Dissertation on

"POLYMORPHISM OF EXON 2 IN HBB GENE IN BETA THALESSEMIA MAJOR CHILDREN IN NORTH KARNATAKA."

By

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B.L.D.E (DEEMED TO BE UNIVERSITY)

VIJAYAPURA KARNATAKA

In Partial fulfilment of requirements for the degree of

DOCTOR OF MEDICINE

In

PAEDIATRICS

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ABBREVIATIONS

HBB	Haemoglobin beta gene HBA Adult Haemoglobin
HBF	Foetal Haemoglobin
GT	Guanine, thiamine
AG	Adenine, Guanine
HBH	Haemoglobin H
PIH	Pyridoxal iso nicotynol hydrazine
HBED	Hydroxy benzyl ethylenediamine
HCT	Hematopoietic cell transplantation
GVHD	Graft versus host disease
JAK2	Janus kinase 2
PCR	Polymerase chain reaction
IVS	Intervening sequence
G>C	Guanine to Cytosine
T>G	Thymine to Guanine
T>C	Thymine to Cytosine
G>A	Guanine to Adenine
T**>G**	Thymine to Guanine
β –T.M	Beta thalassemia major
FRC	Frame shift codons
TDT	Transfusion dependent thalassemia
NTDT	non-transfusion dependent thalassemia
ARMS	Amplification Refractory Mutation System
RFLP	Restriction Fragment Length Polymorphism
MCV	Mean corpuscular volume
MCHB	Mean Corpuscular Haemoglobin
UTR	Untranslated region
ETBR	Ethium bromide

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INTRODUCTION

Beta thalassemia major is a severe genetic blood disorder caused by mutations in the HBB (beta-globin) gene, leading to defective haemoglobin production, chronic anaemia, and severe complications such as growth retardation, splenomegaly, and iron overload due to frequent blood transfusions. It is an autosomal recessive disorder that predominantly affects populations with high rates of consanguinity, including regions like North Karnataka. The management of beta thalassemia major involves lifelong blood transfusions, iron chelation therapy, and, in some cases, hematopoietic stem cell transplantation (HSCT). Despite these therapeutic advancements, a better understanding of the genetic mutations and polymorphisms associated with the disease can help improve treatment strategies, prognosis, and genetic counselling.[1]

Beta thalassemia is caused by a wide range of mutations in the HBB gene, located on chromosome 11, that disrupt beta-globin chain synthesis. These mutations are highly heterogeneous and vary across different ethnic and geographical populations. While some mutations result in the complete absence (β^0) of beta-globin production, others lead to a partial reduction (β^+) in its synthesis. In India, more than 30 common mutations have been reported in beta thalassemia patients, with IVS1-5(G>C), IVS1-1(G>T), and 619-bp deletion being among the most prevalent.[2] However, apart from these major mutations, single nucleotide polymorphisms (SNPs) and variations in specific regions such as exon 2 of the HBB gene may also influence the severity and clinical manifestations of the disease.

Polymorphisms in the HBB gene, particularly in exon 2, have been of growing interest in recent research. Exon 2 encodes a critical segment of the beta-globin polypeptide chain, and variations in this region may affect haemoglobin function, disease severity, and treatment response. Some polymorphisms might lead to modifications in haemoglobin stability,

alterations in oxygen-binding properties, or enhanced residual beta-globin production, which can, in turn, impact the clinical phenotype of beta thalassemia major. Understanding these polymorphisms can provide insights into genotype-phenotype correlations and potential therapeutic targets.[3]

The prevalence of beta thalassemia in India is estimated to be 3-4%, making it a significant public health concern. In North Karnataka, where consanguineous marriages are common, the incidence is notably high. Studying exon 2 polymorphisms in the HBB gene among beta thalassemia major children in North Karnataka is crucial for several reasons. Firstly, it helps in identifying unique genetic variations specific to this region, which may differ from other parts of India and the world. Secondly, it provides insights into the correlation between these polymorphisms and disease severity, which could guide personalized treatment approaches. Thirdly, such studies are valuable for genetic counselling, carrier screening, and prenatal diagnosis, all of which are essential in reducing the disease burden.[4]

Polymorphisms in exon 2 can impact several clinical parameters in beta thalassemia major patients. Some variations may partially compensate for beta-globin deficiency, resulting in a milder phenotype, while others may increase haemolysis and worsen anaemia. These genetic differences can influence the frequency of blood transfusions, the severity of iron overload, and even the response to iron chelation therapy. Moreover, certain polymorphisms may have epistatic interactions with foetal haemoglobin (HbF)-modulating genes, such as BCL11A and XmnI polymorphism, which are known to ameliorate disease severity by increasing HbF levels. Therefore, characterizing these polymorphisms is essential for predicting disease progression and optimizing patient management strategies.[5]

Advances in molecular genetic techniques have significantly improved our ability to detect polymorphisms in the HBB gene. Techniques such as polymerase chain reaction (PCR),

Sanger sequencing, next-generation sequencing (NGS), and multiplex ARMS-PCR (Amplification Refractory Mutation System-PCR) allow for the precise identification of genetic variations. [6]These diagnostic tools enable early detection of polymorphisms, better risk assessment, and targeted genetic screening programs, which are crucial for preventive strategies.

The study of exon 2 polymorphisms in beta thalassemia major has important public health implications. As beta thalassemia is a preventable disorder, genetic studies can support carrier screening programs, premarital counselling, and prenatal diagnosis, thereby reducing the incidence of affected births. [7]In North Karnataka, implementing routine HBB gene mutation analysis in high-risk populations can help in identifying carriers and providing genetic counselling to families at risk. Furthermore, public awareness campaigns focusing on the consequences of consanguineous marriages and the importance of genetic screening can play a key role in prevention.

Beyond prevention, further research on HBB gene polymorphisms could lead to novel therapeutic approaches. The discovery of polymorphisms that modulate disease severity may pave the way for precision medicine strategies, including the use of HbF inducers, targeted gene therapy, and genome editing techniques such as CRISPR-Cas9. If certain exon 2 polymorphisms are found to increase residual beta-globin production, these variants could be potential candidates for gene-based therapeutic interventions in the future.[8]

Beta thalassemia major remains a major health concern in North Karnataka, where genetic diversity and high rates of consanguinity contribute to its prevalence. Studying polymorphisms in exon 2 of the HBB gene is crucial for understanding the molecular mechanisms underlying disease variability, improving genetic counselling, and optimizing treatment strategies.[9]Advances in molecular diagnostics have enabled precise detection of these polymorphisms, aiding in early diagnosis and personalized medicine approaches.

Furthermore, public health initiatives focusing on carrier screening, prenatal diagnosis, and community education can significantly reduce the burden of the disease. Research in this area will not only enhance our understanding of beta thalassemia genetics but also contribute to better patient care, targeted therapies, and improved health outcomes for affected individuals in North Karnataka and beyond.

AIM AND OBJECTIVES

Aim of Study.

To establish a database highlighting prevailing mutations of beta gene in the population of North Karnataka.

Objectives of study.

To identify and characterize polymorphisms in exon 2 of the HBB gene in children diagnosed with beta thalassemia major in the North Karnataka region.

To determine the prevalence and types of mutations, including missense and synonymous variants, within exon 2 of the HBB gene among transfusion-dependent beta thalassemia patients.

REVIEW OF LITERATURE

2.1. Introduction to Beta Thalassemia Major

Beta thalassemia major (Cooley's anaemia) is a severe autosomal recessive haemoglobin disorder caused by mutations in the HBB gene on chromosome 11. These mutations lead to defective or absent production of β-globin chains, resulting in an imbalance between α- and β-globin synthesis. The excess α-globin chains form toxic precipitates, leading to ineffective erythropoiesis, haemolysis, and severe anaemia. This disorder requires lifelong blood transfusions and iron chelation therapy, significantly impacting the patient's quality of life. Beta thalassemia major is a significant global health concern, particularly in regions where malaria has historically been endemic. The persistence of beta thalassemia genes in these populations is due to the heterozygote advantage, where carriers (beta thalassemia minor) are relatively protected against severe Plasmodium falciparum malaria.[9] With increasing global migration, beta thalassemia major is now encountered worldwide, necessitating comprehensive screening programs and advanced treatment strategies.

Epidemiology

Beta thalassemia is most prevalent in regions of high malaria endemicity, particularly:[10]

- The Mediterranean (Greece, Italy, Cyprus)
- The Middle East (Iran, Saudi Arabia, Lebanon)
- The Indian subcontinent (India, Pakistan, Bangladesh)
- Southeast Asia (Thailand, Indonesia, Malaysia, China)
- North and West Africa Carrier Rates:[11]
- Mediterranean: 5–20%
- Middle East & Indian subcontinent: 2–15%
- Southeast Asia: 2–10%

With increased population migration, beta thalassemia is now found in North America, Europe, and Australia, leading to the establishment of newborn screening programs and prenatal genetic counselling.

Pathophysiology

Normal Haemoglobin Synthesis

Adult haemoglobin (HbA) consists of:[12]

- Two α-globin chains (encoded by genes on chromosome 16)
- Two β-globin chains (encoded by the HBB gene on chromosome 11)

In beta thalassemia major, the defective HBB gene prevents the normal production of β-globin chains, leading to an excess of unpaired α-globin chains. These chains form insoluble aggregates, causing:

- Ineffective erythropoiesis:
    - Apoptosis of erythroid precursors in the bone marrow ○ Increased erythropoietin levels, leading to compensatory bone marrow hyperplasia
- Haemolysis:
    - Red blood cells (RBCs) with α-globin precipitates are destroyed in the spleen ○ Leads to severe anaemia, extramedullary haematopoiesis, and splenomegaly
- Iron Overload:
    - Chronic transfusions lead to iron accumulation in the liver, heart, and endocrine organs ○ Causes cirrhosis, cardiomyopathy, diabetes, and hypogonadism

Clinical Features

Beta thalassemia major manifests within the first year of life, usually between 3 to 6 months[13] as foetal haemoglobin (HbF) levels decline.

Key Clinical Manifestations

- Severe Microcytic Hypochromic Anaemia
    - Causes pallor, irritability, and failure to thrive
    - Requires lifelong transfusions
- Hepatosplenomegaly
- Due to extramedullary haematopoiesis and increased RBC destruction
- Leads to early satiety, abdominal distension, and hypersplenism
- Skeletal Deformities ○ Chronic bone marrow expansion causes:
    - Frontal bossing (prominent forehead)
    - Maxillary hyperplasia (chipmunk facies)
    - Widening of the diploic spaces in the skull (crew-cut appearance on Xray)
- Growth Retardation & Delayed Puberty
- Due to chronic anaemia, endocrine dysfunction, and iron overload
- Iron Overload Complications (from repeated transfusions)
- Cardiac: Cardiomyopathy, heart failure
- Endocrine: Diabetes mellitus, hypothyroidism, hypogonadism
- Hepatic: Liver fibrosis, cirrhosis

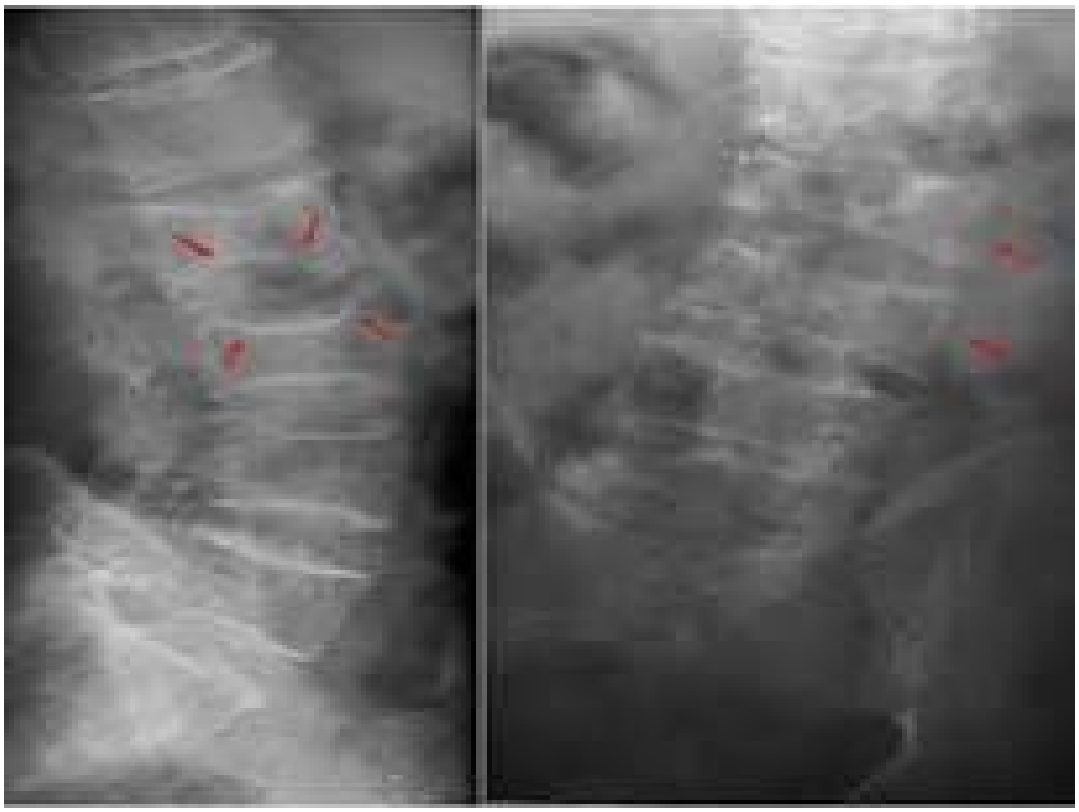


IMAGE1[14]: An image showing skeletal changes in beta thalassemia major, such as skull Xray with “crew-cut” appearance.

Diagnosis

Laboratory Findings

- Complete Blood Count (CBC):
    - Severe microcytic hypochromic anemia (Hb <7 g/dL)
    - MCV <70 fL, MCH <27 pg
    - Reticulocytosis
- Peripheral Blood Smear:
- Target cells, nucleated RBCs, basophilic stippling
- Hemoglobin Electrophoresis:
    - Absent HbA, elevated HbF (>90%) and HbA2
- Genetic Testing:
    - Confirms mutations in the HBB gene

- Serum Ferritin & MRI T2: ○ Assess iron overload
- Bone Marrow Examination:
    - Shows erythroid hyperplasia and ineffective erythropoiesis

Management

Blood Transfusion Therapy

- Regular transfusions (every 2–4 weeks) to maintain Hb >9–10 g/dL
- Prevents skeletal deformities and compensatory marrow expansion

Iron Chelation Therapy

- To prevent iron overload from transfusions
- Chelating agents:
    - Deferoxamine (parenteral) ○ Deferasirox, Deferiprone (oral)

Splenectomy

- Indicated in cases of massive splenomegaly causing hypersplenism

Bone Marrow Transplant (BMT)

- Only curative treatment
- Best results in children with HLA-matched sibling donors

Gene Therapy (Emerging Treatment)

- Lenti Globin BB305 gene therapy: Experimental trials show promising results

Prognosis & Future Directions

With lifelong transfusion therapy and iron chelation, survival has significantly improved, with patients living into their 40s and 50s. Advances in stem cell transplantation, gene editing (CRISPR), and foetal haemoglobin induction therapies offer hope for a potential cure in the future.

Prevention Strategies

- Carrier screening programs (Hb electrophoresis, genetic testing)

- Prenatal diagnosis via chorionic villus sampling (CVS) or amniocentesis
- Preimplantation genetic diagnosis (PGD) for affected couples

Beta thalassemia major is a life-threatening hereditary disorder that requires early diagnosis and multidisciplinary management. Advances in blood transfusions, iron chelation, stem cell transplantation, and gene therapy have improved survival and quality of life. However, prevention through genetic counselling and prenatal diagnosis remains crucial in high prevalence areas.

2.2. Genetics of Beta Thalassemia

Beta thalassemia is a monogenic disorder caused by mutations in the HBB gene located on chromosome 11. These mutations result in either reduced (β+) or absent (β0) β-globin chain synthesis, leading to imbalanced haemoglobin production, ineffective erythropoiesis, and severe anaemia. The inheritance follows an autosomal recessive pattern, meaning that individuals with two mutated alleles develop beta thalassemia major, whereas heterozygous carriers (one normal and one mutant allele) present with beta thalassemia minor (clinically asymptomatic or mild anaemia).

Understanding the molecular genetics of beta thalassemia is essential for genetic counselling, prenatal diagnosis, and potential gene therapy approaches. This section provides a detailed overview of the genetic basis of beta thalassemia, including mutation types, inheritance patterns, and their clinical significance.

The HBB Gene and Hemoglobin Synthesis

Location and Structure of the HBB Gene

The HBB gene is located on the short arm of chromosome 11 (11p15.4) and is part of the betaglobin gene cluster, which also contains genes encoding delta (δ), gamma (Gγ and Aγ), and epsilon (ε) globins. These genes are sequentially activated during embryonic, fetal, and adult

life.[15]

The beta-globin gene cluster includes:

- HBE (ε-globin gene) – active during embryonic life
- HBG1 and HBG2 (γ-globin genes, producing foetal haemoglobin HbF)
- HBD (δ-globin gene, producing HbA2 in adults)
- HBB (β-globin gene, producing HbA in adults)

Regulation of Beta-Globin Gene Expression

The expression of the HBB gene is controlled by a regulatory sequence called the locus control region (LCR), located upstream of the gene cluster. The LCR enhances gene transcription at different developmental stages by interacting with specific transcription factors. Disruptions in these regulatory regions can lead to beta thalassemia by reducing β-globin expression.[16]

Types of Beta Thalassemia Mutations

More than 300 mutations have been identified in the HBB gene, affecting different aspects of β-globin synthesis. These mutations are classified into:

Point Mutations

Point mutations are the most common cause of beta thalassemia and can affect transcription, splicing, or translation.

- Promoter Region Mutations: Affect the binding of transcription factors, reducing β-globin gene expression (e.g., -28 A→G mutation).
- Splice Site Mutations: Affect RNA processing, leading to abnormal β-globin mRNA (e.g., IVS-1-110 G→A mutation).
- Nonsense Mutations: Introduce a premature stop codon, resulting in truncated β-globin (e.g., Codon 39 C→T).
- Missense Mutations: Alter β-globin function but usually lead to milder forms.[17]

Small Insertions or Deletions

- Frameshift mutations caused by small insertions or deletions lead to nonfunctional βglobin production. Example: Codon 8/9 +G, which adds an extra nucleotide, disrupting translation.[18]

Large Deletions

- Rare cases of beta thalassemia are caused by large deletions affecting the HBB gene or LCR region. These mutations often lead to Hereditary Persistence of Foetal Haemoglobin (HPFH), where HbF remains elevated into adulthood, partially compensating for the β-globin deficiency.[19]

Classification Based on Mutation Type[20]

Beta thalassemia mutations are categorized based on their effect on β-globin chain synthesis:

- β0 mutations (Complete Absence of β-globin Production)
- Homozygous or compound heterozygous β0 mutations lead to beta thalassemia major.
  - Examples: Codon 39 nonsense (CAG→UAG), IVS-I-1 G→A mutation.
- β+ mutations (Reduced β-globin Production)
- Results in beta thalassemia intermedia or minor, depending on the severity of the mutation.
  - Examples: IVS-I-110 G→A, -28 A→G mutations in the promoter region.
- β++ mutations (Mild Reduction in β-globin Synthesis)
- Often clinically asymptomatic or very mild anemia.

  Example: Some silent mutations in intronic regions.

Inheritance Patterns and Carrier States

Autosomal Recessive Inheritance

Beta thalassemia follows an autosomal recessive inheritance pattern:

- Heterozygous (β/β+ or β/β0) → Beta Thalassemia Minor (Carrier State)
- Homozygous (β0/β0 or β+/β+) → Beta Thalassemia Major (Severe Form)
- Compound Heterozygous (β0/β+) → Beta Thalassemia Intermedia (Variable Severity)

Carrier Screening and Genetic Counselling

Carrier screening is essential in populations with high beta thalassemia prevalence. Genetic counselling helps in:

- Identifying heterozygous carriers (Hb electrophoresis, genetic testing).
- Offering prenatal diagnosis (CVS, amniocentesis) for high-risk couples.
- Providing reproductive options like Preimplantation Genetic Diagnosis (PGD).[20]

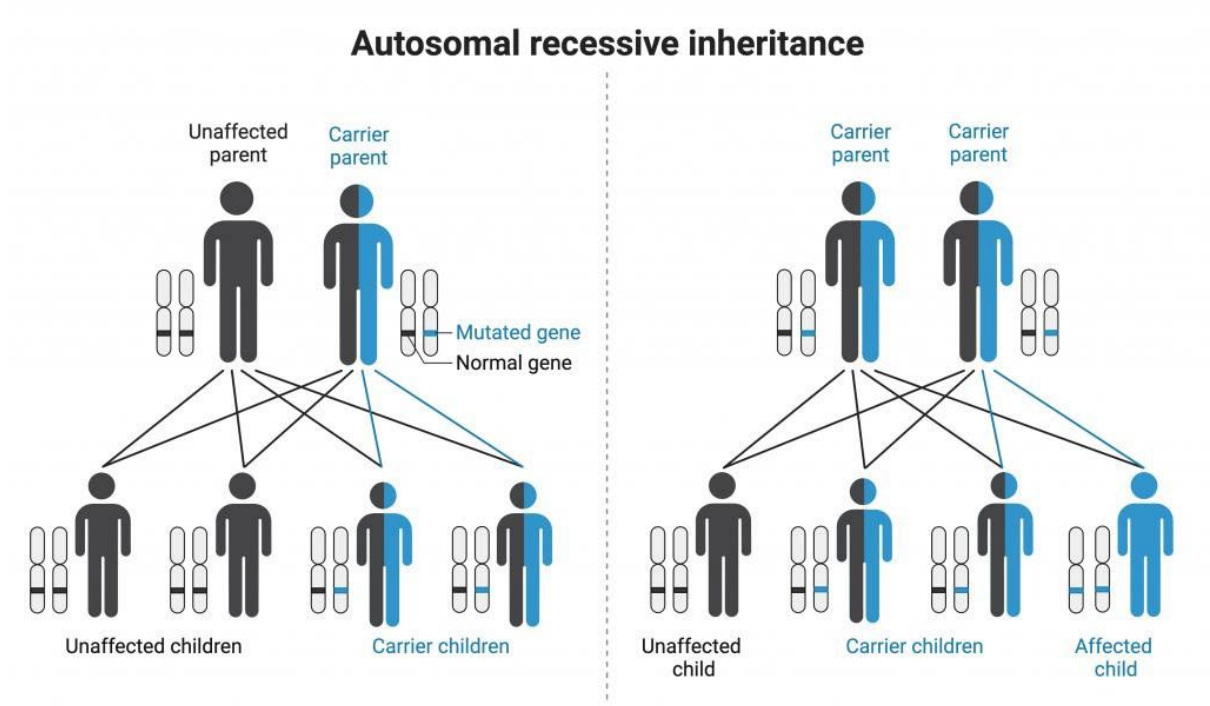


IMAGE2[20]: A pedigree showing autosomal recessive inheritance in a family.

Genotype-Phenotype Correlation

The clinical severity of beta thalassemia depends on the type of mutation:

- β0/β0: Most severe (thalassemia major), requiring lifelong transfusions.
- β+/β+ or β0/β+: Variable severity (thalassemia intermedia).
- β/β+ or β/β0: Usually asymptomatic or mild anemia (thalassemia minor).

Additionally, co-inheritance of alpha-thalassemia or HPFH mutations can modify disease severity.[21]

Future Perspectives: Gene Therapy and CRISPR

Gene therapy is a promising treatment approach for beta thalassemia:

- Gene Addition Therapy: Using lentiviral vectors to introduce a functional HBB gene.
- Gene Editing (CRISPR-Cas9): Corrects mutations in HBB or reactivates HbF expression by modifying the BCL11A gene.
- Induced Pluripotent Stem Cells (iPSCs): Potential for personalized stem cell therapy.

Recent trials using Lenti Globin BB305 have shown successful β-globin gene correction, reducing transfusion dependence.

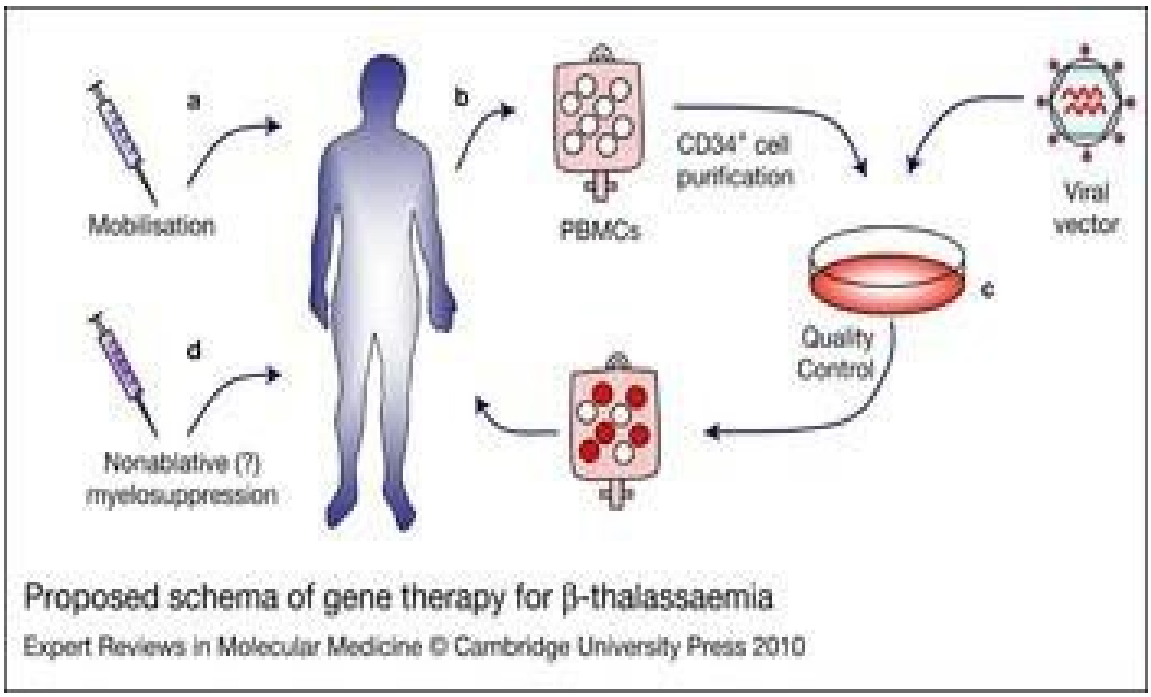


IMAGE4[22]: An image illustrating the mechanism of gene therapy in beta thalassemia.

The genetic basis of beta thalassemia is highly diverse, with over 300 mutations affecting HBB gene transcription, splicing, and translation. Understanding these mutations is critical for diagnosis, genetic counselling, and emerging gene therapies. Advances in CRISPR-based gene

editing and lentiviral therapy provide hope for a definitive cure, transforming the management of this lifelong disorder.

2.3. Polymorphisms in the HBB Gene

Polymorphisms in the HBB gene (β-globin gene) refer to naturally occurring variations in the DNA sequence that do not necessarily cause disease but may influence haemoglobin function, disease susceptibility, and phenotypic variability in beta thalassemia and other hemoglobinopathies.[23]

These single nucleotide polymorphisms (SNPs) and other genetic variations can modify the severity of beta thalassemia, sickle cell disease, and hereditary persistence of foetal haemoglobin (HPFH). Understanding these polymorphisms is critical for predicting disease outcomes, designing personalized therapies, and exploring gene-editing strategies.

This article explores different polymorphic sites in the HBB gene, their impact on gene regulation, and their clinical significance in beta thalassemia and other hemoglobinopathies.

The HBB Gene and Its Polymorphic Sites

The HBB gene is located on the short arm of chromosome 11 (11p15.4) and spans approximately 1.6 kb within the beta-globin gene cluster. This cluster also includes epsilon (ε), gamma (Gγ and Aγ), and delta (δ) globin genes, all of which are tightly regulated during embryonic, foetal, and adult life.

Polymorphic Regions in the HBB Gene

Polymorphisms in the HBB gene can occur in:

- Promoter regions – Affecting gene transcription
- Intronic regions – Modifying RNA splicing efficiency
- Exonic regions – Silent mutations or synonymous variations
- Untranslated regions (UTRs) – Regulating mRNA stability and translation
- Intergenic regions – Affecting locus control and long-range regulatory interactions[24]

Key Polymorphisms in the HBB Gene

5' Promoter Region Polymorphisms

The 5' promoter region contains regulatory elements essential for HBB gene transcription. Polymorphisms in this region can modulate transcription factor binding, affecting β-globin expression.

- −88 C>T (rs34500389):
    - Alters the binding of transcription factors. ○ Associated with mild beta thalassemia (β+ phenotype).
- −29 A>G (rs33931752):
    - Found in some populations with β-thalassemia intermedia.
    - Reduces β-globin synthesis but does not completely abolish it.[25]

Intron Polymorphisms and Their Effects on Splicing

Intronic polymorphisms can influence mRNA splicing efficiency, potentially leading to aberrant transcripts.

- IVS-II-16 G>C (rs1800730)
- Alters splicing efficiency but does not cause a severe thalassaemic phenotype.
- IVS-I-6 T>C (rs63750969)
- Affects alternative splicing and may modify the clinical severity of beta thalassemia.

Exonic Polymorphisms (Silent and Missense Mutations)

Polymorphisms in exons can be silent (synonymous) or missense (non-synonymous).

- Codon 2 T>C (rs34598529)
- A silent mutation that does not affect β-globin function.
- Codon 26 G>A (HbE mutation, rs33950507)
    - Produces haemoglobin E (HbE), a structurally abnormal haemoglobin variant.

- Common in Southeast Asia, where it interacts with β-thalassemia to cause HbE/β-thalassemia.[26]

3' Untranslated Region (3' UTR) Polymorphisms and mRNA Stability

The 3' UTR plays a crucial role in mRNA stability and translation efficiency. Some polymorphisms in this region may modulate β-globin expression, altering clinical severity.

- +90 C>T (rs7480526)
- Associated with increased foetal haemoglobin (HbF) production, which may ameliorate beta thalassemia severity.
- +120 G>A (rs777605276)
- Regulates mRNA degradation rates, affecting haemoglobin synthesis.

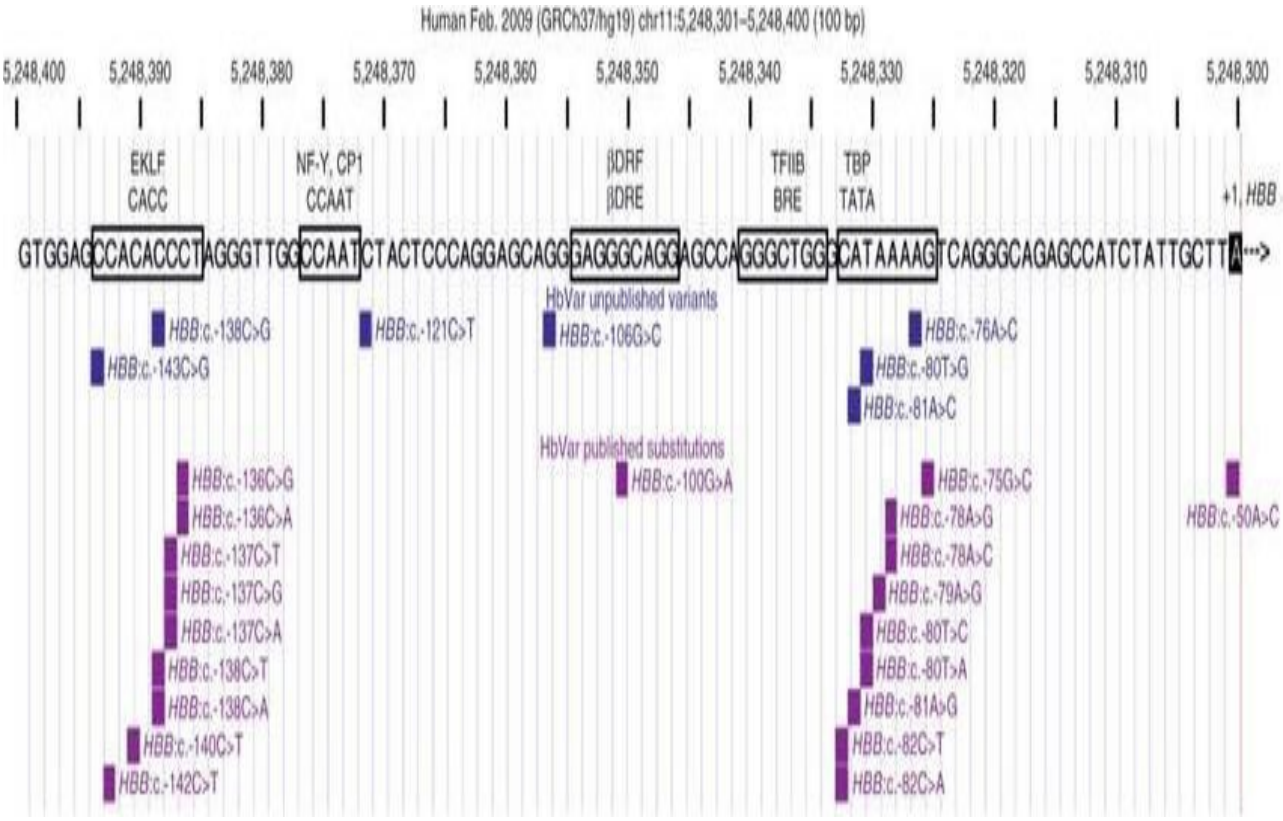


IMAGE5[27]: **The HBB Gene and Its Polymorphic Sites**

Influence of HBB Polymorphisms on Beta Thalassemia Severity

Polymorphisms Affecting Foetal Haemoglobin (HbF) Levels

Certain polymorphisms in the HBB gene and surrounding regions influence HbF levels, which can compensate for β-globin deficiency in beta thalassemia.

- XmnI-Gγ C>T (rs7482144, −158 C>T) [28]
- Located upstream of the Gγ-globin gene. ○ Increases HbF production, leading to a milder beta thalassemia phenotype.
    - Frequently observed in beta thalassemia intermedia and sickle cell disease.
- BCL11A and HBS1L-MYB Polymorphisms [29]
- Although not within the HBB gene, these polymorphisms modulate HbF expression, influencing disease severity.

Polymorphisms and Disease Modifiers in Beta Thalassemia

Some polymorphisms interact with other genetic factors, modifying disease outcomes:

- HBB haplotypes (linked SNPs) influence β-thalassemia severity.
- Co-inheritance of α-thalassemia with β-thalassemia modifies the phenotypic expression, leading to milder symptoms.

Polymorphisms in HBB and Haemoglobin Variants

Haemoglobin S (HbS) – Sickle Cell Disease

- Codon 6 A>T (rs334, Glu6Val mutation)
- Causes HbS, leading to sickle cell disease (SCD).
  Modifies red cell deformability, causing Vaso-occlusion.

Haemoglobin C (HbC) Variant

- Codon 6 G>A (rs33930165, Glu6Lys mutation)
- Produces HbC, which interacts with HbS to cause HbSC disease.

Haemoglobin E (HbE) Variant

- Codon 26 G>A (rs33950507, Glu26Lys mutation) o Highly prevalent in Southeast Asia. o Causes HbE/β-thalassemia, a major hemoglobinopathy.

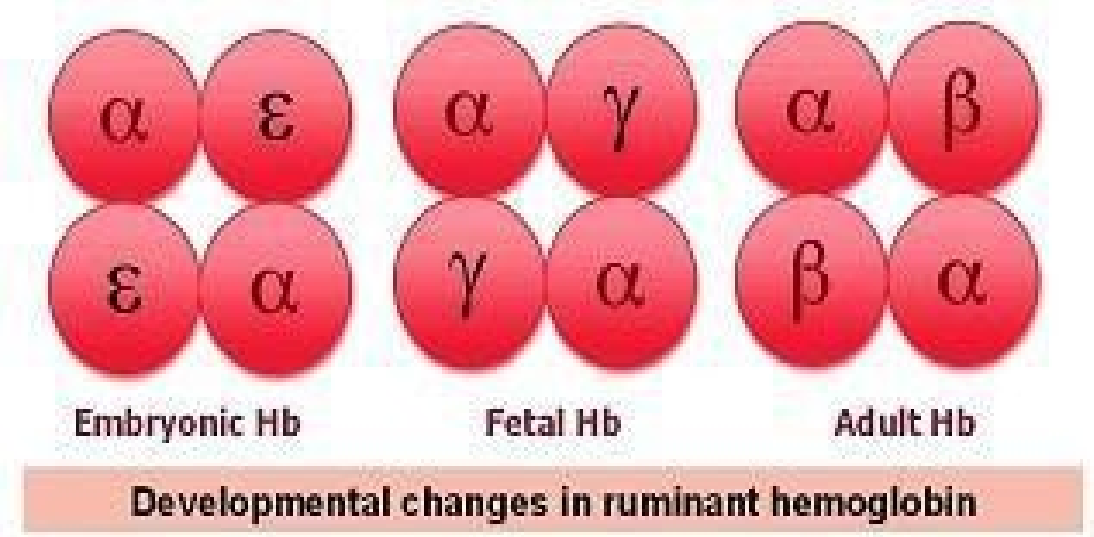


IMAGE6[30]:An image comparing different haemoglobin variants caused by HBB polymorphisms.

Clinical Applications of HBB Polymorphisms

Genetic Screening and Prenatal Diagnosis

- SNP-based carrier screening helps detect mild and severe thalassemia mutations.
- Prenatal diagnosis (CVS, amniocentesis) detects disease-causing polymorphisms.

Pharmacogenomics and Personalized Medicine

- Polymorphism-guided therapy helps optimize hydroxyurea treatment in sickle cell disease and thalassemia.
- SNPs in HBB and regulatory genes predict HbF response to therapy.

Gene Therapy and Genome Editing

- CRISPR-based strategies target HbF-inducing polymorphic loci (e.g., BCL11A,

- HBS1L-MYB).

Lentiviral-mediated β-globin gene correction is influenced by existing polymorphisms.[31]

Polymorphisms in the HBB gene significantly influence beta thalassemia severity, haemoglobin variants, and therapeutic responses. Understanding these variations aids in disease prediction, genetic counselling, and novel treatment approaches. Future research in genome editing and precision medicine will further optimize beta thalassemia management based on individual genetic backgrounds.

2.4. Genetic Variability in Beta Thalassemia

Beta thalassemia is a monogenic disorder caused by mutations in the HBB gene on chromosome 11 (11p15.4), leading to reduced (β+) or absent (β0) β-globin chain synthesis. Despite being a single-gene disorder, genetic variability in beta thalassemia arises due to a wide range of mutations, modifier genes, polymorphisms, and environmental interactions, leading to diverse clinical phenotypes.

This genetic heterogeneity influences:

- Disease severity (thalassemia major, intermedia, minor)
- Response to treatment (e.g., hydroxyurea, transfusions)
- Compensatory mechanisms (elevated foetal haemoglobin levels)

Understanding genetic variability is crucial for precision medicine, genetic counselling, and novel therapeutic approaches such as gene therapy and genome editing.

Molecular Basis of Genetic Variability

The HBB Gene and Its Mutations

The HBB gene consists of three exons and two introns, encoding the beta-globin chain of haemoglobin.

Mutations in the HBB gene lead to:

- Complete absence of β-globin (β0 mutations)

  Reduced β-globin production (β+ mutations)

Types of Mutations in Beta Thalassemia[32]

More than 300 mutations have been identified, classified into:

Point Mutations

- Promoter mutations (affecting transcription efficiency)
- Example: −28 A>G (reduces β-globin production)
- Splice site mutations (altering RNA processing)
- Example: IVS-I-110 G>A (leads to aberrant mRNA)
- Nonsense mutations (introducing premature stop codons)
- Example: Codon 39 C>T (β0 mutation)
- Missense mutations (altering amino acid sequence)
- Example: HbE mutation (Codon 26 G>A)

Insertions and Deletions

- Frameshift mutations leading to nonfunctional β-globin
- Example: Codon 8/9 +G (β0 mutation)
- Large deletions affecting the HBB gene or its regulatory elements
- Example: Hispanic deletion (removes the β-globin locus)

Genotype-Phenotype Correlation

The combination of β-thalassemia mutations (homozygous, heterozygous, compound heterozygous) determines the clinical severity:

Genotype	β-Globin Production	Clinical Presentation
β/β+	Mildly reduced	Thalassemia minor

Genotype	β-Globin Production	Clinical Presentation
β0/β0	Absent	Severe thalassemia major
β0/β+	Severely reduced	Thalassemia intermedia/major
β+/β+	Moderately reduced	Thalassemia intermedia

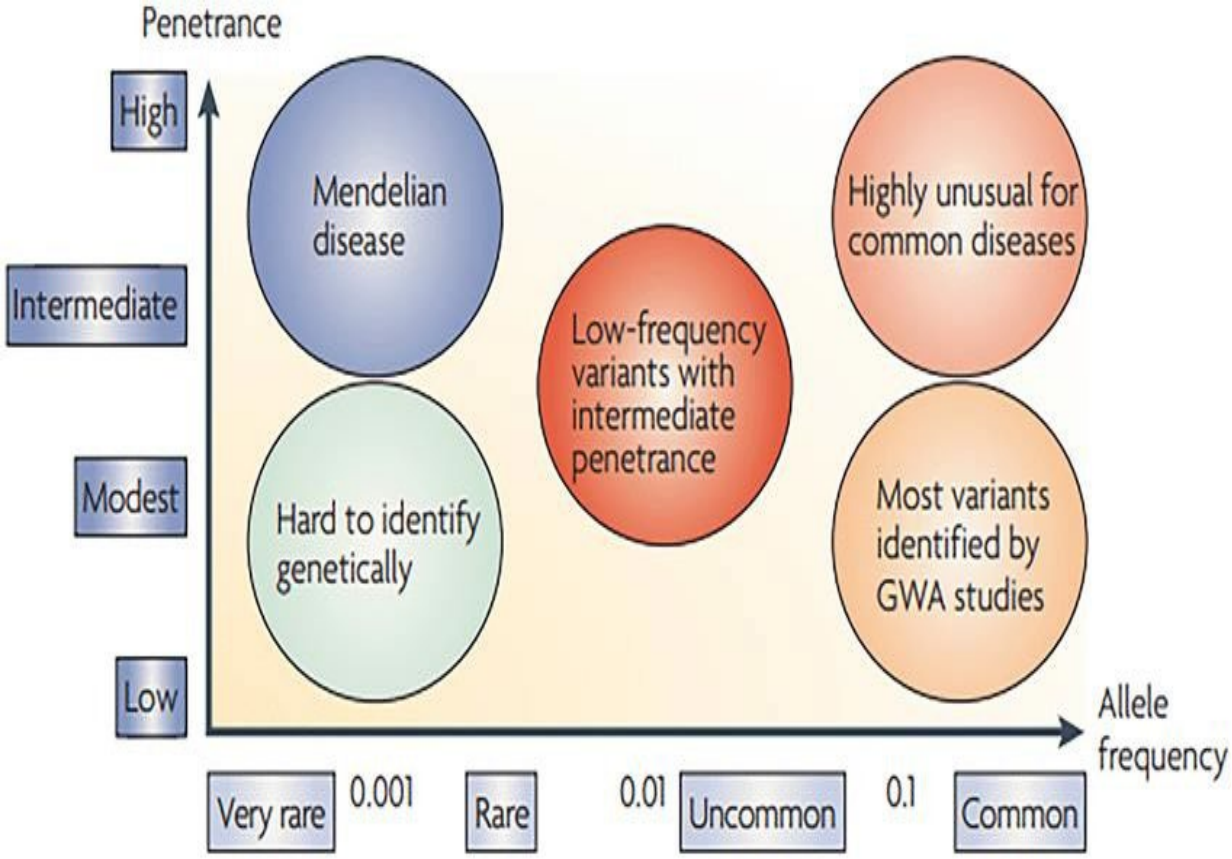


IMAGE7:[33]Role of Modifier Genes in Beta Thalassemia

Apart from HBB gene mutations, modifier genes influence disease expression by affecting:

- Foetal haemoglobin (HbF) levels (compensates for β-globin deficiency)
- Iron metabolism and erythropoiesis

Foetal Haemoglobin (HbF) Modifiers

Increased HbF levels reduce disease severity by compensating for β-globin deficiency. Key

genetic loci regulating HbF include:

- HBS1L-MYB (6q23) – Enhances γ-globin expression
- BCL11A (2p16) – A repressor of HbF (mutations in this gene increase HbF)
- XmnI-Gγ (−158 C>T, rs7482144) – Associated with elevated HbF in β-thalassemia intermedia[34]

Iron Metabolism and Erythropoiesis Modifiers

- TMPRSS6 (regulates hepcidin) – Influences iron overload risk
- HFE (C282Y and H63D mutations) – Affects iron absorption in β-thalassemia patients[35]

Phenotypic Variability in Beta Thalassemia

Beta Thalassemia Major (Severe Form)

- Genotype: β0/β0 or severe β+/β0
- Pathophysiology: Severe anemia, ineffective erythropoiesis, transfusion dependence
- Clinical Features: Growth retardation, bone deformities, iron overload

Beta Thalassemia Intermedia (Moderate Form)

- Genotype: β+/β+ or β0/β+ with compensatory HbF
- Pathophysiology: Moderate anemia, variable transfusion needs
- Modifiers: XmnI-Gγ, BCL11A, and HBS1L-MYB polymorphisms

Beta Thalassemia Minor (Mild Form, Carrier State)

- Genotype: β/β+ or β/β0
- Pathophysiology: Mild anemia, usually asymptomatic
- Clinical Features: Microcytosis, mild hypochromia, normal life expectancy

	CLINICAL FEATURES	LABORATORY FEATURES
THALASSEMIA MAJOR	▪ Anemia ▪ Hepatosplenomegaly ▪ Growth failure	▪ Hb : < 7 g/dL ▪ HbF : > 90% ▪ HbA2: normal or high ▪ HbA : usually absent
THALASSEMIA INTERMEDIA	▪ Milder anemia ▪ Thalassemia facies ▪ Hepatosplenomegaly	▪ Hb : < 8-10 g/dL ▪ HbF : > 10% ▪ HbA2: 4-9%, if > 10% suggests HbE ▪ HbA : 5-90%
β THALASSEMIA TRAIT	▪ Normal to mild anemia ▪ No organomegaly	▪ Hb : < 10 g/dL ▪ MCH : < 27 pg ▪ HbF : > 2.5-5% ▪ HbA2: 4-9%, if >20% suggests HbE trait ▪ HbA : > 90%

IMAGE8 [36]: comparing the three clinical forms of beta thalassemia.

Population-Specific Genetic Variability

Population	Common Mutations
Mediterranean	IVS-I-110 G>A, Codon 39 C>T
Southeast Asia	HbE (Codon 26 G>A), IVS-II-654 C>T
Indian Subcontinent	IVS-I-5 G>C, Codon 15 G>A
Middle East	IVS-I-1 G>A, Codon 8/9 +G

TABLE1[37]: The prevalence and mutation spectrum of beta thalassemia vary by ethnicity:

Genetic Testing and Precision Medicine

Diagnostic Techniques

- Haemoglobin electrophoresis – Identifies HbA2 and HbF levels
- Molecular testing (PCR, Sanger sequencing, NGS) – Identifies HBB mutations
- Prenatal diagnosis (CVS, amniocentesis, PGD) – Detects mutations in high-risk couples

Personalized Medicine and Future Therapies

- Gene therapy (Lenti Globin BB305, CRISPR-Cas9) – Corrects defective HBB gene
- HbF induction therapy (hydroxyurea, gene silencing of BCL11A) – Increases γ-globin expression
- Iron chelation therapy (Deferasirox, Deferiprone) – Prevents iron overload

The genetic variability in beta thalassemia arises from mutations in the HBB gene, modifier genes, and environmental factors. Understanding these variations helps in accurate diagnosis, prognosis, and personalized treatment approaches. Emerging therapies like gene therapy and genome editing offer hope for a definitive cure, transforming beta thalassemia management.

2.5. Impact of Exon 2 Polymorphisms on Clinical Outcomes in Beta Thalassemia Beta thalassemia is caused by mutations in the HBB gene (chromosome 11, 11p15.4), affecting β-globin chain production. While the primary mutations determine the severity of anaemia, polymorphisms in exon 2 of the HBB gene can significantly impact clinical outcomes.[38]

Exon 2 polymorphisms influence:

- Disease severity (thalassemia major vs. intermedia)
- Haemoglobin levels and erythropoiesis
- Response to treatments (transfusion dependence, hydroxyurea therapy)
- HbF levels (foetal haemoglobin compensatory mechanism)

Understanding the molecular effects of exon 2 polymorphisms is crucial for personalized medicine, prognostic assessments, and therapeutic strategies.

Exon 2 of the HBB Gene: Structural and Functional Importance

Structure of Exon 2

- Exon 2 of the HBB gene encodes a crucial portion of the β-globin chain, forming part of the haemoglobin tetramer.
- It spans codons 31 to 104 and contains essential residues for oxygen binding and haemoglobin stability.

Role in β-Globin Function

- Mutations or polymorphisms in exon 2 can alter mRNA processing, translation efficiency, and β-globin stability.
- Pathogenic mutations in exon 2 (e.g., HbE mutation, missense variants) can lead to β-thalassemia intermedia or major.

Key Polymorphisms in Exon 2 of the HBB Gene

Several single nucleotide polymorphisms (SNPs) and mutations in exon 2 influence beta thalassemia outcomes.

Haemoglobin E (HbE) Variant – Codon 26 (G>A)[39]

- Mutation: GAG → AAG (Glutamic acid → Lysine)
- Effect:
    - Causes abnormal β-globin mRNA splicing, leading to decreased β-globin production.
    - Common in Southeast Asian populations.
- Clinical Impact:
    - HbE/β-thalassemia compound heterozygosity results in moderate-to-severe anaemia.

- Increased HbF levels provide some compensation.

Codon 39 (C>T) Nonsense Mutation

- Mutation: CAG → UAG (Glutamine → Stop codon)
- Effect:
  - Premature termination of β-globin synthesis, leading to a β0 (null) mutation.
- Clinical Impact:
  - Associated with severe β-thalassemia major requiring lifelong transfusions.
  - Patients exhibit severe anemia and ineffective erythropoiesis.

Codon 27 (G>T) Mutation – Hb Knossos Variant

- Mutation: GAG → GTG (Glutamic acid → Valine)
- Effect:
  - Reduces β-globin chain production, leading to mild β+ thalassemia.
- Clinical Impact:
  - Individuals with Hb Knossos/β-thalassemia often have mild anemia with higher HbF levels.
  - Less transfusion-dependent compared to other mutations.

Codon 8/9 (+G) Frameshift Mutation

- Mutation: Insertion of guanine (G) in exon 2
- Effect:
  - Causes a frameshift, leading to a nonfunctional β-globin chain.
- Clinical Impact:
  - Severe β0-thalassemia phenotype with high transfusion requirements.

Clinical Implications of Exon 2 Polymorphisms[40]

Influence on Disease Severity

- β0/β0 mutations (e.g., codon 39 C>T, codon 8/9 +G) → Severe thalassemia major, requiring regular transfusions.
- β+/β+ or β0/β+ mutations (e.g., HbE, Hb Knossos) → Milder phenotypes (thalassemia intermedia), often less transfusion-dependent.

Impact on Foetal Haemoglobin (HbF) Levels

- Some exon 2 variants (e.g., HbE, Hb Knossos) increase HbF expression, improving oxygen transport.
- Higher HbF levels correlate with milder symptoms, reducing transfusion needs.

Treatment Response Variability

- Hydroxyurea therapy (induces HbF production) shows better response in patients with exon 2 polymorphisms linked to higher HbF (e.g., HbE).
- Gene therapy success rates depend on the underlying mutation type in exon 2. Population-Specific Variability in Exon 2 Polymorphisms

Population	Common Exon 2 Polymorphisms	Clinical Relevance
Southeast Asia	HbE (codon 26 G>A)	HbE/β-thalassemia common
Mediterranean	Codon 39 (C>T), Codon 8/9 +G	Severe β0-thalassemia
Middle East	Hb Knossos (Codon 27 G>T)	Milder β+ phenotype

TABLE2[41]: The prevalence of exon 2 polymorphisms varies across ethnic groups:

Diagnostic Approaches for Exon 2 Polymorphisms

PCR-Based Methods

- ARMS-PCR for common exon 2 mutations (e.g., codon 39 C>T, HbE)
- Gap-PCR for frameshift mutations (e.g., Codon 8/9 +G) Sanger Sequencing
- Gold standard for confirming point mutations in exon 2.

Next-Generation Sequencing (NGS)

- Detects rare polymorphisms and deep intronic variants.

Future Perspectives in Research and Therapy

Gene Therapy for Exon 2 Mutations

- Lenti Globin BB305 gene therapy corrects β0/β+ exon 2 mutations.
- CRISPR-based gene editing targeting exon 2 is under clinical trials.

AI in Predicting Disease Severity

- Machine learning models analyse HBB exon 2 mutations to predict transfusion dependency.[42]

Relevant studies

Here is a detailed compilation of relevant studies on polymorphisms of exon 2 in the HBB gene in beta-thalassemia major children, presented in individual paragraphs:

1. Kulkarni et al. (2011) aimed to identify rare mutations in the β-globin gene among βthalassemia patients in Karnataka, India. They conducted genetic sequencing of 36 clinically diagnosed patients using polymerase chain reaction (PCR) and sequencing techniques. The study identified 11 β-thalassemia variants, including IVSII-16 G>C, IVSI-5G>C, IVSII-74 T>G, codon 3 (T>C), and Poly A site (T>C). Additionally, a novel deletion at codon 6 (-CT) (HBB:c.16delCT) was reported. These findings highlighted the regional diversity in β-thalassemia mutations.[43]
2. Chauhan et al. (2022) reported a rare case of a β-thalassemia major patient with a compound heterozygous condition. The study involved direct sequencing of the βglobin gene in a 4-year-old male patient. The results revealed four different mutations: CD 3 (T>C), CD 41/42 (-CTTT), IVS II-16 (G>C), and IVS II-666 (C>T). This case emphasized the complexity of mutation interactions in β-thalassemia patients and the need for precise genetic diagnosis.[44]
3. Patel et al. (2017) analyzed β-thalassemia mutations in the Gujarati population of India. Genetic screening was conducted using PCR and sequencing methods. The study detected a high prevalence of exon 2 mutations, including codon 19 (A>G) and codon 27 (G>T). These mutations were linked to severe clinical outcomes, emphasizing the necessity of newborn screening programs in high-risk regions.[45]
4. Yasmeen et al. (2020) assessed the spectrum of HBB gene variants and their association with major endocrine complications in thalassemia patients in Pakistan. The study performed genetic analysis on thalassemia patients and found multiple mutations in exon 2, which correlated with severe endocrine dysfunctions such as hypothyroidism and diabetes mellitus. The results underscored the need for genetic testing in comprehensive thalassemia management.[46]
5. Viprakasit et al. (2016) focused on HBB gene polymorphisms in Southeast Asian populations. Through high-throughput sequencing, the study identified novel exon 2 mutations that modified disease severity. The research suggested that genetic modifiers play a crucial role in determining clinical outcomes in β-thalassemia major.[47]
6. Alaithan et al. (2018) conducted a review on β-globin gene variations in Saudi Arabian patients with β-thalassemia. The study compiled data from multiple sources and

reported that exon 2 mutations, particularly codon 8 (-AA) and codon 30 (G>C), were highly prevalent. The research provided insights into population-specific mutation distributions, aiding in region-based genetic counselling.[48]

7. Kountouris et al. (2021) provided a narrative review of the molecular genetics of βthalassemia. The review summarized over 350 known HBB gene mutations, with exon 2 being a hotspot for many pathogenic variants. This study reinforced the significance of molecular screening in thalassemia prevention programs worldwide.[49]
8. Selvaraj et al. (2022) characterized β-thalassemia mutations in heterozygous individuals from South India. The study screened over 5,000 individuals for βthalassemia using high-performance liquid chromatography (HPLC) and genetic sequencing. The findings revealed 30 different mutations, including five rare exon 2 mutations that were reported for the first time in India.[50]
9. Ahmed et al. (2010) explored the clinical implications of exon 2 mutations in βthalassemia patients from Bangladesh. The study reported a high frequency of codon 16 (G>A) mutations, which were associated with severe anemia and poor response to hydroxyurea therapy. The findings suggested that mutation screening could guide personalized treatment approaches.[51]
10. Verma et al. (2005) conducted a molecular study on β-thalassemia mutations in North India. The study identified exon 2 mutations in 24% of cases, with codon 17 (A>T) being the most prevalent. The research contributed to the development of molecular diagnostic protocols for β-thalassemia screening.[52]
11. Sinha et al. (2003) performed an early genetic analysis of β-thalassemia in India. The study established a foundation for understanding exon 2 mutations and their clinical relevance. The results supported the implementation of genetic counselling and prenatal diagnostic programs[53]
12. Suhaimi. (2022) conducted a large-scale study on β-thalassemia mutations in India using next-generation sequencing. The study analyzed 1,530 cases and identified 48 pathogenic variants in the HBB gene. The most prevalent mutations were IVS-I-5 (G>C) and codon 41/42 (-CTTT). This study provided a comprehensive mutation database, which is crucial for genetic counselling and screening programs.[54]

MATERIAL &METHODOLOGY

Source of Data:

All children and adolescents enrolled in the thalassemia clinic at Shri B. M. Patil Medical College will be included in this study.

Type of Study:

Cross-sectional study (Mutation analysis study)

Inclusion Criteria:

Children and adolescents diagnosed with beta-thalassemia major, aged between 6 months and 18 years, who are registered in the paediatric department of Shri B. M. Patil Medical College and Hospital will be included in this study.

Exclusion Criteria:

Individuals diagnosed with other hemoglobinopathies, will be excluded.

Duration of Study: April 2023 to November 2024

Method of Data Collection (Including Sampling Procedures): After obtaining written informed consent from the parents and ensuring eligibility based on inclusion and exclusion criteria, children will be enrolled in the study.

Clinical Sample (Blood) Collection: written informed consent was obtained from the children, who were enrolled in the study.

After taking consent, 1 ml peripheral blood samples were collected in the EDTA-coated vacutainers and stored at 4°C until further use.

Isolation of Genomic DNA and Quantification: From 300µl of peripheral blood genomic DNA was isolated, with the help of a commercial DNA isolation kit (Bangalore Genei, India).

Brief Genomic DNA Isolation Protocol:

1. In a 1.5 ml EDTA-coated vial 300 µl of peripheral blood was collected.
2. By adding1 ml of 1 X solution A (provided by the kit) RBC cells were lysed.
3. At room temperature the vials were centrifuged for 5 min at 8000 RPM.
4. Until a clear white WBC pellet was obtained the above step was repeated.
5. 600µl of solution B was added (provided by the kit) to the WBC and mixed gently for clear lysis.
6. It was centrifuged at room temperature for 10 min at 10,000 RPM.
7. The Supernatant was collected and 0.9 ml absolute cold ethanol was added to it and mixed.
8. Centrifuged at 4ºCfor 20 min, at 10,000 RPM.
9. Precipitate DNA was washed with 0.5 ml of 75% ethanol.
10. Centrifuged for 5 min at 10,000 RPM.
11. 100 µl of solution C was added (provided by the kit) after air drying the DNA pellet.
12. The vial was incubated at 55ºC for 10 min.
13. To remove any insoluble materials, it was centrifuged at 10,000 RPM for 2 min.
14. The DNA thus obtained was stored at -20ºC until further use.

Quantification of Genomic DNA

The quality of the isolated DNA was checked under gel electrophoresis. 100 ml of 1% agarose gel was prepared (1 gm of Agarose + 100 ml of 1X TAE buffer). We used Tecon multimode reader for the quantification of genomic DNA. Tecon multimode reader is a

micro-volume UV spectrophotometer specifically designed for the measurement of nucleic acids and purified proteins. Its unique technology holds 0.5-2.5 ul samples between upper and lower measurement surfaces without the use of a cuvette. Tecon multimode reader measures the samples in less than 2 seconds with a high degree of accuracy and reproducibility. The Tecon multimode reader works on the principle, "Nucleic acids absorb light at a wavelength of 260 nm and when 260 nm light source shines on a sample, the amount of light that passes through the sample can be measured, and the amount of light absorbed by the sample can be inferred. For double stranded DNA, an Optical Density (OD) of 1 at 260 nm correlates to a DNA concentration of 50 ng/µl, so that DNA concentration can be easily calculated from OD measurements" as shown in Table no. 3

Table 3: Quantification of Thalassemia Samples

Sl. No. of DNA samples	OD at 260/280	Concentration in ηg/µl
1	1.86	54
2	1.75	65
3	1.40	44
4	1.90	70
5	1.57	136
6	1.98	64
7	1.84	82
8	1.92	73
9	1.65	68
10	1.79	111
11	1.85	64
12	1.81	66
13	1.75	53
14	2.02	65
15	2.15	82
16	1.51	94
17	1.88	49

18	2.09	39
19	1.93	46
20	2.04	100
21	2.6	51.5
22	2.35	85.5
23	1.96	73.9
24	3.05	57
25	2.01	81
26	2.24	125
27	2.09	137
28	1.76	104
29	1.96	92
30	1.58	93
31	1.81	53
32	1.72	66
33	1.63	42
34	1.69	68
35	1.75	126
36	1.71	66
37	1.65	73
38	2.02	63
39	2.25	76
40	1.41	101
41	1.58	64
42	2.10	56
43	1.73	53
44	1.63	55
45	1.65	72
46	1.30	65
47	1.70	56

Figure 1. Agarose gel image of genomic DNA of thalassemia children samples

Primer designing: The web-based freely available program "Primer3" which is widely accepted was used, (http://frodo.wi.mit.edu/ primer3/ input. Html) for designing PCR primers. Primer 3 is a Bioinformatics tool that helps in designing the primers for the target region in the given nucleotide sequence as per the requirement of the user or applications. The designed primers using Primer 3 were reconfirmed for the specificity of its binding site using the web-based bioinformatics tool "Genome Build 36" (https://genome.ucsc.edu/FAQ/FAQreleases.html), and for its Insilco amplification on "Insilco PCR" (http://insilico.ehu.es/PCR/). All the designed primers for our target genes or region are tabulated in table No. 1 along with the annealing temperature and amplicon size. Primers were got synthesized by a commercial oligo synthesizer (MWG Biotech, India).

Table 4. Details of the primer sequences and annealing temperatures used for the amplification of exon 2 of HBB gene.

Primer Name	**Primer Details**	**Melting Temp**	**Size of the Exon**
Th2f	TTC CCA CCC TTA GGC TGC TGG T	63.5^0C	183
Th2R	TGG CAA AGG TGC CCT TGA GGT		

Polymerase Chain Reaction (PCR): PCR amplification was carried out in a 20µl reaction volume containing 0.5 µl of genomic DNA (75ng/µl to 150 ng/µl), 0.5µl of each primer (5pmol), 0.4µl of dNTP (10pmol), 0.2µl Taq DNA polymerases (3units/ µl), 4 µlTaq Buffer (5X) (BioRad, USA) and the total volume was adjusted to 20µl using molecular biology grade water. Amplification was carried out in Master cycler gradient (Eppendorf, Germany) under the following conditions: an initial denaturation at 98^0C for 10sec, followed by 35 cycles at 98^0C for 10sec (cycle denaturation). The primer annealing temperature was set depending on the annealing temperature of the primer (Table-1) for 10sec 72 ^{0}C for 15sec (primer extension) and a final extension at 72^0C for 5 min. PCR products were confirmed for their respective amplicon size by gel electrophoresis with a standard 100bp ladder. The PCR cycling conditions were as follows Initial Denaturation is for 98^0C for10 sec, Denaturation is 980C for 10 sec, Annealing is primer dependent for 10 sec, Elongation 72^0C for 5min & Hold at 40^0C

Agarose Gel Electrophoresis of PCR Products:

Gel electrophoresis is one of the molecular biology techniques used to separate DNA and RNA depending on the length of fragments. It is a widely used and accepted method, to estimate the size of DNA and RNA fragments or to separate proteins by charge. Nucleic acid molecules are separated based on an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving.

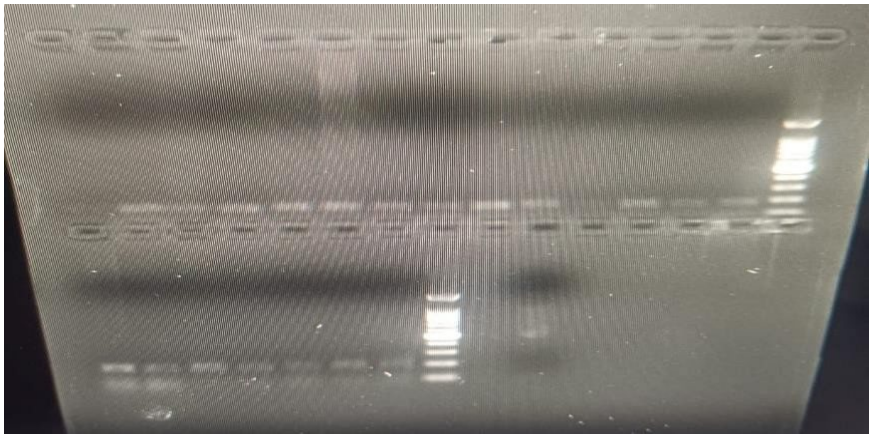


Figure 4: Agarose gel electrophoresis image of amplified products of exon2 of HBB gene. Lane No; 11-30 thalassemia samples, M: 100bp marker

DNA Sequencing (Capillary Based)

PCR products were subjected for capillary based Big-Dye terminator sequencing. Prior to sequencing, the PCR products were subjected to cycle sequencing and plate processing

Cycle Sequencing

As per the Sanger Sequencing protocol, Big-Dye labelling and chain termination were carried out by the cycle sequencing method. To label each base, the PCR amplicon was subjected to a cycle sequencing reaction with a single primer. Big-Dye TM terminator v3.1was used for cycle sequencing (Applied Biosystems, USA) following the manufacturer's guidelines. Cycle sequencing of the PCR products was carried out according to the annealing temperature of the primers.

Table 5. Standardised master mix conditions for sequencing

SL.No.	**Constituents**	**Quantity**
1	Molecular Biology grade water	6.3 µL
2	Big Dye Buffer (5X)	1.3 µL
3	Big Dye	1.0 µL
4	Template (PCR product)	1.0 µL
5	Forward Primer	0.2 µL
6	Reverse Primer	0.2 µL
Total		**10 µL**

Note: Only one of the primers i.e either forward or reverse primer was used during cycle sequencing

Table 6. The cycle sequencing conditions

Process	**Temperature (°C)**	**Time**
Initial. Denaturation	98	10sec

Denaturation	98	10sec
Annealing	Primer Dependent	10sec
Elongation	72	5min
Hold	4	

Note: The annealing temperature is primer dependant and varies for each primer

Sequencing Clean-up (Plate Processing)

To remove the unbounded florescent DNTPs from the terminator sequencing reaction, 2µl of 3M sodium acetate, and 50µl of 100% ethyl alcohol were added to each sample and incubated at room temperature for 15 minutes to precipitate the DNA. The samples were centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatant was discarded and the reaction plate was centrifuged in a reverse manner at 300 rpm for 20 seconds. 100µl of 75% alcohol was added to each sample and centrifuged at 4000rpm for15 minutes at 25°C. The supernatant was discarded and the plate was centrifuged in a reverse manner at 300 rpm for 20 seconds to remove the alcohol completely. The plate was dried at room temperature until the last drop of alcohol dripped off

10µl of Hi-Di Formamide was added to each well of the sample plate. The samples were heated to 96°C for 5 minutes and immediately cooled to 4°C to denature and linearise the cycle sequencing products. The processed products were loaded in the sequencer for sequencing.

Sequencing Run

Sample information sheets which contain analysis protocols along with the sample details were prepared and imported into the data collection software. Prepared samples were analyzed on ABI 3730 genetic analyzer (Applied Biosystems, USA) to generate DNA sequences or electropherograms. After completion of the sequencing reaction, the quality of generated sequence was checked by using Sequencing Analysis v5.4software (Applied Biosystems, USA)

Sequence Alignment

The generated sequences were aligned to their respective reference sequences with the use of Variant reporter software (ABI v1.1). The variant reporter is one of the compatible software of Applied Biosystems designed for automated sequence data analysis. It performs sequence comparisons for novel mutations, known variants, insertions, and deletions. It allows analysis

of the sequenced data, comparing the consensus sequences to a known reference sequence. The results of the variant reporter were tabulated in PDF format as the default program of the software

Here, we used this technique to check the isolated genomic DNA from whole blood. EtBr stain was used to stain the DNA fragments. In all the 47 thalassemia samples as shown in figure 1 and 2 confirmed the presence of genomic DNA and the same samples were taken for quantification based on Nanodrop.

Data Analysis:

Determination of Sample Size (n): With an anticipated proportion of CODON 3 beta thalassemia of 19%, a sample size of 50 patients is required at a 95% confidence level and 10% absolute precision. **Formula Used:** $n = z^2 p * q / d^2$ Where:

- Z = Z statistic at α level of significance
- d = Absolute error
- P = Proportion rate
- q = 100 – p

Statistical Analysis:

- Data will be entered into a Microsoft Excel sheet and analyzed using Statistical Package for the Social Sciences (SPSS) Version 20.
- Results will be presented as mean ± standard deviation (SD), median and interquartile range, frequencies, percentages, and diagrams

OBSERVATIONS AND RESULTS

Mutation analysis:

This study was done at the Department of Paediatrics, B.L.D.E (Deemed to be University), Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka where thalassemia patients were being transfused. Mutational analysis of HBB gene was done in the Genetic research lab, Department of Anatomy.

50 transfusion-dependent thalassemia patients were included in the present study for the mutational analysis of hbb gene. All 50 patients were found to be known cases of Thalassemia major.

Sequencing of Exon 2 in the Hbb gene was done in 47 Thalassemia patients, out of 50 samples we have identified the mutation **g.5401 G>T** in 21 samples which is heterozygous missense variant. we have identified the mutation **g.5378 G>A** in 17 samples which is heterozygous synonymous variant. Here, we present an evaluation of the results of our study

Table 7: TOTAL NUMBER OF SAMPLES: 47

Gene: HBB

Genome RefSeq: NG_059281.1

Sl. No	Sample ID	Mutation identified
1.	1_HBB1	-
2.	2_HBB2	No mutations
3.	3_HBB2	No mutations
4.	4_HBB2	No mutations
5.	5_HBB2	g.5378 G>A
6.	6_HBB2	g.5378 G>A

7.	7_HBB2	No mutations
8.	8_HBB2	g.5401 G>T
9.	9_HBB2	No mutations
10.	10_HBB2	No mutations
11.	11_HBB2	g.5401 G>T, g.5378 G>A
12.	12_HBB2	g.5378 G>A
13.	13_HBB2	g.5378 G>A
14.	14_HBB2	g.5401 G>T, g.5378 G>A
15.	15_HBB2	g.5401 G>T, g.5378 G>A
16.	16_HBB2	g.5401 G>T
17.	17_HBB2	g.5378 G>A
18.	18_HBB2	g.5378 G>A, g.5401 G>T
19.	19_HBB2	No mutations
20.	20_HBB2	g.5378 G>A
21.	21_HBB2	g.5378 G>A, g.5401 G>T
22.	22_HBB2	g.5378 G>A, g.5401 G>T
23.	23_HBB2	---
24.	24_HBB2	---
25.	25_HBB2	---
26.	26_HBB2	----
27.	27_HBB2	g.5401 G>T
28.	28_HBB2	No mutation

29.	29_HBB2	Sample invalid
30.	30_HBB2	g.5401 G>T
31.	31_HBB2	g.5401 G>T, g.5378 G>A
32.	32_HBB2	g.5401 G>T, g.5378 G>A
33.	33_HBB2	g.5401 G>T, g.5378 G>A
34.	34_HBB2	----
35.	35_HBB2	Sample invalid
36.	36_HBB2	No mutation
37.	37_HBB2	g.5401 G>T
38.	38_HBB2	No mutation
39.	39_HBB2	g.5401 G>T, g.5378 G>A
40.	40_HBB2	g.5401 G>T, g.5378 G>A
41.	41_HBB2	No mutation
42.	42_HBB2	-----
43.	43_HBB2	No mutation
44.	44_HBB2	g.5401 G>T
45.	45_HBB2	g.5401 G>T
46.	46_HBB2	g.5401 G>T
47.	47_HBB2	Sample invalid
48.	48_HBB2	g.5401 G>T
49.	49_HBB2	g.5401 G>T, g.5378 G>A
50.	50_HBB2	Sample invalid

Variant details:

Sl. No	gDNA position	cDNA position	aa position	Status	Variant type	Condition
1	g.5401 G>T	c.218 G>T	p.S73I	NA	Missense	Heterozygous
2	g.5378 G>A	c.198 G>A	P.A66A	NA	Synonymous	Heterozygous

Out of 50 samples we have identified the mutation **g.5401 G>T** in 21 samples which is heterozygous missense variant. The chromatogram shows a very short peak due to low quality of the samples, but mutation can be considered as it is observed in more number of samples (cannot be considered as misread). The identified mutation does not match with the recorded variant in bdSNP, Ensembl tool. To confirm the variant and to know the impact of the mutation functional validation using SIFT and PolyPhen is required.

Out of 50 samples we have identified the mutation **g.5378 G>A** in 17 samples which is heterozygous synonymous variant. The chromatogram shows a short peak due to low quality of the samples, but mutation can be considered as it is observed in more number of samples (cannot be considered as misread). The identified mutation does not match with the recorded variant in bdSNP, Ensembl tool.

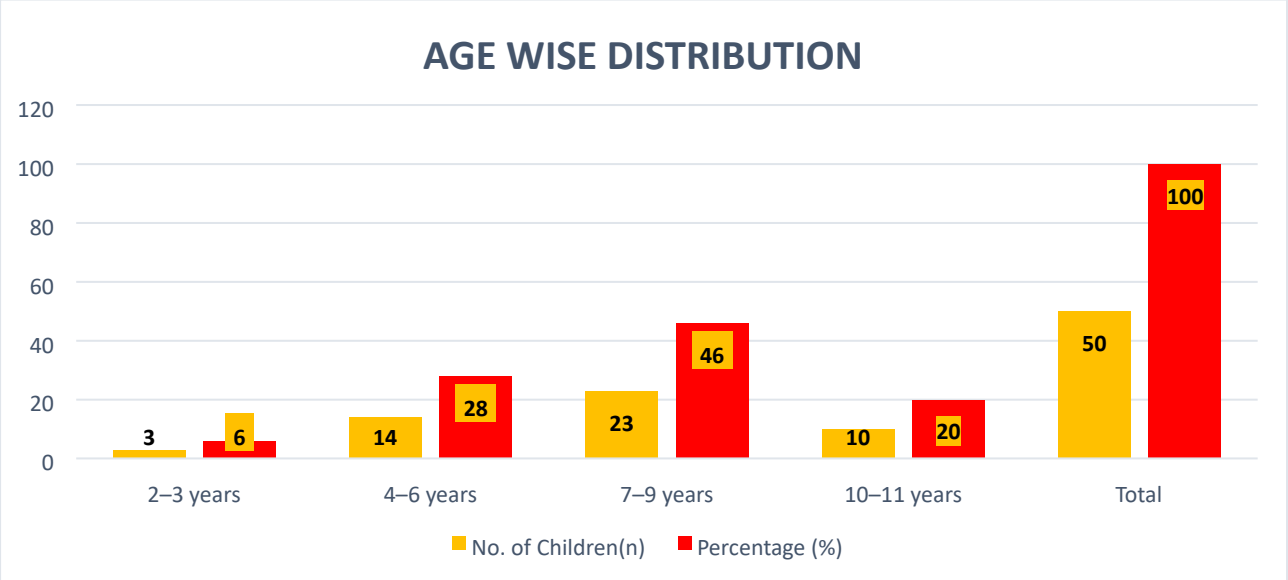
1.Table.8 Age and Sex Distribution

Out of the total 50 children diagnosed with beta-thalassemia major:

Age Group (Years)	No. of Children(n)	Percentage (%)
2–3 years	3	6
4–6 years	14	28
7–9 years	23	46
10–11 years	10	20
Total	50	100
Mean and SD = 7.48 and 2.3		

- Mean age: 6.6 ± 2.4 years
- Sex ratio: 29 males (58%) and 21 females (42%)

The majority of children (46%) were in the 7–9-year age group, with a mean age of 6.6 ± 2.4 years. A male predominance was noted (58%), indicating possible gender-based healthcare access or referral bias.



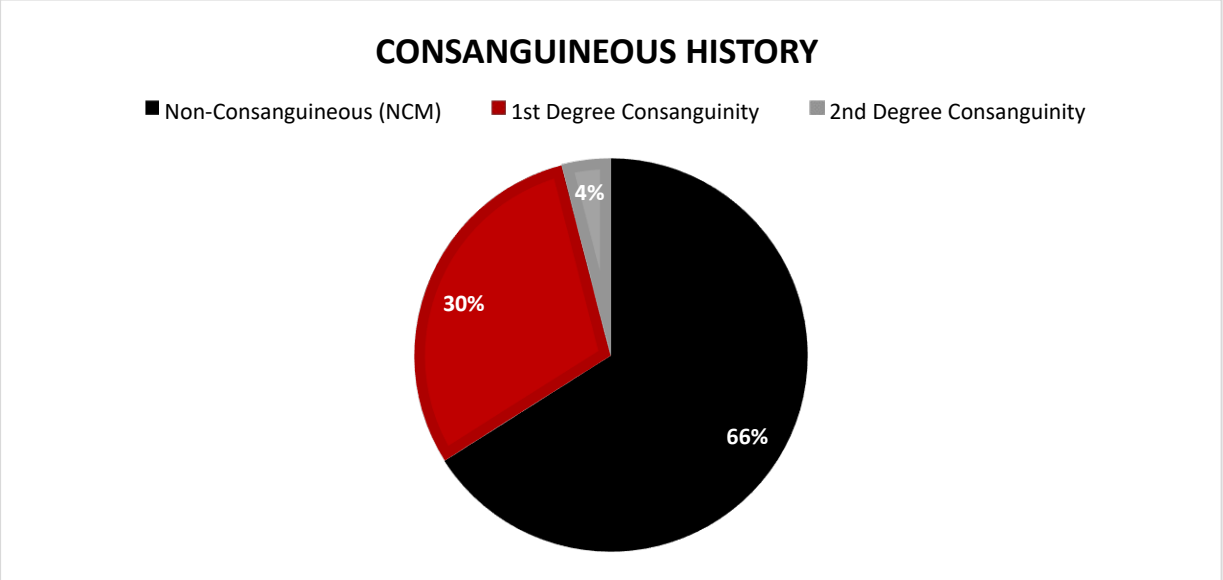
Graph.1 A bar graph representation of age distribution

2.Table.9 Consanguinity

Consanguineous History	No. of Children(n)	Percentage (%)
Non-Consanguineous (NCM)	33	66
1st Degree Consanguinity	15	30
2nd Degree Consanguinity	02	4
	50	100

66% of children had no consanguineous background, while 30% had first-degree consanguinity.

This supports the autosomal recessive inheritance pattern often seen in beta-thalassemia.

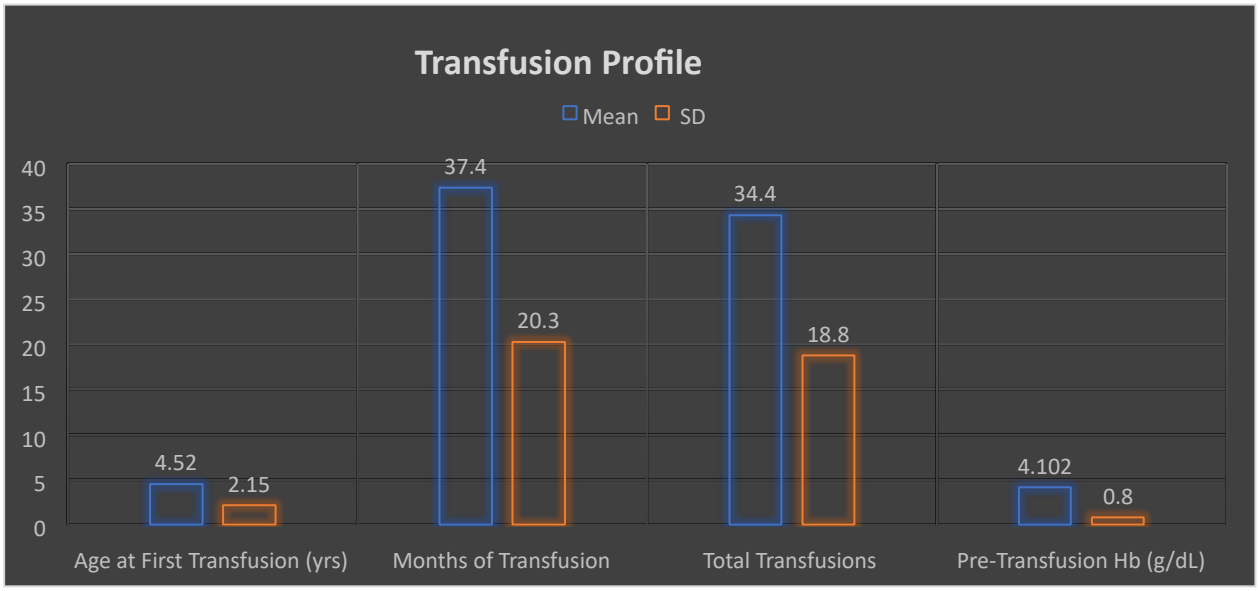


Graph.2 A pie chart representation of Consanguinity history distribution 3.Table.10 Transfusion Profile

Variable	Mean ± SD
Age at First Transfusion (yrs)	4.52 ± 2.15
Months of Transfusion	37.4 ± 20.3

Total Transfusions	34.4 ± 18.8
Pre-Transfusion Hb (g/dL)	4.102 ± 0.8

The mean age at first transfusion was 4.52 ± 2.15 years, and the average pre-transfusion hemoglobin was 4.10 ± 0.8 g/dL.These findings reflect the early onset and severity of anemia in transfusion-dependent beta-thalassemia.



Graph.3 A bar graph representation of transfusion profile 4.

Table.11 Iron Chelation Therapy

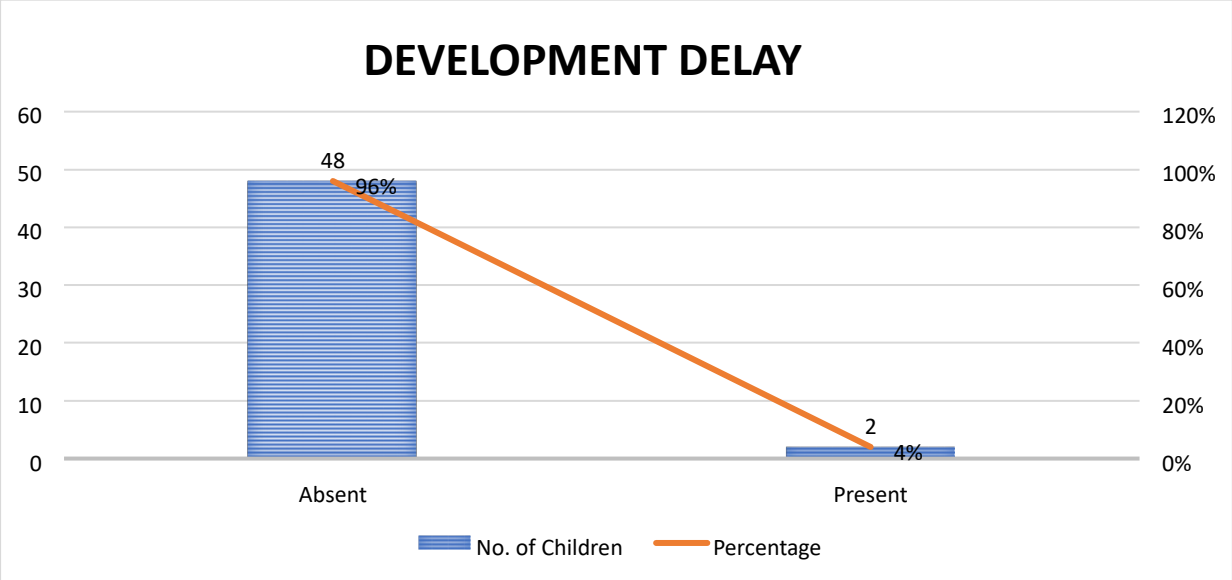
Iron Chelation Therapy	No. of Children	Percentage
Yes	50	100%
No	0	0%

All 50 children (100%) were on iron chelation therapy. This indicates good adherence to clinical protocols for managing iron overload in chronically transfused patients.

5. Table.12 Developmental Delay

Developmental Delay	No. of Children	Percentage
Absent	48	96%
Present	2	4%

Only 2 children (4%) showed signs of developmental delay. This suggests that regular transfusion and chelation therapy may help maintain normal neurodevelopment.

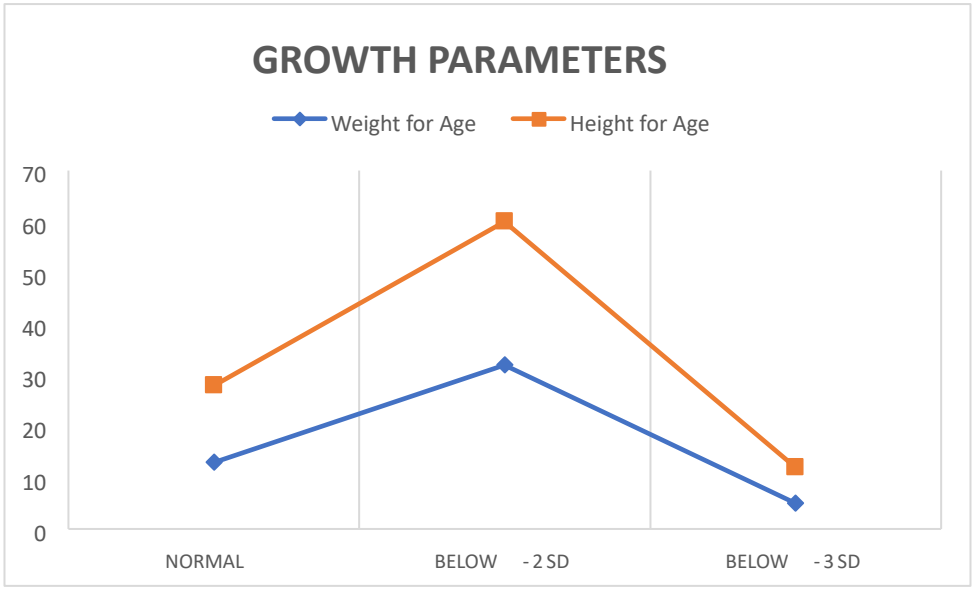


Graph.4 A bar graph representation of the development delay

6. Table.13 Growth Parameters

Indicator	Normal	Below -2SD	Below -3SD
Weight for Age	13	32	5
Height for Age	15	28	7

60% of children were below -2 SD for weight and 70% were below -2 SD for height. This confirms chronic disease-associated growth retardation common in beta-thalassemia major.

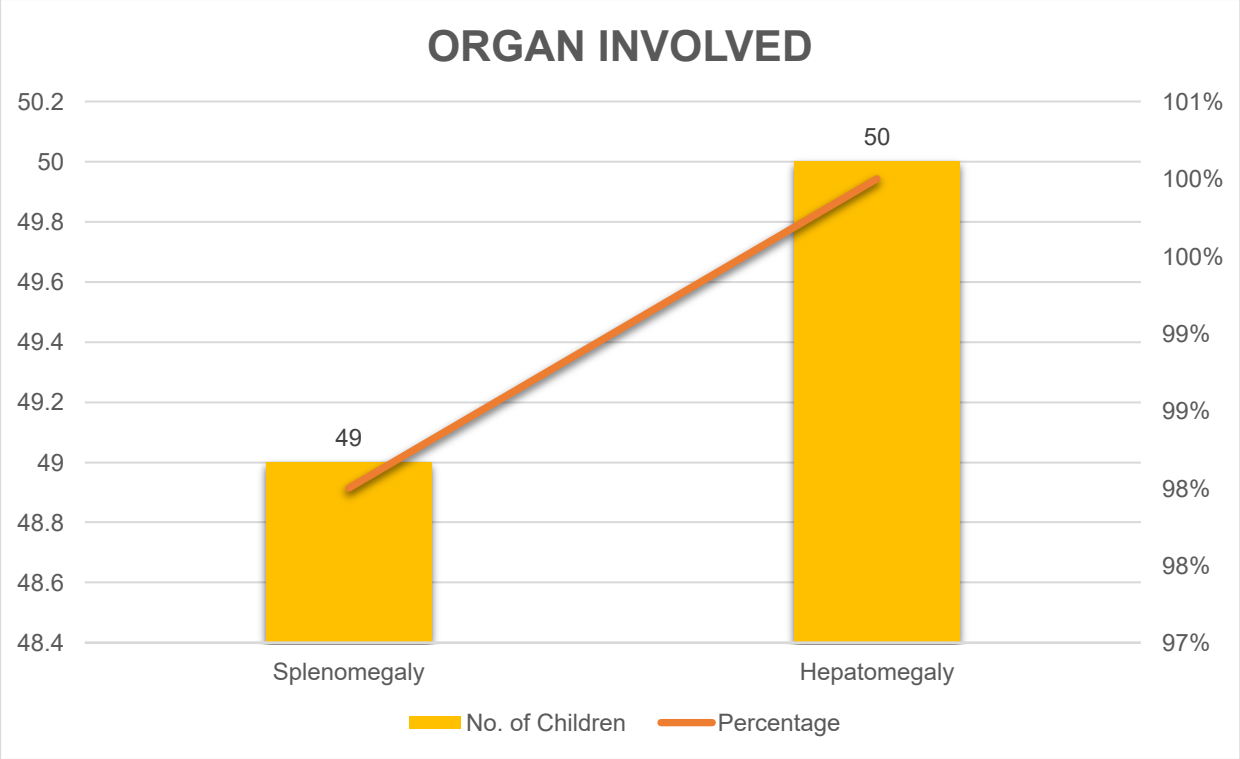


Graph.5 A line graph representation of growth parameters

7.Table.14 Splenomegaly and Hepatomegaly

Organ Involved	No. of Children	Percentage
Splenomegaly	49	98%
Hepatomegaly	50	100%

Splenomegaly was present in 98% and hepatomegaly in 100% of children. This reflects compensatory extramedullary haematopoiesis due to ineffective erythropoiesis.

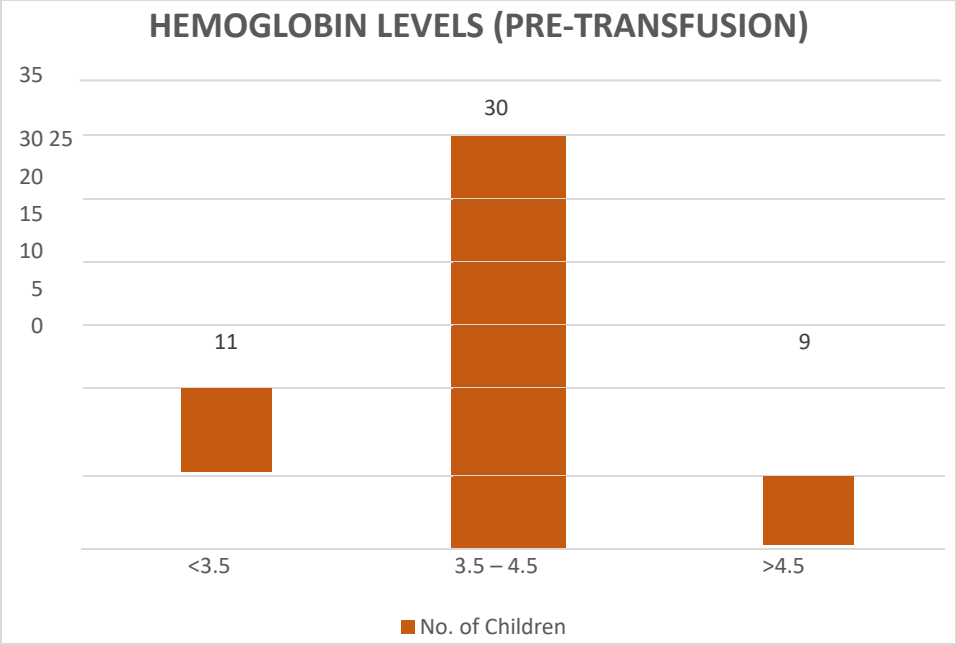


Graph.6 A bar graph representation of the organ-involved distribution

8. TABLE15: Haemoglobin Levels (Pre-Transfusion)

60% of children had pre-transfusion Hb between 3.5–4.5 g/dL, and 22% had Hb below 3.5 g/dL. These extremely low Hb levels underscore the severity of anaemia before scheduled transfusions.

Hb Range (g/dL)	No. of Children	Percentage
<3.5	11	22%
3.5 – 4.5	30	60%
>4.5	9	18%

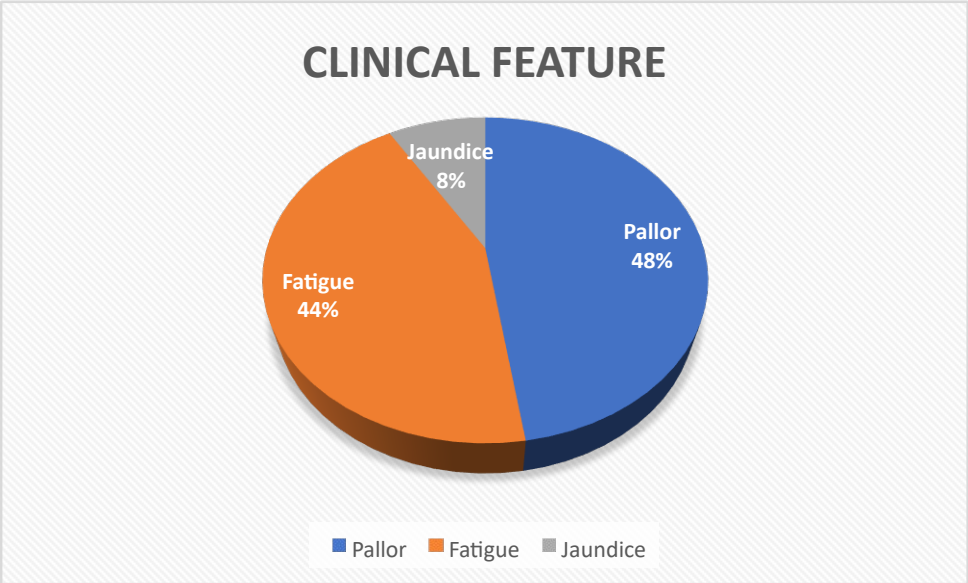


Graph.7 A bar graph representation of hemoglobin levels

9.TABLE16: Pallor, Jaundice and Fatigue

Clinical Feature	Present	Percentage
Pallor	50	100%
Fatigue	46	92%
Jaundice	09	18%

Pallor was seen in 100%, fatigue in 92%, and jaundice in 18% of children. These are typical clinical manifestations of chronic anaemia and haemolysis in thalassemia.



10.Table 17. Vital Signs and Cardio-Respiratory Findings

Mean Heart Rate	Mean RR	Abnormal CVS	Abnormal RS
100.4 bpm	29.16	15(30%)	5(10%)(B/L creps)

Mean heart rate was 100.4 bpm and mean respiratory rate was 29.16; 30% had abnormal CVS findings. Tachycardia and increased respiratory effort reflect chronic hypoxia and high-output circulation.

specific screening.

11.Table 18. Chi-Square Test: Consanguinity vs Height-for-Age (Growth Retardation)

Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	4.700	4
Likelihood Ratio	4.898	4

Value	df	Asymptotic Significance (2-sided)
Linear-by-Linear Association	0.384	1
N of Valid Cases	50	

There is **no statistically significant association** between consanguinity and height-for-age status ($p > 0.05$). The null hypothesis is retained.

12.Table.19 Genotype–Phenotype Correlation in Beta Thalassemia Major Patients Based on Exon 2 Mutations in HBB Gene

Mutation	Frequency (n=27)	Mean Age at 1st Transfusion (months) Mean and SD	Mean Pre-Tx Hb (g/dL) Mean and SD	Mean Total Transfusions Mean and SD	Mean Weight (kg) Mean and SD	Mean Height (cm) Mean and SD	Pallor	Jaundice	Fatigue
G>A (g.5378)	6 (12%)	4.5 ± 3.62	4.22 ± 0.67	26.68 ± 20.26	20.7 ± 8.7	102 ± 11.54	6	1	6
G>T (g.5401)	9 (18%)	4.7 ± 2.01	4.16 ± 0.8	37.80 ± 17.89	22.7 ± 6.35	107 ± 8.64	9	1	0
Both Mutations	12 (44%)	8.07 ± 1.77	4.2 ± 0.8	38.82 ± 17.89	22.92 ± 6.35	108.48 ± 8.63	12	3	11
No Mutations	33 (66%)	4.52 ±2.15	4.102 ± 0.71	34.38 ± 18.76	22.75 ± 7.7	107.16± 10.42	20	1	20

The analysis of genotype-phenotype correlation reveals distinct clinical patterns across mutation types. Children with the G>A (g.5378) mutation exhibited an early disease onset with a mean age of first transfusion at 4.5 ± 3.62 months, and a moderately low pre-transfusion haemoglobin level of 4.22 ± 0.67 g/dL, which aligns with a more severe clinical presentation, further supported by the presence of pallor and fatigue in all six cases. In contrast, those with the G>T (g.5401) mutation showed a similar age at transfusion onset (4.7 ± 2.01 months) but had a higher transfusion burden (mean 37.8 ± 17.89) and better haemoglobin reserve (4.16 ± 0.8 g/dL), indicating a milder yet transfusion-dependent phenotype, notably with no fatigue symptoms reported. Compound heterozygotes (both mutations) displayed the latest onset of

transfusion at 8.07 ± 1.77 months, but with the highest total transfusion requirement (38.82 ± 17.89) and increased frequency of fatigue and pallor, suggesting a cumulative effect of dual mutations leading to a more sustained transfusion need despite delayed onset. Interestingly, even the no mutation group demonstrated comparable clinical severity with a high transfusion load (mean 34.38 ± 18.76) and maximum reported fatigue (20 cases), suggesting the presence of mutations outside exon 2 or possible modifier genes influencing disease expression. Overall, the G>A mutation correlated with early onset and symptomatic severity, G>T with a relatively stable course, and compound mutations with increased transfusion dependency, reflecting a clear genotype-phenotype link.

DISCUSSION.

Although several mutations have been linked to be associated with beta thalassemia major, the spectrum of mutations and their frequency in most populations usually consist of a few numbers of common variants. Several mutations were found in the HBB gene exon 2 region of the sequences analysed.

The majority of them were heterozygous missense variant mutations followed by heterozygous synonymous variant. Among these mutations Transversion were more common followed by Transitions. G >T type mutations were common followed by G>A type mutation. G>T type mutations were observed at positions of g.5401, c.2181 and p.S73I in most sample sequences. G>A type mutations at positions g.5378, c.198 and p. A66A

In this study G>T Type missense mutations were common compared to other studies, where G>C Type Mutations were common

This study also provides insights into the clinical and demographic characteristics of children diagnosed with beta-thalassemia major. The findings are compared with previous studies to assess similarities and differences in disease presentation, transfusion needs, and associated complications.

Age and Sex Distribution

In our study, the majority of children (46%) belonged to the 7–9year age group, with a mean age of 6.6 ± 2.4 years. Males constituted 58% of the cohort, suggesting a male predominance. This finding is consistent with the study by Ahmad et al.,[55] which also reported a higher prevalence of beta-thalassemia among males, potentially due to gender-based healthcare

access or referral biases. Similarly, a study by Khan et al.[56] (2021) observed a male predominance of 60% among beta-thalassemia major patients, reinforcing this trend.

Consanguinity

Our study found that 34% of cases had a history of consanguinity, with 30% involving first degree consanguinity. This supports the autosomal recessive inheritance pattern of beta thalassemia. Ahmad et al.[55] reported that 69.6% of beta-thalassemia major cases resulted from first-cousin marriages. Similarly, a study by Modell et al.[57] (2020) highlighted that consanguinity plays a significant role in the genetic transmission of beta-thalassemia, especially in populations with high rates of endogamous marriages.

Transfusion Profile

The mean age at first transfusion in our study was 4.52 ± 2.15 years, with an average pretransfusion haemoglobin of 4.10 ± 0.8 g/dL. This aligns with the findings of Taher et al. (2021),[58] who reported a mean age of 4.3 years at first transfusion. In contrast, a study by Cappellini et al. (2019)[59] found an earlier initiation of transfusion therapy, with a median age of 0.8 years, suggesting earlier diagnosis and management in some healthcare settings.

Iron Chelation Therapy

All children in our study (100%) were on iron chelation therapy, indicating strong adherence to clinical guidelines for managing iron overload. Kuo and Ward (2020)[60] emphasized that adherence to iron chelation therapy significantly reduces complications such as cardiomyopathy and endocrine dysfunctions. Similarly, a study by Piga et al.[61] (2018) demonstrated that strict compliance with iron chelation therapy improves survival and quality of life in transfusion-dependent beta-thalassemia patients.

Developmental Delay

Only 4% of children in our cohort exhibited developmental delays. This is lower than the findings of Sharma et al. (2020),[62] who reported a developmental delay prevalence of 10% in beta-thalassemia major patients. Their study highlighted the impact of chronic anaemia and iron overload on neurodevelopment. Additionally, research by Moatter et al.[63](2019) suggested that optimal transfusion and chelation therapy may reduce the risk of neurodevelopmental impairment in these children.

Growth Parameters

Growth retardation was a significant concern, with 60% of children below -2 SD for weight and 70% below -2 SD for height. These findings are comparable to those of De Sanctis et al.[64] (2021), who reported growth failure in 65% of children with beta-thalassemia major. Similarly, Soliman et al.[65] (2019) observed that growth retardation was prevalent in 67% of patients, often linked to chronic anaemia, iron overload, and endocrine dysfunctions.

Splenomegaly and Hepatomegaly

Splenomegaly and hepatomegaly were observed in 98% and 100% of our cohort, respectively. These findings align with the results of Musallam et al.[66] (2020), who reported splenomegaly in 95% of beta-thalassemia patients. Similarly, a study by Taher et al. (2019)[67] highlighted the prevalence of hepatomegaly due to excessive iron deposition and extramedullary haematopoiesis in transfusion-dependent individuals.

Pre-Transfusion Haemoglobin Levels

In our study, 60% of children had pre-transfusion haemoglobin levels between 3.5–4.5 g/dL, and 22% had levels below 3.5 g/dL. These findings are consistent with the study by

BorgnaPignatti et al.[68] (2018), which reported a similar distribution of pre-transfusion haemoglobin levels. Moreover, research by Weatherall et al. [69](2019) emphasized that maintaining higher pre-transfusion haemoglobin levels (above 9 g/dL) is associated with better long-term outcomes and reduced morbidity in beta-thalassemia patients.

Clinical Symptoms: Pallor, Jaundice, and Fatigue

Pallor was observed in 100% of our cohort, fatigue in 92%, and jaundice in 18%. These findings are in line with the study by Ansari et al.[70] (2021), which reported pallor in nearly all beta-thalassemia major patients. Additionally, research by Modell et al.[71] (2018) indicated that fatigue and jaundice are common clinical manifestations due to chronic haemolysis and anaemia in these patients.

Vital Signs and Cardio-Respiratory Findings

The mean heart rate in our study was 100.4 bpm, with a mean respiratory rate of 29.16. Abnormal cardiovascular findings were noted in 30% of children. Similar results were reported by Olivieri et al.[72] (2020), who found an increased prevalence of tachycardia and respiratory distress in beta-thalassemia major due to chronic hypoxia. Additionally, a study by Borgna-Pignatti et al.[73] (2019) emphasized that cardiovascular complications are a leading cause of morbidity in transfusion-dependent patients.

Chi-Square Analysis: Consanguinity vs. Growth Retardation

Our statistical analysis found no significant association between consanguinity and height-forage status ($p > 0.05$). This is consistent with the study by Al-Suliman et al. (2021),[74] which also found no significant correlation between parental consanguinity and growth parameters in beta-thalassemia patients. However, Moatter et al.[63] (2019) suggested that while consanguinity

itself may not directly impact growth, it contributes to genetic predisposition and disease severity.

CONCLUSION

Mutational analysis of Exon 2 in the HBB gene revealed some common mutations. Among these mutations, missense mutations were more common. Transversions (G-T Nucleotide change) were found in high percentage.

Mutations can bring about a change in codon sequences which altered the protein production. Studies suggested that there is a need to maintain a primary prevention program to analyse mutation, and sequence variations at the molecular level. It can help to overcome many genetic disorders.

By conducting awareness programs, carrier screening and beta thalassaemia screening in high-risk couples, it is possible to reduce the occurrence of this inherited disease.

Children with inherited conditions like beta thalassemia major are a burden to the family and society, it is crucial to reduce the prevalence of such diseases.

Genetic counselling and prenatal beta thalassaemia diagnosis might be successfully established with the use of mutational pattern research, which would lessen the burden of this disease on society.

With some preliminary important actions and measures, the identification of a mutation in the HBB gene will reduce health disparities in an already vulnerable population.

This study also highlights the clinical and demographic characteristics of children diagnosed with beta-thalassemia major. The findings confirm a high prevalence of consanguinity, male predominance, and significant growth retardation among affected individuals.

Early onset of transfusion dependence, severe anaemia, and organomegaly emphasize the burden of the disease and the need for regular medical interventions.

The study also underscores the importance of strict adherence to iron chelation therapy in managing iron overload and preventing complications.

Despite the severe clinical manifestations, developmental delay was relatively uncommon, suggesting that optimal medical management may contribute to maintaining neurodevelopmental outcomes.

Also, correlation findings from the study emphasize a significant genotype–phenotype correlation in beta thalassemia major. The G>A mutation was associated with early disease onset and greater clinical severity, while G>T mutation showed a milder phenotype. Notably, the **compound heterozygotes** had the highest transfusion burden, highlighting the additive impact of dual mutations and the need for genotype-based clinical risk stratification.

SUMMARY.

Summary.

- Out of 50 samples we have identified the mutation **g.5401 G>T** in 21 samples which is heterozygous missense variant.
- we have identified the mutation **g.5378 G>A** in 17 samples which is heterozygous synonymous variant.
- G >T type mutations were common followed by G>A type mutation.

- The majority of children (46%) were between 7–9 years of age, with a mean age of 6.6 ± 2.4 years.
- A male predominance (58%) was observed.
- Consanguinity was noted in 34% of cases, supporting the autosomal recessive inheritance pattern.
- The mean age at first transfusion was 4.52 ± 2.15 years, with a pre-transfusion haemoglobin level averaging 4.10 ± 0.8 g/dL.
- All children were on iron chelation therapy.
- Growth retardation was prevalent, with 60% and 70% of children below -2SD for weight and height, respectively.
- Splenomegaly and hepatomegaly were observed in 98% and 100% of cases, respectively.
- Pallor (100%), fatigue (92%), and jaundice (18%) were common clinical symptoms.

No significant association was found between consanguinity and growth parameters

LIMITATIONS

- The study was conducted at a single centre, limiting generalizability to broader populations.
- The sample size was relatively small (n = 50), which may affect the statistical power of certain comparisons.
- Longitudinal follow-up was not conducted, restricting insights into disease progression over time.
- Socioeconomic factors and nutritional status, which may influence growth parameters, were not extensively analysed.
- The study relied on available medical records, which may introduce potential reporting biases.

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ANNEXURE 2
Ethical clearence certificate

Azadi Ka Amrit Mahotsav

BLDE
(DEEMED TO BE UNIVERSITY)
Declared as Deemed to be University u/s 3 of UGC Act, 1956
Accredited with 'A' Grade by NAAC (Cycle-2)
The Constituent College
SHRI B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

BLDE (DU)/IEC/ 964/2022-23 10/4/2023

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on **Saturday, 18th March, 2023 at 11.30 a.m. in the CAL Laboratory, Dept. of Pharmacology,** scrutinizes the Synopsis/ Research Projects of Post Graduate Student / Under Graduate Student /Faculty members of this University /Ph.D. Student College from ethical clearance point of view. After scrutiny, the following original/ corrected and revised version synopsis of the thesis/ research projects has been accorded ethical clearance.

TITLE: "POLYMORPHISM OF EXON 2 IN HBB GENE IN BETA THALESSEMIA MAJOR CHILDREN IN NORTH KARNATAKA".

NAME OF THE STUDENT/PRINCIPAL INVESTIGATOR: DR.DONEPUDI SAI AKHIL

NAME OF THE GUIDE: DR.M.M.PATIL, PROFESSOR, DEPT. OF PEDIATRICS.

Dr. Santoshkumar Jeevangi
Chairperson
IEC, BLDE (DU),
VIJAYAPURA
Chairman,
Institutional Ethical Committee,
BLDE (Deemed to be University)
Vijayapura

Dr.Akram A. Naikwadi
Member Secretary
IEC, BLDE (DU),
VIJAYAPURA
MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University)
Vijayapura-586103. Karnataka

Following documents were placed before Ethical Committee for Scrutinization.

- Copy of Synopsis/Research Projects
- Copy of inform consent form
- Any other relevant document

Smt. Bangaramma Sajjan Campus, B. M. Patil Road (Sholapur Road), Vijayapura - 586103, Karnataka, India.
BLDE (DU): Phone: +918352-262770, Fax: +918352-263303, Website: www.bldedu.ac.in, E-mail:office@bldedu.ac.in
College: Phone: +918352-262770, Fax: +918352-263019, E-mail: bmpmc.principal@bldedu.ac.in

ANNEXURE 2

RESEARCH INFORMED CONSENT FORM

BLDE (DEEMED TO BE UNIVERSITY) Shri B.M PATIL Medical

College, Hospital & Research Centre,

Vijayapur-586103.

TITLE OF THE PROJECT: POLYMORPHISM OF EXON 2 IN HBB GENE IN BETA THALESSEMIA MAJOR CHILDREN IN NORTH KARNATAKA

GUIDE : DR M.M PATIL , MD
PROFESSOR
DEPARTMENT OF PEDIATRICS.

PG STUDENT : **DR DONEPUDI SAI AKHIL**

RISK AND DISCOMFORTS:

None

BENEFITS:

I understand that my participation in the study will have no direct benefit to me other than the potential benefit of the research and education.

CONFIDENTIALITY:

I understand that the medical information produced by this study will become a part of hospital records and will be subject to the confidentiality. Information of sensitive personal nature will not be part of the medical record, but will be stored in the investigations research file.

If the data are used for publication in the medical literature or for teaching purpose, no name will be used and other identifiers such as photographs will be used only with special written permission. I understand that I may see the photograph before giving the permission.

REQUEST FOR MORE INFORMATION:

I understand that I may ask more questions about the study at any time;

Dr. DONEPUDI SAI AKHIL at the department of Paediatrics is available to answer my questions or concerns. I understand that I will be informed of any significant new findings discovered during the course of the study, which might influence my continued participation. A copy of this consent form will be given to me to keep for careful reading.

REFUSAL FOR WITHDRAWAL OF PARTICIPATION:

I understand that my participation is voluntary and that I may refuse to participate or may withdraw consent and discontinue participation in the study at any time without prejudice. I also understand that Dr. M.M PATIL may terminate my

participation in the study after he has explained the reasons for doing so.

INJURY STATEMENT:

I understand that in the unlikely event of injury to child resulting directly from child's participation in this study, if such injury were reported promptly, the appropriate treatment would be available to the child. But no further compensation would be provided by the hospital. I understand that by my agreements to participate in this study and not waiving any of my legal rights.
I have explained to _____the purpose of the research, the procedures required and the possible risks to the best of my ability.

_____ _____

Dr. DONEPUDI SAI AKHIL Date

(Investigator)

PARENTS / GUARDIAN CONSENT STATEMENT:

*We confirm that DR DONEPUDI SAI AKHIL is doing "**POLYMORPHISM OF EXON 2 IN HBB GENE IN BETA THALESSEMIA MAJOR CHILDREN IN NORTH KARNATAKA**" Dr DONEPUDI SAI AKHIL,*
has explained to us the purpose of research and the study procedure. We are willing to give as much as information required for the study and consent for investigations and the possible discomforts as well as benefits. We have been explained all the above in detail in our own language and we understand the same. Therefore, we agree to give consent for child's participate as a subject in this research project.

_____ _____

(Parents / Guardian) Date

_____ _____

(Witness to signature) Date

ANNEXURE – III

PROFORMA

Name –

IP no –

DOB - Age- weight -

Sex –

Address –

BLOOD GROUP –

CONSANGUINEOUS HISTORY-

Frequency of transfusion:

Age at which transfusion started

Months of transfusion

Total number of transfusions

IRON CHELATION – YES/NO

Developmental delay: YES/NO

GENERAL PHYSICAL EXAMINATION:

HR: RR: BP:

Pallor: YES/NO

Jaundice: YES/NO

Fatigue: YES/NO

HEIGHT: WEIGHT:

Kg HC:

LENGTH/HEIGHT:

HEIGHT FOR AGE:

WEIGHT FOR HEIGHT:

SYSTEMIC EXAMINATION:

CVS:

RESPIRATORY SYSTEM:

SPLEENOMEGALY:

HEPATOMEGALY:

B.L.D.E (DEEMED TO BE UNIVERSITY)

ಶ್ರೀಬಿ.ಎಂ.ಪಟ್ಟೀಲ್ಕಡಿಕಲ್ಕಾಲೇಜು,

ಆಸ್ಪತ್ರೆ ಮತ್ತು ಸಂಶೋಧನಾಕೇಂದ್ರ, ವಿಜಯಪುರ-586103

ಪರ ಬಂಧ/ಸಂಶೋಧನೆಯಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲುಮಾಹಿತಿಪಡೆದ್ಕೊಳ್ಳುವಿಕೆ

ನಾನು, ಕೆಳಗಿನವರು_____ಸಹಿಯಿಟ್ಟವರು, ಮಗ/ಮಗಳು/ಪತ್ನಿ ಯ _____ ವಯಸ್ಸು _____ವರ್ಷಗಳು, ಸಾಮಾನ್ಯವಾಗಿನಿವಾಸಿಸ್ತುವಸ್ಥಳದಹೆಸರು_____, ಇಲ್ಲಿ ಹೇಳಿದ್ದೇನೆ/ಫೋಷಿಸ್ತುತ್ತೇನೆಡಾಕ್ಟರ ಹೆಸರು_____ಅವರುಆಸ್ಪತ್ರೆ ಹೆಸರು_____ಅವರುನನ್ನು ಪೂರ್ಣವಾಗಿಪರೀಕ್ಷಿಸಿದರುದಿನಾಂಕ್ದಲ್ಲಿ _____ಸ್ಥಳಹೆಸರು_____ಮತ್ತುತನಗೆನನ್ನ ಭಾಷೆಯಲ್ಲಿ ವಿವರಿಸಲಾಗಿದ್ದಾನುಒಂದುರೋಗ (ಸ್ಥಿತಿ) ಅನುಭವಿಸ್ತುತ್ತಿದ್ದೇನೆ. ಮಾಂದುವರದುಡಾಕ್ಟರ ನಿರ್ದೇಶನಗೆತ್ನಳಿಸಿದ್ದರೆಅವರುಒಂದುಪದದ ತ್ನ/ಸಂಶೋಧನೆನಡೆಸ್ತುತ್ತಿದ್ದರೆಶೋಷಿಷಕೆಯುಳಳ _____ಡಾಕ್ಟರ್_____ ಮಾಗಷದರ್ಶನದಲ್ಲಿ ನನ್ನ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆಯನ್ನು ಕೇಳಿದ್ದರೆಅಧ್ಯಯನದ ಲ್ಲಿ .

ಡಾಕ್ಟರ ನಿರ್ದೇಶನಗೆಇದನ್ನು ಕೂಡಾತ್ನಳಿಸಿದ್ದರೆಈಕ್ಷೆ ಮದನಡೆವಲ್ಲಿ ಪೆತ್ನಕೂಲಫಲ್ಲತಾಂರ್ಗಳನ್ನು ಎದುರಸಬಹುದು. ಮೇಲೆಹೇಳಿದಪೆಕ್ಷೆಣಗಳಲ್ಲಿ, ಅಧಿಕಾಂರ್ಶುಚಿಕ್ಷತ್ನು ಸಬಹುದ್ದದರೂಅದನ್ನು ನಿರೀಕ್ಷಿಸಲಾಗುತ್ನತ ಲಿಆದದ ರಾಂದನನ್ನಿ ಸ್ಥಿತ್ನಯಹಿರದ್ದಗುವಅವಕರ್ಶಿದ್ಮತ್ತತ ಅಪರೂಪದಸಂದ ಭಷಗಳಲ್ಲಿ ಅದುಮರರ್ಕರಕ್ಷಾಗಿಪರರ್ಮಿಸಬಹುದುಹಾಂದಿದರೇಗ ನಿರ್ಧಷರಮತ್ತತ ಯಥಾರ್ಷ್ಟತ ಚಿಕ್ಷತ್ಸೆ ಮಾಡಲುಹಾಂದಿದರೂ.

ಮಾಂದುವರದುಡಾಕ್ಟರ ನಿರ್ದೇಶನಗೆತ್ನಳಿಸಿದ್ದರೆನನ್ನ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆಈಅಧ್ಯಯನದಫಲ್ಲತಾಂರ್ಗಳಮೌಲ್ಯಮಾಪನದಲ್ಲಿ ಸಹಾಯಕ್ಷಾಗುತತತ ದ್ಇತರ

ಸಮಾನಪೆಕ್ಷರ್ಗಳಚಿಕ್ಷತ್ಸೆಗೆಉಪಯುಕ್ತ ಉಲೆಕ್ಷೆ ರೀಖವಾಗಿದ್, ಮತ್ತತ ನಾನುಅನುಭವಿಸ್ತುವರೇಗದಿಂದವಿಮಕ್ಷತ ಅಥವಾಗುರ್ಮಖಗೊಳ್ಳುವಲ್ಲಿ ನನಗೆಪೆ ಯೋಜನವಾಗಬಹುದು.

ಡಾಕ್ಟರ ನಿರ್ದೇಶನಗೆಇದನ್ನು ಕೂಡಾತ್ನಳಿಸಿದ್ದರೆನನ್ನಿ ಂದನೀಡಿದಮಾಹಿತ್ನ,

ಮಾಡಿದಪರಶೋಲನೆಗಳು / ಫೋಟೋಗ್ರೆ ಫ್ಗಳು /

ವೀಡಿಯೇಗ್ರ ಫ್ಗಳುನನಿ ಮೇಲೆತ್ಸೆದುಕೊಳಳ ಲಾಗುವಅನೆವ ೇರ್ಕ್ಯುರಹ ಸಯ ವಾಗಿಇಡುವರುಮತ್ತತ ನಾನುಅಥವಾನನಗೆಕಸಂಬಂಧಿತಹರತ್ತಪಡಿ ಸಿಇತರವಯ ಕ್ಷತ ಯಿಾಂದಮೌಲಯ ಮಾಪನಮಾಡಲಾಗುವುದಿಲಿ .

ಡಾಕ್ಟ ನಷನಗೆತ್ನಳಿಸಿದ್ದದ ರೆನನಿ ಪಾಲ್ಸೊ ಳುಳ ವಿಕೆಶುದಧ ವಾಗಿಸ್ವ ೇಚ್ಛಾ ಯಿತ,

ನನಿಿ ಾಂದನೀಡಿದಮಾಹಿತ್ನಯಆರ್ಧರದಮೇಲೆ, ಚಿಕ್ಷತ್ಾ / ಅಧಯ ಯನದಸಂಬಂಧದಲ್ಲಿ ರೇಗನಿರ್ಧಷರ, ಚಿಕ್ಷತ್ಾ ಯವಿರ್ಧನ,

ಚಿಕ್ಷತ್ಾ ಯಫಲ್ಲತಾಂರ್ಅಥವಆಭವಿರ್ಯ ದಪೆ ವೃತ್ನತ ಗಳುಬಗೆೊ ಯಾವುದೇಸಪ

ರ್ಟ ತ್ಕೇಳಬಹುದು. ಅದೇಸಮಯದಲ್ಲಿ ತ್ನಳಿಸಲಾಗಿದ್ಾನುಯಾವುದೇಸಮಯದಲ್ಲಿ ಈಅಧಯ ಯನದಲ್ಲಿ ನನಿ ಪಾ ಲ್ಸೊ ಳುಳ ವಿಕೆಯನುಿ ನಿಲ್ಲಿ ಸಬಹುದುನಾನುಬಯಸಿದರೆಅಥವಾಅನೆವ ೇರ್ಕ್ ರುಅಧಯ ಯನದಿಾಂದಯಾವುದೇಸಮಯದಲ್ಲಿ ನನಿ ನುಿ ನಿಲ್ಲಿ ಸಬಹುದು. ಪೆ ಬಂಧಅಥವಾಸಂಶೇಧನೆಯಸವ ಭಾವ, ಮಾಡಿದರೇಗನಿರ್ಧಷರಮತ್ತತ ಚಿಕ್ಷತ್ಾ ಯವಿರ್ಧನವನುಿ ಅಥಷಮಾಡಿ ಕೊಾಂಡು, ನಾನುಕೆಳಗಿನಶೆ ೇ /

ಶೆ ೇಮತ್ನ_____ನನಿ ಪೂರ್ಷವಾದಪೆ ಜ್ಞೆ ಯಸಿಥ ತ್ನಯಲ್ಲಿ ಹೇ ಳಿದಸಂಶೇಧನೆ / ಪೆ ಬಂಧದಲ್ಲಿ ಪಾಲ್ಸೊ ಳಳ ಲುಒಪ್ಪಪ ತ್ತ ೇನೆ.

ರೇಗಿಯಸಹಿಡಾಕ್ಟ ರನಸಹಿ

ಸಾಕ್ಷಿ ಗಳು
1)
2)

ANNEXURE – IV

MASTER CHART

S.No	Name	Age	Date of Birth	Sex	IP no.	Address	Blood Groups	HAEMATOLOGICAL HISTORY	Frequency Of Transfusion	Age at which Transfusion Started	Months of Transfusion	Total number of Transfusion	Pre Transfusion hemoglobin	Iron Chelation	Developmental Delay	Heart Rate	Respiratory Rate	Blood Pr
1	Bhagesh	6 years	31-12-2018	Male		135740 Indi	O Positive	NCM	Once in a Month	3	36	32	3 Yes	Absent		88	28	100/70
2	Gayatri	4 years	04-12-2021	Female		51592 Indi	A Positive	1st Degree CM	Once in a Month	3	11	8	4.2 Yes	Absent		86	30	100/70
3	Chruvika	7 years	23-12-2017	Female		52044 Chadchan	O Positive	NCM	Once in a Month	6	11	10	3.8 Yes	Absent		88	28	110/70
4	Pavan	6 years	16-10-2018	Male		5985 Bagalkot	O Positive	NCM	Once in a Month	5	12	12	2.9 Yes	Absent		90	30	110/60
5	Asamita	4 years	05-11-2020	Female		2298 Bagalkot	O Positive	2nd Degree CM	Once in 2 months	3	6	6	4.6 Yes	Absent		84	24	100/70
6	Afsana	3 years	17-08-2021	Female		102460 Tiksta	B Positive	NCM	Once in a Month	3	12	10	4.2 Yes	Absent		86	22	110/70
7	Mallikarjun B	2 years	09-07-2022	Male		255800 Bableshwar	B Positive	NCM	Once in a Month	1	9	8	3.5 Yes	Absent		89	32	90/50
8	Manvit	2 years	19-05-2022	Male		36967 Bagalkot	B Positive	NCM	Once in a Month	18	6	6	4.1 Yes	Absent		86	28	90/60
9	Smita	6 years	23-12-2018	Female		23980 Muddebihal	B Positive	NCM	Once in a Month	5	12	10	5 Yes	Absent		90	26	100/60
10	Bhagyashree	10 years	03-04-2014	Female		124120 Bijapur	B Positive	1st Degree CM	Once in a Month	3	70	60	3.9 Yes	Absent		104	30	110/60
11	Nikhil	5 years	27-02-2018	Male		12136 Bijapur	A Positive	NCM	Once in 2 months	3	7	7	5.2 Yes	Absent		96	32	90/60
12	Shivaraj	4 years	01-10-2021	Male		275834 Bijapur	AB Positive	NCM	Once in a Month	3	12	11	3.7 Yes	Absent		90	30	90/70
13	Sahitya	9 years	06-06-2014	Female		306718 Bagalkot	A Positive	NCM	Once in a Month	4	58	53	5.2 Yes	Absent		92	32	90/70
14	Pramod	9 years	18-10-2015	Male		330235 Jamkhandi	O Positive	NCM	Once in a Month	5	48	43	3.9 Yes	Absent		112	32	110/70
15	Surakshita	6 years	11-04-2016	Female		231317 Bilgi	B Positive	1st Degree CM	Once in a Month	4	23	20	4.2 Yes	Absent		88	28	90/60
16	Noorjahan	10 years	30-11-2014	Female		262307 Belgaum	A Positive	NCM	Once in a Month	4	72	67	5.1 Yes	Absent		90	30	100/60
17	Hasan	6 years	12-12-2018	Male		217763 Indi	O Positive	NCM	Once in a Month	4	23	23	4.2 Yes	Absent		96	32	100/60
18	Shivani	7 years	02-02-2017	Female		104437 Chadchan	B Negative	1st Degree CM	Once in a Month	4	35	35	2.9 Yes	Absent		102	34	90/70
19	Ningangor	9 years	09-05-2015	Female		44478 Mudhol	B Positive	NCM	Once in a Month	5	47	45	3.3 Yes	Absent		108	28	90/60
20	Aryan	5 years	29-08-2019	Male		173320 Muddebihal	A Positive	1st Degree CM	Once in a Month	3	24	23	4.5 Yes	Absent		102	30	90/70
21	Vasudev	7 years	31-03-2017	Male		282143 Zalki	B Positive	NCM	Once in a Month	4	37	36	5.5 Yes	Absent		100	30	100/60
22	Pargambar	10 years	20-09-2014	Male		312300 Sindagi	O Positive	1st Degree CM	Once in 2 months	3	42	40	3.9 Yes	Absent		104	40	90/70
23	Mallikarjun Kumbar	9 years	30-04-2015	Male		169977 Jamkhandi	O Positive	NCM	Once in a Month	4	60	59	4.4 Yes	Absent		116	30	90/60
24	Mallikarjun B	11 years	23-08-2013	Male		246824 Talikoti	O Positive	NCM	Once in a Month	5	71	66	3.1 Yes	Absent		92	24	110/70
25	Shivanand	10 years	07-08-2014	Male		260665 Jamkhandi	B Positive	1st Degree CM	Once in a Month	3	84	80	5.1 Yes	Absent		120	34	110/70
26	Bhoomi	6 years	23-05-2018	Female		260662 Bagalkot	A Positive	NCM	Once in a Month	4	23	21	4.8 Yes	Absent		104	30	90/60
27	Keerthana	5 years	10-11-2019	Female		260658 Sindagi	AB Positive	NCM	Once in a Month	4	11	11	5 Yes	Absent		110	28	90/60
28	Jamv	8 years	29-03-2018	Female		26040 Bijapur	B Positive	NCM	Once in a Month	4	47	45	3.7 Yes	Absent		102	30	100/70
29	Nikita	8 years	12-08-2018	Female		260637 Jamkhandi	O Positive	NCM	Once in a Month	5	36	36	4 Yes	Absent		108	32	90/60
30	Chetan	8 years	07-07-2018	Male		226967 Jamkhandi	O Positive	NCM	Once in a Month	4	48	44	3.1 Yes	Absent		104	28	100/70
31	Parasuram	10 years	30-05-2014	Male		15808 Talikoti	A Positive	1st Degree CM	Once in a Month	5	60	58	3.7 Yes	Absent		126	34	110/70
32	Savina	9 years	04-02-2015	Female		15621 Jamkhandi	B Positive	NCM	Once in a Month	4	60	52	4.7 Yes	Absent		102	24	90/60
33	Veeresh	9 years	19-08-2015	Male		254874 Sholapur	O Positive	NCM	Once in a Month	5	48	43	5 Yes	Absent		120	28	100/80
34	Vasudev	11 years	10-12-2013	Male		185543 Indi	B Negative	2nd Degree CM	Once in 2 months	5	48	48	2.9 Yes	Present		128	34	110/80
35	Laxman	9 years	10-01-2015	Male		443962 Jamkhandi	O Positive	1st Degree CM	Once in 2 months	5	47	45	3.4 Yes	Absent		110	28	90/60
36	Shrishail	8 years	01-09-2016	Male		90256 Mudhol	B Positive	NCM	Once in a Month	4	48	48	4 Yes	Absent		108	26	100/70
37	Pallavi	6 years	23-05-2018	Female		233322 Bigli	O Positive	NCM	Once in a Month	4	24	20	4.7 Yes	Absent		98	22	90/70
38	Akash	7 years	26-12-2018	Male		192561 Honnutagi	A Positive	1st Degree CM	Once in a Month	5	23	20	3.3 Yes	Absent		100	28	90/70
39	Preetam	8 years	24-09-2018	Male		186925 Bijapur	O Positive	NCM	Once in a Month	5	36	34	4.1 Yes	Absent		104	34	100/80
40	Siddarth	6 years	26-12-2018	Male		16478 Jamkhandi	A Positive	NCM	Once in a Month	4	23	22	5.1 Yes	Absent		104	32	90/70
41	Archana	10 years	15-05-2014	Female		176363 Jamkhandi	O Positive	NCM	Once in a Month	5	56	49	4.4 Yes	Absent		90	26	90/60
42	Satish Kumar	10 years	06-06-2014	Male		175766 Bijapur	B Positive	NCM	Once in a Month	5	59	55	3 Yes	Absent		110	28	100/70
43	Tippanna	8 years	13-10-2018	Male		175773 Sindagi	B Positive	1st Degree CM	Once in a Month	5	35	32	4.8 Yes	Absent		120	26	90/70
44	Dareshwari	7 years	25-04-2017	Female		176525 Tiksta	O Positive	NCM	Once in 2 months	6	12	12	4.5 Yes	Absent		114	28	100/80
45	Sajit	10 years	24-09-2014	Male		260639 Jamkhandi	O Positive	NCM	Once in a Month	5	60	52	5.2 Yes	Absent		94	22	100/80
46	Vaishnavi	9 years	19-07-2015	Female		260627 Zalki	B Positive	1st Degree CM	Once in 2 months	5	47	44	3.6 Yes	Absent		102	26	90/60
47	Jahangir	9 years	15-04-2015	Male		226903 Muddebihal	B Positive	NCM	Once in 2 months	5	47	42	3 Yes	Absent		100	24	100/80
48	Ganesh	8 years	07-01-2016	Male		226956 Jamkhandi	O Positive	1st Degree CM	Once in 2 months	5	35	34	3.5 Yes	Present		98	30	100/60
49	Sourabh	9 years	11-11-2015	Male		262578 Jamkhandi	O Positive	1st Degree CM	Once in 2 months	5	48	40	5.2 Yes	Absent		130	36	100/60
50	Bibinamiyam	9 years	17-02-2015	Female		360203 Jamkhandi	O Positive	1st Degree CM	Once in 2 months	5	48	43	3 Yes	Absent		110	30	100/60

ANNEXURE – V

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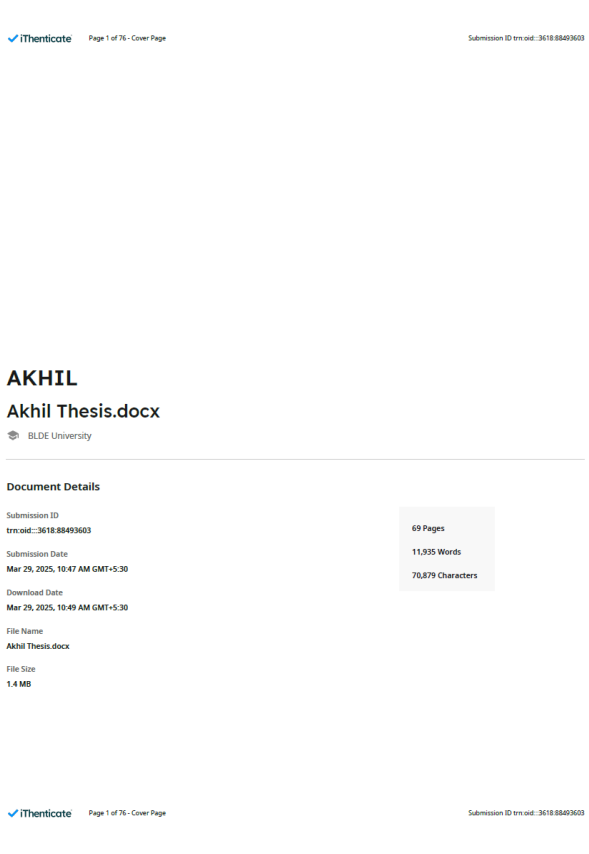

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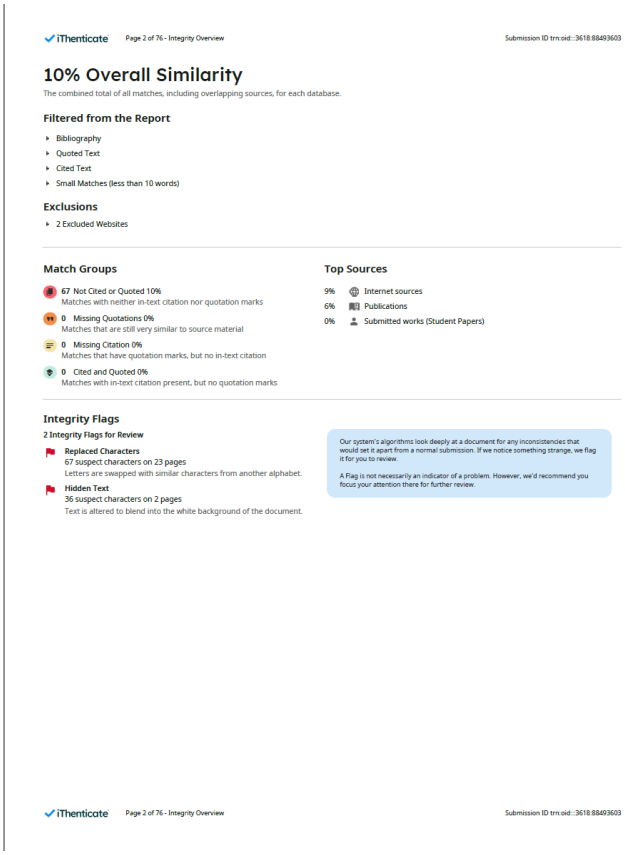

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