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Research Article

To Govern the Pervasiveness of Xmn-1Gγ Gene Polymorphism: A Significant Determinant in Children with β-Thalassemia Major and its Effect on Clinical Phenotype

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Abstract

Background: Beta-thalassemia is the most common monogenic blooddisorder with >200 mutations. The severity of β-thalassemia is directly related to globlin chain imbalance. This imbalance can be reduced by γ-chain production, which combines of with excess α chain to form fetal Hb (HbF). The increase HbF is genetically determined as well as partiality associated with β-haplotypes which shows particular microsatellite sequence or the Xmn 1γG gene polymorphism sequence. In this study, we aim to evaluate the prevalence and impact of Xmn-1 γG gene polymorphism on the clinical phenotype of these children. **Methods:** In this prospective observational study, total 101 diagnosed Beta thalassemia children were included. For five common mutations [IVS I-5 (G→C), -619 bp deletion, IVS I-1 (G→T), Cd 41/42 (-CTTT) and Cd 8/9 (+G)] screening multiplex ARMS PCR was used.The polymerase chain reaction–restriction fragment-length polymorphism (PCR–RFLP) was genotype of Xmn 1 gene. The Chi-square test and t- test were used for statistical analysis. **Results:** Out of 101, Total 79 had Thalassemia major, while 22 had Thalassemia intermedia. Among the 22 children with Thalassemia Intermedia, 12 had E-beta thalassemia and 6 had S-beta thalassemia and 4 had others.Out of 79 children with thalassemia major, a common mutation could be identified in 53 (67.08%) in which IVS I-5 G→C was the commonest mutation. Average transfusion (per year) was significantly lower in homozygous mutated TT genotype as compared to homozygous CC and heterozygous CT genotypes. The C and T allele frequencies of Xmn-1γG gene polymorphism were 85 (80.19%) and 21 (19.81%) in common Mutation. **Conclusion:** The common mutation could be identified in 61.3% children with beta thalassemia and IVS I-5 G→C was the most prevalent mutation. Among the homozygous mutant TT genotype, the average transfusion frequency was significantly lower as compared to CT and CC genotypes.

Keywords: Beta-Thalassemia; Globlinchain; Mutation; Gene polymorphism; PCR

Introduction

Thalassemia syndromes are a group of hereditary blood disorders and the most common form of chronic hemolytic anemia. It is characterized by impaired synthesis of globlin chain that is either absent or reduced in amount. It is one of most common autosomal recessive disorders found worldwide. Children with beta (β) thalassemia have a defect in beta globin chain synthesis, while those with alpha (α) thalassemia have impaired synthesis of α globin chain [1].

Beta-thalassemia is majorly divided into three forms: Thalassemia Major, variably known as "Cooley's Anemia" and "Mediterranean Anemia", Thalassemia Intermedia and Thalassemia Minor also reffered as beta-thalassemia carrier/beta-thalassemia trait. Apart from the rare dominant forms, subjects with thalassemia major may be homozygotes or compound heterozygotes for beta0 or beta+ genes. Individuals with thalassemia intermedia are mostly homozygotes or compound heterozygotes and persons with thalassemia minor are mostly heterozygotes [2].

It is the most common monogenic disorder and more than 200 mutations have been so far identified as causative for Thalassemia. It has been observed that the large majority are point mutations in functionally important regions of the beta globin gene [2,3]. Deletions of the beta globin gene are rare. Population migration and intermarriage between different ethnic and religion groups has introduced thalassemia in almost every corner of the world. In Indian population, a total number of about 30 different mutations have been reported till date. Studies from several parts of India showing that IVS-1-5 (G-C), IVS-1-1 (G-T), 619 bp deletion, CD 8/9 (+G) and CD41/42 (-CTTT) are the common mutations, present in varying frequencies among different states. It has been roughly estimated that about 3% of the Indian population are carriers of beta-thalassemia, with about 10,000-15,000 symptomatic subjects born annually [5]. The most common combination of beta-thalassemia with abnormal Hb or structural Hb variant is HbE/beta-thalassemia, which is most prevalent in Southeast Asia where the estimated carrier frequency may reach as high as 50% [6].

Individuals with thalassemia major usually come to medical attention in early life mostly within the first 6 months to 2 years of life and require frequent RBC transfusions to survive. Thalassemia intermedia include patients who usually present later and do not require regular transfusion for survival [2]. Severity of β-thalassemia is directly related to globlin chain imbalance. This imbalance can be reduced by γ-chain production, which combines of with excess α chain to form fetal Hb (HbF) [7,8]. This happens inheritably in all patients of β thalassemia syndrome resulting in relatively high HbF level in patients of β thalassemia. Children with β thalassemia differ considerably in their ability to synthesize γ-chain leading to variability in levels of HbF. Factors, which lead to elevated HbF decrease the globin imbalance thus leading to milder clinical phenotype of thalassemia [8].

Many studies are in progress to understand the molecular basis of β –thalassemia to help in predicting possible phenotype from genotype [9]. It has been found that variable phenotypes may develop depending on the type of β-globlin gene mutation, α gene interaction or difference in the amount of fetal Hb production.Increase in HbF has an ameliorating effects so despite being homozygous (β^o,β^o) and compound heterozygous (β^+,β^o) patients shows milder form of disease. This increase in HbF is genetically determined as well as partiality associated with β-haplotypes which shows particular microsatellite sequence or the Xmn 1 polymorphism sequence. The Xmn 1Gγ polymorphism (HBG2c211 C→T) that is C>T polymorphism at 158 bp upstream Gc gene may affect the production of HbF in β thalassemia patients. During the haemopoietic stress the presence of Xmn polymorphic allele may activate the production of HbF leading to amelioration of phenotypes [9,10].This polymorphism (Xmn-1) has not been well studied in children with beta thalassemia from Uttar Pradesh and North India. With this study, we attempt to find the molecular basis of beta thalassemia in children from North India and observe the prevalence and impact of Xmn-1 γG gene polymorphism on the clinical phenotype of these children.

Materials and Methods

This prospective observational study was conducted in the Thalassemia Clinic of the Department of Pediatrics in our University. A written informed consent was obtained from patients, as per the institute guidelines. Ethical approval from Institutional Ethics Committee at K. G. Medical University, Lucknow was obtained before the commencement of study (Ethical approval ref no. 93rd ECM II B-Thesis/P41). Patients with ≤21 years age, haematological diagnosis of chronic hemolyticanemia based on complete blood count and general blood picture and confirmed diagnosis of beta thalassemia on haemoglobin analysis by Capillary electrophoresis or High power liquid chromatography (HPLC) were included in this study. Patients with alpha thalassemia and other haemolytic anaemias were excluded from the study. The sample Size was calculation on the basis of prevalence of minor allele frequency (homozygous 'TT') of Xmn-1 genotype of 6.9% (Sharma et al), precision of 5%, 95% confidence, and sample size calculated was 99.

Sample collection and DNA isolation

Blood samples were collected by venipuncture in EDTA tubes. DNA was extracted from whole blood using the DNA extraction kit according to the manufacturers' instructions.

Molecular Screening of beta globin gene

For five common mutations [IVS I-5 (G→C), -619 bp deletion, IVS I-1 (G→T), Cd 41/42 (-CTTT) and Cd 8/9 (+G)] screening multiplex ARMS PCR was used. The primers details are shown in Table 1. Polymerase Chain Reaction (PCR) was performed in a total volume of 15 µl in 0.2-ml PCR tube consisting 1x Master mixture, 1µl template DNA (~40 ng/µl), 1 µl of each primer (Both internal controls primers, one reverse primer and one mutation specific primer) (10 µM), 1 µl $MgCl_2$ and DNase-free water. The PCR amplification (gradient PCR) was carried out by an initial denaturation at 96°C for 3 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 63.9°C for 45 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The amplified ARMS PCR products were analyzed by electrophoresis on ethidium bromide (0.5 µg/ml) containing 1.5% agarose gel and visualized under gel documentation.

Mutation	Nucleotide sequence	Tm (°C)	Product Size
IVS1-1 M	5'-TTAAACCTGTCTTGTAACCTTGATACGAAA	56	281 bp
IVS1-5 M	5' -CTCCTTAAACCTGTCTTGTAACCTTGTTAG	59	285 bp
Cd 8/9 M	5'-CCTTGCCCCACAGGGCAGTAACGGCACACC	70	225 bp
Cd 41/42 M	5'-GAGTGGACAGATCCCCAAAGGACTCAACCT	64	439 bp
619 bp del	5′-GAG TCA AGG CTG AGA GAT GCA GGA-3	61	242 bp
Internal Control C1	5'-CAATGTATCATGCCTCTTTGCACC	56	861 bp
Internal Control C2	5'-GAGTCAAGGCTGAGAGATGCAGGA	59	
Reverse Primer B1	5'-ACCTCACCCTGTGGAGCCA	58	
Reverse Primer B2	5' -CCCCTTCCTATGACATGAACTTAA	54	

Table 1: Primers for β-globin common mutations [26].

Genotype of Xmn 1 gene

Genomic DNA was analyzed by polymerase chain reaction–restriction fragment-length polymorphism (PCR–RFLP). The primer set used for DNA amplification was 5′-AAC TGT TGC TTT ATA GGA TTT T-3′ and 5′-AGG AGC TTA TTG ATA ACT CAG AC-3′. The PCR products were digested with the *Xmn1* restriction enzyme. Digestion products were electrophoresed on a 3% agarose gel. Amplification with the primers produced a 650 bp fragment in the wild genotype; the heterozygous genotype gave 2 bands at 450 bp and 200 bp (Figure 1).

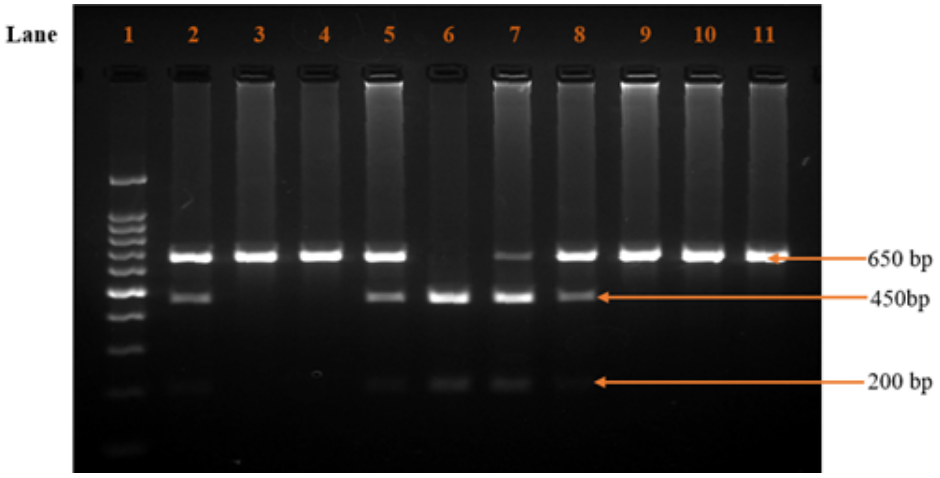

Figure 1: GγXmnI polymorphism: polymerase chain reaction–restriction fragment-length polymorphism (PCR–RFLP) products were 650 bp, 450 bp and 200 bp.Lane 1: DNA ladder; Lane 3, 4, 9-11: Homozygous CC genotype; Lane 2,5, 7, 8: Heterozygous CT genotype; Lane 6: Homozygous Mutation TT genotype.

Statistical Analysis

Normally distributed data were summarised by using mean and standard deviation and data which are not normally distributed were summarised by median and interquartile range. Categorical variables in two groups were compared using the Chi-square test. Continuous variables in two groups were compared by t- test. For comparing mean between more than two groups ANOVA was used. The p-value<0.05 was considered significant. All the analysis was carried out using SPSS 16.0 version (Chicago, Inc., USA).

Results

Total 115 children were assessed for eligibility, and of these 101 children were diagnosed as Beta thalassemia and were included in the study after taking their informed consent. Of the remaining 14 children, 2 were diagnosed as Congenital DyserythropoeiticAnemia (CDA), 1 was diagnosed as Sickle Cell Disease (SCD), 1 was diagnosed as Nutritional anaemia, and one as Transient Erythroblastopenia of Childhood (TEC). The remaining 9 children were diagnosed as chronic hemolyticanemia, but the aetiology could not be established.

Table 2 summarizes the baseline characteristics of the 101 children with Beta Thalassemia included in the study. Out of 101, 98 children had received blood transfusion at least once. The mean age at 1st blood transfusion was 15.37±22.21 months. Mean age of patients at included in this study was 30.81±33.48. A total of 76 (75.25%) children were male and 25 (24.75%) were female. Out of 101, total 61 (60.40%) patients belonged to Hindu religion, whereas 40 (39.60%) belonged to Muslim religion. Total 79 had Thalassemia major, while 22 had Thalassemia intermedia. Among the 22 children with Thalassemia Intermedia, 12 had E-beta thalassemia and 6 had S-beta thalassemia and 4 had others. The phenotypic distribution of enrolled children with Thalassemia is shown in Table 3 and Figure 1.

	Mean±SD
Age at 1st blood transfusion (months)	15.37±22.21
Age (months)	30.81±33.48
Sex	**n (%)**
Male	76 (75.25%)
Female	25 (24.75)
Religion	
Hindu	61 (60.40%)
Muslim	40 (39.60%)
Beta Thalassemia major	79 (78.2%)
Beta Thalassemia Intermedia	22 (21.8%)
E-beta Thalassemia	12 (54.55%)
S-beta Thalassemia	6 (27.27%)
Others	4 (18.18%)

Table 2: Basic Characteristics of Patients (n=101).

Table 3 shows the frequency distribution of the identified common mutations in children with beta thalassemia major and inermedia. Out of 79 children with thalassemia major, a common mutation could be identified in 53 (67.08%). Out of 53 common mutations, 40 (75.47%) were homozygous mutations and 13 (24.53%) were heterozygous mutations. IVS I-5 G→C was the commonest mutation identified both in the homozygous as well as the heterozygous state. It accounted for 34 of the 40 identified mutations in homozygous state, and all the children with heterozygous mutations had IVS I-5 G→C on one allele. Of all the 53 children beta thalassemia major with a common mutation,

IVS I-5 G→C alone accounted for 47 (88.68%). Other common mutations which were identified in a homozygous state were: Cd 8/9 +G (3.77%), Cd 41/42 (-TCTT) (3.77%), IVS 1-1 G→T (1.89%) and -619 bp Deletion (1.89%). Out of 22 children with thalassemia intermedia, a common mutation could be identified in 9 (40.9%).

Beta Thalassemia major (n=79)			
		Types of total mutation	**Number of patients n (%)**
Common Mutation			**53 (67.08)**
	Homozygous n=40 (75.47%)	IVS 1-1 G→T	1 (1.89%)
		IVS I-5 G→C	34 (64.15%)
		Cd 8/9 +G	2 (3.77%)
		Cd 41/42 (-TCTT)	2 (3.77%)
		-619 bp Deletion	1 (1.89%)
	Heterozygous n=13 (24.53%)	IVS I-5 G→C and Cd 8/9	5 (9.43%)
		IVS I-5 G→C and Cd 41/42	1 (1.89%)
		IVS I-5 G→C and IVS 1-1 G→T	4 (7.54%)
		IVS I-5 G→C and unidentified mutation	3 (5.66%)
Unidentified Mutation			26 (32.92%)
β-Thalassemia Intermedia (n=22)			
Common Mutation (n=9)	**Homozygous (n=1)**	**IVS I-5 G→C**	1 (11.11%)
	Heterozygous (n=8)	S-Beta Thalassemia IVS I-5 G→C and HbS	2 (22.22%)
		E-Beta Thalassemia IVS I-5 G→C and HbE	6 (66.67%)
Unidentified Mutation (n=13)		**β-Thal**	3 (23.07%)
		HbS	4 (30.77%)
		HbE	6 (46.15%)

Table 3: Distribution of common mutation in Beta Thalassemia major and Intermedia patients.

Out of 9 common mutations, 8 were heterozygous mutations and 1 was homozygous mutation. Like beta thalassemia major, IVS I-5 G→C was the commonest mutation identified in the Thalassemia intermedia subgroup as well. Apart from IVS I-5 G→C, other 4 common mutations were not found in children with thalassemia intermedia.

Table 4 shows the distribution of Genotype frequencies of Xmn-1 γG gene polymorphism in Beta Thalassemia Major and Intermedia Patients. The frequency distribution of homozygous CC, heterozygous CT and homozygous mutated TT genotypes of Xmn-1 gene polymorphism in enrolled children with beta thalassemia was 69 (68.3%), 28 (27.7%) and 4 (3.9%) respectively. It was 54 (68.35%), 21 (26.59%) and 4 (5.06%) in thalassemia major children, whereas 15 (68.18%), 7 (31.82%) and 0 (0.0%) in beta thalassemia intermedia, respectively. The genotype frequencies of Xmn-1 gene polymorphism were not statistically different in Beta Thalassemia major and Intermedia. The allele frequency of wild allele 'C' was 166 (82.2%), while that of the mutated allele 'T' was 36 (17.8%) for the entire cohort comprising of 202 alleles of Xmn-1 gene. The C and T allele frequencies were 129 (81.65%) and 29 (18.35%) in Beta Thalassemia Major whereas 37 (84.01%) and 7 (15.91%) in Beta Thalassemia Intermedia, respectively. The allele frequencies of Xmn-1 gene polymorphism were not statistically different in Beta Thalassemia major and Intermedia.

	Beta Thalassemia Major (n=79)	Beta Thalassemia Intermedia (n=22)	OR (95%CI)	p-Value
Homozygous CC	54 (68.35%)	15 (68.18%)	-	Ref.
Heterozygous CT	21 (26.59%)	7 (31.82%)	1.20 (0.43-3.36)	0.936
Homozygous Mutation TT	4 (5.06%)	0 (0.0)	0.39 (0.020-7.66)	0.682
Allele 'C'	129 (81.65%)	37 (84.01%)	-	Ref.
Allele 'T'	29 (18.35%)	7 (15.91%)	0.84 (0.34-2.08	0.879

Table 4: Distribution of Genotype frequencies of Xmn-1 γG gene polymorphism in Beta Thalassemia Major and Intermedia Patients.

Table 5 shows the effect of Xmn-1 genotype on phenotype in Beta Thalassemia Major and Intermedia Patients. The homozygous CC genotype of Xmn-1 gene polymorphism, the average transfusion (per year), age of first transfusion (months), Hb and HbF were 18.00±2.06, 8.30±10.24, 4.84±1.51 and 60.64±30.59. In Heterozygous CT genotype of Xmn-1 gene polymorphism, the average transfusion (per year), age of first transfusion (months), Hb and HbF were 16.76±2.14, 11.24±12.55, 4.74±1.19, and 68.23±25.06, respectively. In Homozygous mutated TT genotype of Xmn-1 gene polymorphism, the average transfusion (per year), age of first transfusion (months), Hb and HbF were 12.75±1.50, 11.25±6.95, 4.00±1.29 and 90.23±5.64, respectively. Average transfusion (per year) was significantly lower in homozygous mutated TT genotype as compared to homozygous CC and heterozygous CT genotypes. The C and T allele frequencies of Xmn-1 gene polymorphism were 85 (80.19%) and 21 (19.81%) in Common Mutation whereas 44 (84.62%) and 8 (15.38%) in Unidentified Mutation of Beta Thalassemia Major, respectively. The allele frequencies of Xmn-1 gene polymorphism were not statistically different in Common Mutation and Unidentified Mutation ofBeta Thalassemia major.

	Homozygous CC [n=54 (68.35%)	Heterozygous CT [n=21 (26.59%)]	Homozygous Mutation TT [n=4 (5.06%)]	p-Value
Beta Thalassemia Major				
Common Mutation	36 (67.92%)	13 (24.53%)	4 (7.55%)	0.338
Unidentified Mutation	18 (69.23%)	8 (30.77%)	0 (0.0%)	
Average Transfusion (per year)	18.00±2.06	16.76±2.14	12.75±1.50	**<0.001***
Age of First Transfusion (months)	8.30±10.24	11.24±12.55	11.25±6.95	0.532
Hb	4.84±1.51	4.74±1.19	4.00±1.29	0.521
HbF	60.64±30.59	68.23±25.06	90.23±5.64	0.109
Beta Thalassemia Intermedia				
Common Mutation	7 (100%)	0 (0.0%)	0 (0.0%)	0.029*
Unidentified Mutation	8 (53.33)	7 (42.67%)	0 (0.0%)	

Average Transfusion (per year)	8.40±7.26	9.57±7.02	-	0.726
Age of First Transfusion (months)	31.69±20.35	60.83±54.82	-	0.103
Hb	5.75±1.60	6.20±1.20	-	0.514
HbF	19.36±11.17	28.35±20.13	-	0.286
[1]=ANOVA, *=Significant ($p<0.05$)				

Table 5: Effect of Xmn-1 genotype on phenotype in Beta Thalassemia Major and Intermedia Patients.

Among the 22 children with thalassemia intermedia, no patient with homozygous mutated allele TT was identified. While 15 children had the genotype homozygous CC, the remaining 7 had the genotype CT. The frequency of homozygous CC and heterozygous CT genotype of Xmn-1 gene polymorphism were 7 (100%) and 0 in common mutation whereas 8 (53.33) and 7 (42.67%) in unidentified mutation. The genotypes of Xmn-1 gene polymorphism were statistically different in between common and unidentified mutation. However, there was no difference between the CC and CT groups with respect to average transfusion per year, age at first transfusion, Hb and HbF levels in children with thalassemia intermedia. The C and T allele frequencies of Xmn-1 gene polymorphism were 14 (100%) and 0 (0.0%) in Common Mutation whereas 23 (76.67%) and 7 (23.33%) in Unidentified Mutation of Beta Thalassemia intermedia, respectively as shown in Table 5. The allele frequencies of Xmn-1 gene polymorphism were not statistically different in Common Mutation and Unidentified Mutation of Beta Thalassemia Intermedia.

Discussion

The present study was carried out to know the prevalence of common beta globin mutations (IVS I-5 (G→C), IVS I-1 (G→T), Cd 41/42 (-CTTT), Cd 8/9 (+G), -619 bp deletion) in 101 children with Beta thalassemia and to study the prevalence of Xmn-1 γG gene polymorphism in these children. Mutation could be identified in 62 (61.4%) of children with beta thalassemia. IVS I-5 (G→C) was the most common mutation identified, seen in 59.5% (47out of 79) of Thalassemia Major and 41% (9 out of 22) of Thalassemia Intermedia children. On studying the Xmn-1 polymorphism, we found that the allele frequency of the mutated phenotype 'T' was 17.8%, while that of the wild phenotype 'C' was 82.18% Children with homozygosity for the mutated gene (TT) had significantly lower blood transfusion requirement as compared to the homozygous wild (CC) and compound heterozygotes (CT).

In this study the maximum numbers of beta thalassemia patients (67.32%) were in 1-24 month age group. Similarly, a study reported that the mean age was17.2±19.9 months, with 50% being diagnosed within the first year of life in Indian population [11]. Our study is also supported by Cao and Galanello, (2000), who reported that the mean age of children with thalassemia to be 8.4±9.1 months [12]. In our study cohort, the mean age of 1st blood transfusion was 9.2 months for children with Thalassemia major. This is in concordance with reports from worldover, which suggest that children with thalassemia major start needing blood transfusions from infancy. In a study it was found that 60% of thalassemia major patients presented by 6 months of age [13]. The reason for this age of presentation in children with beta thalassemia major is the deficient production of beta globin chain. After 6 months of age HbA becomes the predominant type of hemoglobin. This HbA is comprised of 2 alpha and 2 beta chains. In children with beta thalassemai major, as the beta chain is deficient so HbA is not formed and hence anemia develops by 6 months of age.

In our study, we found that the Male: Female ratio was 3:1. There have been similar reports of male preponderance from other parts of India as well. Previous study reported a Male: Female ratio was 2:1 in their patient cohorts [14,15]. Another study from Western India also reported that the Beta thalassemia was more common in male 62% as compare to female 38% [16]. A study reported that the prevalence of beta thalassemia was slightly greater in male. There is no genetic basis for thalassemia being more common in males [17]. The probable reason for higher Male: Female ratio is the general social outlook in India, where female children are ignored and boys are given more attention by the family than girls.

In our study cohort, out of the 101 children with beta thalassemia, 22 had thalassemia intermedia. While children with beta thalassemia major require lifelong regular blood transfusion for survival, children with thalassemia intermedia may require occasional or frequent transfusions in certain clinical settings for limited period of time. Thalassemia intermedia can be caused due to mutations in both the beta globin genes or due to co-inheritance of one beta globin gene mutation with a structural variant of Hb

like HbE (E-Beta thalassemia), HbS (S-Beta thalassemia), HbC (C-Beta thalassemia). In India, E-Beta and S-Beta thalassemia are the most common causes of thalassemia intermedia phenotype.

E-Beta thalassemia is particularly common in South-East Asian countries like: India, Bangladesh, Indonesia, Laos, Cambodia, Sri Lanka where the prevalence of carrier rate of HbE may reach as high as 80% [18-20]. Because of increased frequency of beta thalassemia mutation and HbS in people of African and South East Asian ancestry, inheritance of both these defects together is common in these regions. This co-inheritance gives rise to S-Beta thalassemia. Among the 101 children we studied, there were 12 children with E-beta thalassemia, and 6 with S-beta thalassemia.

In our study the age of first transfusion was significantly lower in beta thalassemia major (9.23±10.74 months) as compared to thalassemia intermedia (40.89±36.12 month). The average transfusion (per year) was significantly higher in beta thalassemia major (17.41±2.37) as compared to thalassemia intermedia (8.77±7.04). Muncie et al. (2009), observed that the need for transfusions started as early as six months in children with thalassemia major [21-24].

On testing the 101 children for the 5 common mutations, we were able to identify a common mutation in 61.3% children. In beta-thalassemia major patients, a common mutation could be identified in 67.08% patients. While in thalassemia intermedia patients, a common mutation could be identified in 40.9% patients. Agrawal et al. (2000) from SGPGI Lucknow used the same 5 common mutations to screen 376 carriers of thalassemia. [25] They were able to identify a mutation in 88% of the carriers. In another study from Indian subcontinent, where 702 carriers from seven different regions were screened using these 5 common mutations, a muation could be identified successfully in 93.6% carriers [26, 27].

In our cohort, the detection rate of common mutations was lower. This points out to a need for increasing the number of mutations from 5 to include other rare mutations. An alternative strategy could be use of beta globin sequencing by NGS (next generation sequencing). By using NGS, a child can be tested for common as well as uncommon mutations simultaneously. Large deletion/duplications can be missed by NGS, for which MLPA (Multiplex Ligation-dependent Probe Amplification) may be needed.Among the children with thalassemia major, the IVS I-5 G→C mutation was the most common mutation identified, seen in 34 (64.15%) children, followed by Cd 41/42 and Cd 8/9, seen in 2 (3.8%) children, followed by 619 bp deletion and IVS I-1 G→T (seen in 1 child). Similarly, a reported that the 92+5 G>C (IVS-1–5) mutation is the most common mutation seen in 60.29% cases from Rajasthan and Gujarat followed by deletion 619 bp [16]. Various previous studies from Gujarat, Maharashtra and Rajasthan have reported similar findings [12,28-30]. Whereas, Hassan et al. (2013), reported that the cd26 (A-G) HbE and cd41/42 (−TTCT) were higher in their studies in Thailand population [31]. The cd41/42 (−TTCT) and IVS-2 654 (C-T) were greater in Chinese population [32]. The variation in occurrence of these mutations is related to regional, ethical, migration, interracial marriages, and other factors as mentioned by other researchers [12,33,34]. Christopher et al (2013)among beta thalassemia patients from western Uttar Pradesh, India have shown that IVS 1-5 (G-C) was found to be the most common mutation with a frequency of 46% and the 2nd most common mutation was Fr8/9 (+G) with a frequency of 21% followed by IVS1-1 (12%) and Cd 41/42 (4%) [35]. In their patient cohort HbE, which is prevalent in northeast India was not detected in a single patient. A study on five common mutations including IVS 1–5(G→C), Fr 41/42(-CTTT), Fr 8/9 (+G), IVS 1–1 and Del 619 [36. These accounted for 90% of the total beta thalassemia genes in Pakistan. The IVS 1–5(G→C) was found to be the most common beta thalassemia gene in the Pakistani population with a frequency of 44.4% present in all major ethnic groups. The genotype frequency of the 619-bp deletion was 33.3% among the migrants from Pakistan, 8-17% in the northern states, and less than 5% in the other states [37]. Among non-migrant subjects, the commonest mutation was IVS-I-5 (G-->C), varying from 85% in the southern states and 66-70% in the eastern states to 47-60% in the northern states. The mutation IVS-I-1 (G-->T) was found mostly among the migrants from Pakistan (26.2%), but with very low/zero frequency in the other states. Allele codons 8/9 (+G) and codons 41/42 (-CTTT) were distributed in all regions of India with a frequency varying from 3% to 15%. Overall, 91.8% of the subjects had one of the five commonest mutations [IVS-I-5 (G-->C), 34.1%; 619-bp deletion, 21.0%; IVS-I-1 (G-->T) 15.8%; codons 8/9 (+G), 12.1%, and codons 41/42 (-CTTT), 8.7%], 5.9% of the subjects had a less common mutation, while 1.8% of the carriers remained uncharacterized. The IVS I-5 (G>C) was the most common β-thalassaemia mutation in all India, Pakistan and Sri Lanka, but its national prevalence differed markedly from 64.6% in Sri Lanka to 56.3% in India and 36.5% in Pakistan [38]. Thereafter, the pattern of national similarities in allele type, ceased, while the second most common allele in India was a 619-bp deletion (9.2%), in Pakistan it was Codon 8/9 (+G) (31.2%), and in Sri Lanka IVS I-1 (G>T) (17.5%).

In our study the Xmn-1 γG gene polymorphism was observed in 31.7% (32/101) children with beta thalassemia. While 4 children were homozygous for the mutated gene (TT), 28 were heterozygotes (CT). Our results are supported by similar findings of previous study by Kumar et al. (2010), who found that the mutant allele frequency of X*mn* 1^{G}γ polymorphism was 27.7% in Northern India [8]. Similarly, Nadkarni et al. (2001), who found that the frequency of X*mn* 1^{G}γ gene polymorphism was 25% in western India [39]. In another studyalso observed the mutant allele

frequencyX*mn* 1^{G}γ gene polymorphism was 32% in Canadian infants [40]. In Southern Iran study reported that the mutant allele frequencyX*mn* 1^{G}γ gene polymorphism was 41% [41].

Presence of homozygous mutant X*mn* 1^{G}γ (+/+) genotype in a patient with thalassemia is expected to reduce the globin chain imbalance thereby changing the severity of the disease from severe to less severe. We found 4 thalassemia major cases in which X*mn* 1^{G}γ polymorphism was present in homozygous mutant state. In these children, average transfusion per year was significantly lower in as compared to homozygous (CC) and heterozygous (CT) patients. The age of first transfusion and HbF were not statistically different in the three groups. Similarly, Bandyopadhyay et al. 2001 reported that the homozygosity of the X*mn* 1^{G}γ site (+/+) was strongly correlated with a mild β-thalassemia phenotype and its absence (−/−) with a severe phenotype [42]. The heterozygotes for Xmn I polymorphism (+/-) had later onset (>3 yrs) of symptoms as compared to homozygotes (-/-) (0.5-2.8 yrs) [43]. Heterozygosity of Xmn I polymorphism also delays disease onset. In thalassemia major patients, the average age at onset of disease is increased in presence of X*mn* 1^{G}γ polymorphic site on two or one alleles. The presence of X*mn* 1^{G}γ polymorphic site on both alleles (+/+), the level of HbF was increased compared to the absence of X*mn* 1^{G}γ (−/−) [44]. They also reported that the Xmn1 polymorphic site 5' to the (G) gamma gene and its correlation to the (G)gamma:(A)gamma ratio, age at first blood transfusion and clinical features in beta-thalassemia patients from Western Iran. The impact of Xmn-1 γG gene polymorphism on the severity of beta thalassemia and sickle cell anemia [45]. They found that HbF levels were significantly higher in patients with TT and CT genotype as compared to wild CC genotype of Xmn-1. They also found that this elevation of HbF ameliorates the disease severity in children beta thalassemia and sickle cell anemia with Xmn-1 polymorphism. The Hb F level was significantly higher in patients with at least one Xmn-1 allele than those without the polymorphism in thalassemia intermedia [46]. The Xmn-1(G)gamma status was -/- in 66.9%, +/- in 26.1% and +/+ in 6.9% patients. Xmn-1(G)gamma-/- presented before 1 year of age [47]. The mean age of presentation with +/+ was 18.3 months. Eight patients could be reclassified as thalassemia intermedia on follow up according to expression of Xmn1 gene polymorphism. A study carried out by Pandey et al. 2012 found that the presence and absence of XmnI polymorphism caused extremely significant differences in haematological parameters among patients with beta-thalassemia [48]. High HbF levels were found in Xmn I carriers. In contradictory, there was no significant difference between the Xmn1 Gγ polymorphism and severity of thalassemia, age at onset of symptoms, age at diagnosis, age at first transfusion, transfusion frequency or average hemoglobin levels [49]. However HbF level was significantly higher in Xmn1 Gγ +/+ and Xmn1 Gγ +/− patients.

Conclusion

The results of present study reveal that a common mutation was identified in 61.3% children with beta thalassemia and IVS I-5 G→C was the most prevalent mutation identified in our thalassemia cohort. The most prevalent variant of Xmn-1 γGgene polymorphism was wild type (CC) followed by heterozygous mutant (CT) and least was homozygous mutant (TT). The prevalence of homozygous mutated TT genotype was 3.9% in our cohort. Among the homozygous mutant TT genotype, the average transfusion frequency was significantly lower as compared to CT and CC genotypes.

The results of present study reveal that a common mutation was identified in 61.3% children with beta thalassemia and IVS I-5 G→C was the most prevalent mutation identified in our thalassemia cohort. The prevalence of homozygous mutated TT genotype was 3.9% in our cohort. .The average transfusion frequency was significantly lower as compared to CT and CC genotypes.

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