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ORIGINAL ARTICLE

Molecular basis of beta thalassaemia intermedia in Pakistan.

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ABSTRACT… Objective: To investigate the primary β-globin gene mutations and their association with secondary genetic modifiers—Xmn-1 polymorphism and BCL11A variant—in patients diagnosed with β-thalassemia intermedia in Pakistan. **Study Design:** Descriptive Cross-sectional study. **Setting:** Fauji Foundation Hospital, Islamabad. **Period:** June 2021 to January 2022. **Methods:** Seventy patients with β-TI were enrolled. DNA was extracted using the Chelex method, and molecular analysis was performed using PCR-RFLP and ARMS-PCR to detect BCL11A (rs11886868) and Xmn-1 polymorphisms, respectively. Statistical analysis was carried out using SPSS version 17. **Results:** The most frequent primary mutations included Cd-15 (14.3%), IVSI-5 (11.4%), and Fr 8-9 (12.9%). Xmn-1 and BCL11A polymorphisms were identified in 37.1% and 71.4% of patients, respectively. Statistically significant associations were found between certain primary mutations (e.g., IVSI-5 and IVSI-5/cap +1) and both secondary modifiers ($p < 0.001$ and $p = 0.005$, respectively). Dual modifier presence was observed in 26% of patients. **Conclusion:** This study reveals considerable molecular diversity in β-thalassemia intermedia in Pakistan. The significant association between specific β-globin mutations and secondary genetic modifiers highlights the complex genotype-phenotype interplay. These findings underscore the need for larger, multi-centric genetic studies to enhance predictive accuracy for clinical management and personalized therapy in thalassemia.

Key words: HBF, Secondary Modifiers, Thalassaemia Intermedia.

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INTRODUCTION

Thalassemia syndrome is the commonest autosomal recessive genetic disorder which is caused due to mutations that resulted in reduction of (β°) or (β+) production of β-globin chains of hemoglobin, a combination of two alpha and two beta globin chains (α2 & β2) required for formation of HbA. There is great molecular heterogeneity having more than 300 different molecular defects.[1] It's a global health problem most commonly affecting Asia, Mediterranean regions and Middle East.[2] The severity of the condition is determined by the type and existence of mutations in either one allele (thalassemia minor) or both alleles (thalassemia major as β-thalassemia).

Patients diagnosed having Beta Thalassemia Major require regular blood transfusions for their survival. Recurrent transfusions bring these patients at great risk for heart problems or liver cirrhosis, and iron overload frequently results in death at age 30 or younger.[3] Thalassemia intermedia is a clinical condition with less severity which is also called as non-transfusion dependent thalassemia (NTDT) and ranges in intensity from transfusion-dependent patients to symptomatic carriers.[4]

Pakistan has a total population of about 225,633,392 (225 million). Both the federal and provincial governments govern Pakistan's healthcare delivery system. In Pakistan, around 5000 children are diagnosed with β-thal major (β-TM), and the prevalence of β-thalassemia (β-thal) trait ranges from 5.0 to 7.0%.[5] Based on inequality between the α/β-globin chain production, thalassemia syndrome are broadly classified as minor, major and intermedia.[3] In β-thalassaemia intermedia combination of different genetic defects leads variable clinical manifestations few combinations are compound heterozygotes of β- and α-thalassemia gene and β+/β+ compound heterozygotes.[4]

Beside β globin chain deficiency imbalance between level of α and gamma (γ) globin chain, co-inheritance

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of α thalassemia or various genetic factors can also lead to phenotypic variability in thalassemic patients. These genetic factors which influence the phenotype of β thalassemia are known as "Genetic Modifiers". The "primary modifier" signifies the varying expression of β thalassemia alleles resulting in phenotypic variation. Whereas secondary modifiers are genetic factors comprised of Co inheritance of alpha (α) globin gene copy or which augment fetal hemoglobin (Hb F) production. Tertiary modifiers are genetic factors which do not have any bearing directly on the globin chain balance but influence the clinical phenotype.[5]

Xmn1 polymorphism is one of the most important secondary modifier.[6] A major challenge faced in most under resourced countries in effective management of β-thalassemia intermedia is the early identification of the phenotype of patients.[7] Predicting phenotypic variability from several genotypic variants in patients with thalassemia has advanced globally.[8]

Although, different studies at international level have emphasized the incidence of different genetic mutations but scarcity in literature have been observed at national level in this context. Our goal in this investigation is to determine the molecular underpinnings of beta thalassemia intermedia, with a focus on the interplay between primary beta globin gene mutations and secondary genetic modifiers.

METHODS

Descriptive cross-sectional study was carried out at the Fauji Foundation Hospital Islamabad. Seventy individuals with a diagnosis of thalassemia intermedia were included in this study. The Fauji Foundation Hospital's ethical review committee granted ethical approval (Ref No.975/RC/FFH/RWP). The study took place between June 2021 and January 2022. Patients with a diagnosis of beta thalassemia intermedia are eligible to apply. Both sexes and all ages were represented. Exclusion criteria: beta thalassemia intermedia was the only thalassemia type that was not included. Five milliliters of venous blood were drawn in an EDTA (Ethylene Diamine Tetra Acetic Acid) container following the acquisition of informed consent and a thorough medical history from each patient. The Chelex method was used to extract deoxyribonucleic acid (DNA). PCR was performed. BCL11A polymorphism: The PCR-RFLP technique was used to identify the single nucleotide polymorphism of rs11886868 in the BCL11A gene (T→C).

The primers 5'-TTTGGTGCTACCCTGAAAGAC-3' and 5'-ACTCAACAGTAGCAGAATGAAAGAG-3 were used to the amplification of a 548 bp fragment. After five minutes of incubation at 37°C, the 548-bp product was digested using the MboII restriction enzyme, and the fragments were separated on 6% polyacrylamide gels. The MboII restriction site is present in the C allele but absent in the T allele. Two 470 bp and 70 bp segments of the C allele were found.Xmn-1 Polymorphism: The Amplification Refractory Mutation System (ARMS) approach was used to accomplish the Xmn-1 polymorphism.

The primers listed below were utilized:Xmn-I-Normal5'-TGCAAATATCTGTCTGAAAC-GATCXmn-I-Mutant5'-TGCAAATATCTGTCT-GAAACGATT Xmn-I-Common5'-CCCATGGC-GTCTGGACTAGA 25 μl reaction mixture comprising 5 pM of each primer, 0.5 units of Taq polymerase (Thermo Fisher Scientific, USA), 30 μM of each dNTP (Thermo Fisher Scientific, USA), 10 mM Tris HCl (pH 8.3), 50 mM KCL, 1.5 mM MgCl2, 100 mg/ml gelatin (Sigma, UK), and 0.3-0.5 μg of genomic DNA was used when performing the PCR for ARMS. Macrogen (Korea) manufactured the primers. The Gene Amp 9700 (ABI, USA) automated DNA thermal cycler was utilized for thermal cycling.

Each of the 25 cycles in the regimen included one minute of denaturation at 94°C, one minute of primer annealing at 65°C, and one and a half minutes of DNA extension at 72°C. The extension reaction was extended for an additional three minutes in the last cycle. For testing, Mini Poly Acrylamide Gels (PAGE) were used. Analysis of statistics: SPSS version 17 was utilized to analyze the data. For qualitative variables such as patient gender and secondary modifiers, frequency and percentages were evaluated. For quantitative variables such as the patient's age, the mean and standard deviation were calculated.

RESULTS

TABLE-I

Baseline characteristics of studied patients (N=70)

Characteristics		n	%
Age Group	<5 years	9	12.9
	5 - 10 years	23	32.9
	11 - 15 years	15	21.4
	16 - 20 years	8	11.4
	21 - 25 years	9	12.9
	>25 years	6	8.6
	Mean (±SD)	13.2	±8.0
Gender	Male	32	45.7
	Female	38	54.3
Age at diagnosis (years)	<5 years	43	61.4
	5 - 10 years	16	22.9
	11 - 15 years	6	8.6
	16 - 20 years	5	7.1
	Median (Range)	4	1 – 25

Table-I reports the baseline characteristics of studied patients, in the present study among seventy patients mean age was 13.2 (SD=±8.0) years, Age group less than five years were (12.9%), from 5 - 10 years were (32.9%), from 11 - 15 years were (21.4%), from 16 - 20 years were (11.4%),from 21 - 25 years were (12.9%), and aged >25 years were (8.6%), Male were (45.7%), Female were (54.3%), patients with age at diagnosis less than 5 years were (61.4%), from 5 - 10 years were (22.9%), from 11 - 15 years were (8.6%), from 16 - 20 years were (7.1%), median age of diagnosis was 4 years with range from 1 – 25 years.

FIGURE-1

Molecular basis of beta globin gene mutations in thalassemia intermedia phenotype

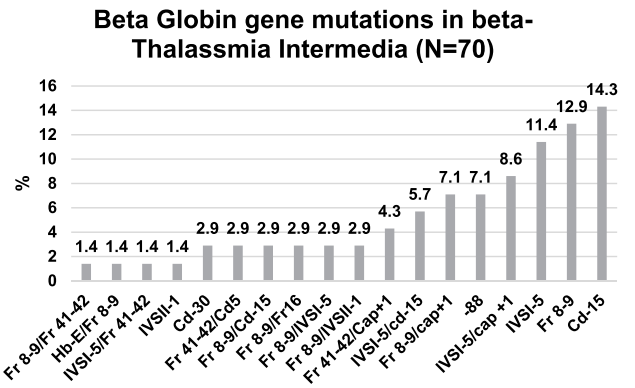

Figure-1 reports the frequency of primary mutations among beta thalassmia intermedia patients, -88 were (7.1%), Cd-15 were (14.3%), Cd-30 were (2.9%), Fr 41-42/Cap+1 were (4.3%), Fr 41-42/Cd5 were (2.9%),Fr 8-9 were (12.9%), Fr 8-9/cap+1 were (7.1%), Fr 8-9/Cd-15 were (2.9%), Fr 8-9/Fr 41-42 were (1.4%),Fr 8-9/Fr16 were (2.9%),Fr 8-9/IVSI-5 were (2.9%), Fr 8-9/IVSII-1 were (2.9%), Hb-E/Fr 8-9 were (1.4%), IVSI-5 were (11.4%), IVSI-5/cap +1 were (8.6%), IVSI-5/cd-15 were (5.7%), IVSI-5/Fr 41-42 were (1.4%), IVSII-1 were (1.4%), and Undetermined were (5.7%) cases.

FIGURE-2

Secondary Modifiers in beta-Thalassmia Intermedia

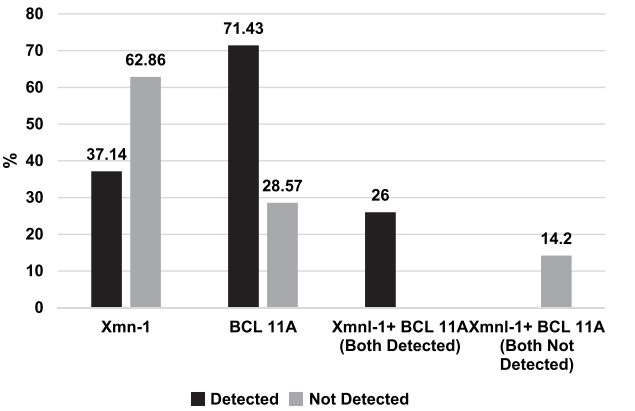

Figure-2 reports the frequency of secondary modifiers in beta Thalassemia Intermedia, results showed among 70 patients 26(37.14%) Xmn-1 and 50(71.43%) BCL 11A were detected, among 18(26%) cases both Xmn-1 and BCL 11A were detected whereas among 8(14.2%) none of the modifier was detected.

Table-II reports the association of primary mutation with Xmn-1, results showed in Xmn-1 detected cases in primary mutations Fr 41-42/Cd5 were (7.7%), Fr 8-9/cap+1 were (7.7%), Fr 8-9/Fr16 were (7.7%), Fr 8-9/IVSII-1 were (7.7%), Hb-E/Fr 8-9 were (3.8%), IVSI-5 were (30.8%), IVSI-5/cap +1 were (15.4%), IVSI-5/Fr 41-42 were (3.8%), IVSII-1 were (3.8%), and Undetermined were (11.5%). Fishers exact test did give significant association of Xmn-1 detected cases with primary mutations (p<0.001)

TABLE-II

Association of primary mutation with XMN-1

Primary Mutation	Xmn-1				P-Value
	Detected		Not Detected		
	n	%	n	%	
-88	0	0.0	5	11.4	
Cd-15	0	0.0	10	22.7	
Cd-30	0	0.0	2	4.5	
Fr 41-42/Cap+1	0	0.0	3	6.8	
Fr 41-42/Cd5	2	7.7	0	0.0	
Fr 8-9	0	0.0	9	20.5	
Fr 8-9/cap+1	2	7.7	3	6.8	
Fr 8-9/Cd-15	0	0.0	2	4.5	
Fr 8-9/Fr 41-42	0	0.0	1	2.3	
Fr 8-9/Fr16	2	7.7	0	0.0	<0.001*
Fr 8-9/IVSI-5	0	0.0	2	4.5	
Fr 8-9/IVSII-1	2	7.7	0	0.0	
Hb-E/Fr 8-9	1	3.8	0	0.0	
IVSI-5	8	30.8	0	0.0	
IVSI-5/cap +1	4	15.4	2	4.5	
IVSI-5/cd-15	0	0.0	4	9.1	
IVSI-5/Fr 41-42	1	3.8	0	0.0	
IVSII-1	1	3.8	0	0.0	
Undetermined	3	11.5	1	2.3	

*p<0.05 was considered statistically significant using Fishers Exact test

TABLE-III

Association of primary mutation with BCL 11A

Primary Mutation	BCL 11A				P-Value
	Detected		Not Detected		
	n	%	n	%	
-88	4	8.0	1	5.0	
Cd-15	8	16.0	2	10.0	
Cd-30	2	4.0	0	0.0	
Fr 41-42/Cap+1	2	4.0	1	5.0	
Fr 41-42/Cd5	0	0.0	2	10.0	
Fr 8-9	5	10.0	4	20.0	
Fr 8-9/cap+1	2	4.0	3	15	
Fr 8-9/Cd-15	2	4.0	0	0.0	
Fr 8-9/Fr 41-42	0	0.0	1	5.0	
Fr 8-9/Fr16	0	0.0	2	10.0	0.005*
Fr 8-9/IVSI-5	2	4.0	0	0.0	
Fr 8-9/IVSII-1	0	0.0	2	10.0	
Hb-E/Fr 8-9	1	2.0	0	0.0	
IVSI-5	8	16.0	0	0.0	
IVSI-5/cap +1	6	12.0	0	0.0	
IVSI-5/cd-15	4	8.0	0	0.0	
IVSI-5/Fr 41-42	1	2.0	0	0.0	
IVSII-1	1	2.0	0	0.0	
Undetermined	2	4.0	2	10.0	

*p<0.05 was considered statistically significant using Fishers Exact test

Table-III reports the association of primary mutation with detected cases of BCL 11A, results showed among detected cases of BCL 11A in primary mutation -88 were (8%), Cd-15 were (16%), Cd-30 were (4%), Fr 41-42/Cap+1 were (4%), Fr 8-9 were (10%),Fr 8-9/cap+1 were (4%), Fr 8-9/Cd-15 were (4%), Fr 8-9/IVSI-5 were (4%), Hb-E/Fr 8-9 were (2%), IVSI-5 were (16%), IVSI-5/cap +1 were (12%), IVSI-5/cd-15 were (8%), IVSI-5/Fr 41-42 were (2%), IVSII-1 were (2%) and Undetermined were (4%). The association between these two was considered statistically significant with p=0.005 was obtained using Fisher's Exact test.

DISCUSSION

β-thalassemia intermedia comprised of genetically and phenotypically heterogeneous clinical conditions with severity ranges from mild-to-moderate anemia and variability in transfusion requirement.[9] Primary determinant of clinical severity of Thalassemia intermedia is β-globin gene mutation. However, clinical heterogeneity is also dependent on many genetic modifiers that modulates the level of globin chain imbalances, and therefore, the degree of ineffective erythropoiesis.[10,11]

Severity of illness of B-Thalassemia syndrome is also affected by improvement in α-globin chain to β-globin chain balance, either by the production of β-like globin or by reducing the production of α-globin genes. It has been discovered that co-inheritance of the α-thalassemia trait (-α/αα, --/αα, -α/-α) improves the clinical symptoms of β-thalassemia and, in rare instances, eliminates the requirement for lifelong transfusions in thalassemia.[12,13] Genetic modifiers improve clinical symptoms by increasing the production of the γ-globin chain, which protects against the harmful effects of an excess α-globin gene.[13,14]

Numerous genome editing techniques, which have been widely used in recent decades, have been shown to either reactivate HbF[15] or fix mutations that cause thalassemia. Among them, certain DNA double-strand breaks (DSBs) are produced by three generations of programmable nucleases: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-associated nuclease Cas9 (CRISPR-Cas).[16] Clinical

trials are showing encouraging outcomes from advanced gene therapies that aim to reactivate HbF in autologous HSPCs by ex vivo CRISPR/Cas9 gene-editing targeting the erythroid enhancer region of BCL11A.[17]

Current research findings not only reflect the frequency of primary mutations and secondary modifiers present in thalassemia intermedia in Pakistan but it signifies the association of primary mutations with secondary modifiers. There are currently no research available worldwide that offer statistics regarding the relationship between primary and secondary modifiers. The age range in our study was 3–30 years old, with a mean age of 13.2 ± 8.0 years. The majority of the 23 patients (32.9%) were in the 5–10 age range. Thirty-two (45.7%) and thirty-eight (54.3%) of the 70 thalassemia intermedia cases were male.

In a mutational analysis conducted by Hariharan et. al commonest mutation found was IVS 1–5, mutation experienced in both the β-thalassemia homozygous group (TM: 32%, TI: 28%). Other mutations including codons 8/9, 619 bp deletion IVS 1-1, codon 15 and codons 41/42 considered for 82.5% of molecular lesions (TM: 44.5%, TI: 38%).[18] It is in contrast to our study in which commonest mutation found was Cd-15 (14.3%). Other mutations found were -88 (7.1%), Cd-30 (2.9%), Fr 41-42/Cap+1 (4.3%), Fr 41-42/Cd5 (2.9%), Fr 8-9 (12.9%), Fr 8-9/cap+1 (7.1%), Fr 8-9/Cd-15 (2.9%), Fr 8-9/ Fr 41-42 (1.4%), Fr 8-9/Fr16 (2.9%), Fr 8-9/IVSI-5 (2.9%), Fr 8-9/IVSII-1 were (2.9%), Hb-E/Fr 8-9 were (1.4%), IVSI-5 were (11.4%), IVSI-5/cap +1 were (8.6%), IVSI-5/cd-15 (5.7%), IVSI-5/Fr 41-42 (1.4%), IVSII-1 (1.4%), and Undetermined were (5.7%) cases.

Study conducted by Hassan et al showed that commonest mutations were Cap +1, Fr 8-9, IVS1-5 and del 619, respectively which were identified as homozygosity and compound heterozygous mutations. It is also in contrast to our study according to which most common primary mutation is Cd-15.[19] This difference may be due different sample size and different sample population.

In our study mutational analysis was performed only in thalassemia intermedia patients whereas the study conducted by Priya Hariharan included patients of thalassemia major as well as of sickle cell anemia. Also in study by Hariharan[18], the homozygosity for the mutant T allele T/T, Xmn I+/+] was found to be substantially greater in TI (44.0%) than in TM (28.0%) in the β-thalassemia homozygous group (P: 0.01). Whereas in our study frequency of secondary modifiers Xmn-1 in beta Thalassemia Intermedia found out to be 37.14%.[18]

In a study conducted in District Bannu total 250 patients of beta thalassemia intermedia were checked by ARMSPCR for six B-thalassemia mutations.

Results of study revealed that frame shift codons (FSC) 8/9, the most common mutation. Other mutations found to be Codons 41/42, IVS-I-5 and FSC 5 having frequencies of 42%, 26%, 19% and 13% respectively. However, this study could not report codon 15 & IVS-I- which was found to be the most common mutation according to our study.[20]

83.3% of β-thalassemia intermedia patients were heterozygous for the XmnI polymorphism, according to an Egyptian study. These patients with a single T allele of XmnI had milder disease, a delayed diagnosis, and higher HbF levels than patients who were negative for the XmnI polymorphism.[21] Whereas in our study secondary mutations Xmnl-I Homozygous were (10%), Heterozygous were (25.7%).[24]

A research in northern Iran found that 86% of people had Xmn1 gene polymorphism, either at one locus (-/+, 21%) or both loci (+/+, 65%).[22] The frequency of Xmn1 gene polymorphism in the southeast of Iran was 62%, according to another Iranian study by Miri-Moghaddam et al.[23] This may be because patients from different countries and even different regions within the same countries have varied geographical distributions of the Xmn1 polymorphism.

In our study it has been found that primary mutations highly associated with Xmn-1 polymorphism are IVSI-5 (30.8%), IVSI-5/cap +1 (15.4%), whereas association of other primary mutations was low with Xmn-1 polymorphism. These mutations are Fr

41-42/Cd5 (7.7%), Fr 8-9/cap+1 (7.7%), Fr 8-9/Fr16 (7.7%), Fr 8-9/IVSII-1 (7.7%), Hb-E/Fr 8-9 (3.8%), IVSI-5/Fr 41-42 (3.8%), IVSII-1 (3.8%), and Undetermined (11.5%).

In our study Primary mutations which are highly associated with BCL 11 A are IVSI-5 (16%), Cd-15 (16%) and IVSI-5/cap +1 (12%). Whereas other primary mutations associated with BCL 11 A were found to be Cd-30 (4%), Fr 41-42/Cap+1 (4%), Fr 8-9 (10%),Fr 8-9/cap+1 (4%), Fr 8-9/Cd-15 (4%), Fr 8-9/IVSI-5 (4%), Hb-E/Fr 8-9 (2%), IVSI-5/cd-15(8%), IVSI-5/Fr 41-42 (2%), IVSII-1(2%) and Undetermined were (4%).

Our findings indicate that certain primary mutations, particularly IVSI-5 and its combinations, show a relatively higher association with both Xmn-1 polymorphism and BCL11A variants, suggesting a potential modifying effect on clinical phenotype. However, the overall distribution of secondary modifiers across other mutations appears scattered and inconsistent. This variability, alongside the notable proportion of undetermined cases, highlights the complexity of genotype-modifier interactions and suggests that additional genetic or epigenetic factors may be involved. The limited dataset underscores the need for larger cohort studies and functional analyses to better elucidate the role of secondary modifiers in modulating disease severity, especially in compound heterozygotes or less common mutation profiles.

CONCLUSION

In summary, while our current understanding of the interplay between the primary mutation and potential secondary modifiers remains limited, the available evidence highlights the complexity of genotype-phenotype relationships. The scarcity of comprehensive data underscores the need for larger, well-characterized cohorts and integrative approaches that combine genetic, epigenetic, and environmental factors. Future studies are essential to unravel the modifying effects that may influence disease presentation and progression, ultimately guiding more precise diagnostic and therapeutic strategies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORSHIP AND CONTRIBUTION DECLARATION	
1	**Fariha Nasreen:** Literature search, Conceptualization of study.
2	**Asma Shaikh:** Literature search.
3	**Madeeha Rehan:** Data collection.
4	**Fatima Iqbal:** Data analysis.
5	**Zareen Irshad:** Proof reading.
6	**Aqsa Noureen:** Literature search.