(41).

In the coming years, we must tackle the challenges of understanding disease mechanisms, gene involvement, and preventing inborn errors. Advancements in genomics will make it easier to analyze individual genomic DNA and detect connections between diseases and genetic variations, leading to fewer affected births. To achieve this, we must conduct population-based studies in countries like Pakistan.

Key initiatives to improve the management of hereditary genetic disorders like β-thalassemia in Pakistan include:

- Genetic education and awareness
- Accessible and affordable genetic testing services
- Collaboration with international organizations
- Integration of genetic counseling into healthcare services

CONCLUSION

In conclusion, this study sheds light on genotyping results for three unique families, revealing the intricate connections between parental genotypes, fetal phenotypes, and pregnancy outcomes. Utilizing the ARMS PCR diagnostic method, the research identified varying genetic variations and inheritance patterns within the families.

These findings emphasize the significance of genetic testing in predicting pregnancy outcomes and evaluating the risk of inheriting genetic conditions. This knowledge allows healthcare providers to better guide and support families during crucial decision-making processes, ultimately improving health outcomes and quality of life for both affected individuals and their families. As genetic testing technology advances, it is essential to continue investigating and exploring how these diagnostic tools can contribute to personalized medicine and preventative healthcare strategies.

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