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ORIGINAL ARTICLE

Spectrum of Mutations of Beta Thalassemia

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ABSTRACT

Objective: To identify gene mutations known to cause thalassemia major and intermedia amongst patients coming to thalassemia Centre of Pakistan Institute of Medical Sciences(PIMS).

Patients and Methods: Hundred transfusion dependent thalassemia patients were recruited from PIMS. Genome DNA was isolated by using phenol-chloroform method. Allele specific PCR was performed by using primers specific for twelve known disease causing mutations, prevalent in Pakistan. The PCR product was run on 6% polyacrylamide gel electrophoresis and visualized by silver staining technique. Results were recorded and data were entered and analyzed using SPSS version 16.

Results: Total Number of patients included in the study was 100, among them 46% were males and 54% were females. Parenteral consanguinity was seen in 95% cases. Most common homozygous mutations were Fr 8-9 [23(28.7%)], followed by IVSI-5 [17(21.3%)] cases. Compound heterozygous mutations were seen in 20% cases, among them the most common was Fr 8-9/IVS1-5 (5/20 cases), and Fr 8-9/del 619 (3/20 cases). Analysis of type of mutation in different ethnic groups showed that Fr 8-9 was the most common mutation in Punjabis and Pathans seen in 14/63 and 6/28 cases respectively, followed by IVS1-5 seen in 11/63 and 5/28 cases respectively. The most common mutation in Thalassemia major was Fr 8-9 seen in 22 (25%) cases followed by IVS1-5 seen in 15 (17%) cases and Fr 41-42 seen in 10 (11.4%) cases. The number of patients of Thalassemia Intermedia was low in this study (n=12), however among these the commonest mutations were Cap +1, Fr 8-9, IVS1-5 and del 619, presenting as homozygous or compound heterozygous mutations.

Conclusion: Molecular characterization of Thalassemia major and intermedia patients is very essential so that we can set trigger of hemoglobin level accordingly before putting them on regular transfusion. Less frequent transfusion, iron chelation and HU therapy will significantly reduce serum ferritin, liver and spleen size of this group of patients and thus significantly improve their quality of life.

Key words: Beta Thalassemia, Spectrum, Mutation, Thalassemia major.

Author`s Contribution
[1] *Conception, synthesis, planning of research and review*
[2] *Data analysis, interpretation*
[3] *manuscript writing and review*
[4,5] *Data collection*

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Article info.
Received: October 21, 2017
Accepted: December 5, 2017

Cite this article. *Hassan K, Rasheed M, Asif N, Anwar T, Tahir M. Spectrum of Mutations of Beta Thalassemia. JIMDC.2017;6(4):*

Funding Source: *Nil*
Conflict of Interest: *Nil*

Introduction

Beta Thalassaemia is one of the most common monogenic disorders in Pakistan with career rate of 5-8%.[1] Every year, almost 5000 new children are born with thalassemia major.[2] Among haemoglobin disorders, β

thalassemia is one of the most common causes of morbidity and mortality worldwide.[3] Beta thalassemia is inherited in autosomal recessive pattern and almost 300 mutations have been identified in α or β globin gene which impairs transcription, mRNA processing or its translation.[4] With exception of a few deletions, vast majority of β thalassaemias are caused by point mutations within the gene or its immediate flanking sequences.[5] Depending on whether thalassemia is heterozygous or homozygous, the phenotypic form varies from asymptomatic state known as thalassemia minor to a transfusion dependent form known as thalassemia major. This clinical variability mainly depends on β-globin genotype. About 20 mutations account for 90 percent of β-globin genes effects in the world and it is noted that each ethnic population has its own unique set of most frequent mutations.[6] $\beta l^0/\beta^0$ is the severest-manifesting genotype, in which no β-chain production occurs; in β+/β + or $\beta l^0/\beta^+$ genotype, β-chains are produced in small quantities, allowing a small amount of HbA production. A small percentage of patients have silent β thalassemia; these patients have β+ thalassaemia in which the deficit in β globin chains production is minimal. Such individuals present with normal HbA2 levels on haemoglobin electrophoresis, making their identification difficult with conventional methods, thus making genetic analysis mandatory for detection of thalassemia.[2,7] Further, a co-inheritance of α-thalassemia or various hemoglobinopathies with β-thalassemia may manifest as thalassemia intermedia, in which anemia is moderate, and the patient is usually not transfusion dependent, except in stress like various types of infections.[8]

Epidemiological data about prevalence and distribution of mutations plays a very crucial role in development of effective disease management strategies. Previous studies conducted in different ethnic groups of Pakistan showed prevalence of six mutations, Fr 8-9 (+G), IVSI-5 (G-C), Fr 41-42 (–TTCT), Del 619 bp, Cd 15 (G-A) and IVSI-1 (G-T). Similarly, Thalassemia Intermedia (TI)encompasses a wide clinical spectrum of beta thalassemia phenotype and various genetic factors affect the severity of disease and response to treatment, particularly in TI. This study was aimed to identify disease causing mutations in selected Pakistani patients.

Patients and Methods

The study was designed to identify underlying beta globin gene mutation in patients with beta thalassemia major. The study protocol was approved by the institutional review board and informed written consent was obtained before sample collection from patients. A total of 100 transfusion dependent beta thalassemia cases were recruited from Pakistan institute of medical sciences, Islamabad. Clinical details of the patients were obtained and blood samples of the selected patients were collected in EDTA vacutainer tubes. Genomic DNA was isolated by using Phenol-Chloroform method.[9] The isolated DNA was then amplified to detect 12 previously known mutations in Pakistani subjects [IVSI-5 (G-C), Fr 8-9 (+G), IVSI-1 (G-T), Fr 41-42 (–TTCT), Del 619 bp, Cd 15 (G-A), Cd 5 (–CT), Cd30 (G-C), Cd 30 (G-A), Fr 16 (–C), IVSII-1 (G-A), Cap +1 (A-C)] by using mutation specific primers.[1] Multiplex amplification refractory mutation system (ARMS) PCR was performed in three separate reaction mixtures. The first reaction mixture contained primers specific for amplification of Fr 8-9 (+G), Fr 41-42 (-TTCT), IVSI-5 (G-C), IVSI-1 (G-T) and Del 619. The second reaction mixture contained primers specific for Cd 5 (-CT), Fr 16(-C), IVSI-1 (G-T), Cd30 (G-C), Cd 30 (G-A), and IVSII-1 (G-A) while the third reaction mixture contained primers specific for Cd 15 (G-A) and Cap +1 (A-C).

PCR reaction mixture was prepared by using 5 pmol of each primers, 0.2 units of Taq Polymerase (company name), 10 mM of each dNTP (company name), 25 mmol $MgCl_2$, 100 mM Tris-HCl and 500 mM KCl (pH 8.3) and 30-50 ng of DNA. The PCR amplification process consisted initial denaturation at 94°C for 10 minutes followed by of 25 cycles of denaturation at 94°C for one minute, primer annealing at 65°C for one minute, and DNA extension reaction at 72°C for 1.5 minute. Final extension was done at 72°C for 3 minutes. The amplified product was resolved by using 6% mini polyacrylamide gel at 180 V for 20 minutes. After electrophoresis, gels were stained by dipping it in 0.1% silver nitrate solution for 15 mins and later treating with a freshly prepared solution of 1.5% NaOH and 0.15% formaldehyde until the bands appear. Gels were interpreted and images were recorded. If a single mutation was obtained, a second PCR was performed with a set of primers specific for the normal

allele of the respective mutation to detect the presence of wild type sequence. If the normal allele was amplified, the patient was considered heterozygous carrier. However, if the normal counterpart was not detected, the patient was considered homozygous recessive the respective mutation.

Results

Total Number of patients included in the study was 100, among them 46% were males and 54% were females. Mean Age (in months) at diagnosis was 13.48±11.65 (3-66 Months) and median Age (in months) at diagnosis was 8 (2-156 months). Mean age at diagnosis of Thalassemia Major was 8.09±3.30 months and mean age at diagnosis of Thalassemia Intermedia was 44.50±39.5. As shown in table 1, the most common ethnic groups were Punjabis (63%) and Pathans (29%). Parenteral consanguinity was seen in 95% cases; out of these 92% being 1st cousins.

Table 1: Demographic characteristics of patients

		n (%)
Gender	Male	46 (46)
	Female	54 (54)
Ethnicity	Hinko	1 (1)
	Punjabi	63(63)
	Potohari	1 (1)
	Pathan	29 (29)
	Kashmiri	4 (4)
	Other	3 (3)
Parental Consanguinity	Yes	95 (95)
	No	5 (5)
Relationship between parents	1st cousins	97 (97)
	More distant relationship	3 (3)

Homozygous mutations were seen in 76% cases; the most common homozygous mutations were Fr 8-9 [23(28.7%)], followed by IVSI-5 [17(21.3%)] cases, table 2. In 4% cases no mutation was found. Compound heterozygous mutations were seen in 20% cases, among them the most common was Fr 8-9/IVS1-5 (5/20 cases), and Fr 8-9/del 619 (3/20 cases) Table 3.

Table 2: Frequency of homozygous mutations (n=80)

S.No	Mutation	Frequency (%)
1	Fr 8-9	23 (28.7)
2	Fr 41-42	10 (12.5)
3	Fr 16	6 (7.5%)
4	Del 619	10 (12.5)
5	Cd 30	4 (5%)
6	Cap +1	2 (2.5)
7	IVSI-1	3 (3.8)
8	IVSII-1	1 (1.2)
9	IVSI-5	17 (21.3)
10	No mutation	4 (5)

Table 3: Compound heterozygous mutations in beta-globin gene (n=20)

S. No	Mutation	Frequency
1	Cap+1/IVSII-1	1 (5)
2	Cd 30/Fr 16	1 (5)
4	Fr 8-9/ Fr 16	1 (5)
5	Fr 8-9/ Fr 41-42	1 (5)
6	Fr 8-9/Cd 5	1 (5)
7	Fr 8-9/del 619	3 (15)
8	Fr 8-9/IVSI-5	5 (25)
10	Fr 8-9/Cap+1	2 (10)
11	Fr 41-42/ IVSI-5	2 (10)
12	Fr 41-42/ IVSI-1	1 (5)
13	IVSI-5/ IVSII-1	1 (5)
14	IVSII-1/ Cd 5	1 (5)

Analysis of type of mutation in different ethnic groups showed that Fr 8-9 was the most common mutation in Punjabis and Pathans seen in 14/63 and 6/28 cases respectively, followed by IVS1-5 seen 11/63 and 5/28 cases respectively (table 4). As shown in the table 5, the most common mutation in Thalassemia major was Fr 8-9 seen in 22 (25%) cases followed by IVS1-5 seen in 15 (17%) cases and Fr 41-42 seen in 10 (11.4%) cases. The number of patients of Thalassemia Intermedia was low in this study (n=12), however among these the commonest mutations were Cap +1, Fr 8-9, IVS1-5 and del 619, presenting as homozygous or compound heterozygous mutations.

Table 4: Types of mutations in different ethnic groups (n=100)						
	Hinko (n=1)	Kashmiri (n=4)	Pathan (n=28)	Pothohari (n=1)	Punjabi (63)	Others (n=3)
Fr 8-9 (n=23)	-	2	6	1	14	-
IVSI-5 (n=17)	-	1	5	-	11	-
del 619 (n=10)	-	1	2	-	6	1
Fr 41-42 (n=10)	1	-	4	-	5	-
Fr-16 (n=6)	-	-	2	-	4	-
Cd-30 (n=4)	-	-	2	-	2	-
IVSI –1 (n=3)	-	-	-	-	2	1
Cap +1 (n=2)	-	-	1	-	1	-
IVSII-1 (n=1)	-	-	-	-	1	-
Cap +1 /IVS11-1 (n=1)	-	-	1	-	-	-
Cd-30/Fr 16 (n=1)	-	-	-	-	1	-
Fr 16 / Fr 8-9 (n=1)	-	-	1	-	-	-
Fr 41-42 / Fr 8-9 (n=1)	-	-	-	-	1	-
Fr 41-42 / IVSI-1 (n=1)	-	-	-	-	1	-
Fr 41-42/IVSI-5 (n=2)	-	-	1	-	1	-
Fr 8-9 / IVSI-5 (n=5)	-	-	1	-	4	-
Fr 8-9 / del619 (n=3)	-	-	-	-	3	-
Fr 8-9 /Cd-5 (n=1)	-	-	1	-	-	-
Fr 8-9/ cap+1 (n=2)	-	-	-	-	1	1
IVSI-5 /IVSII-1 (n=1)	-	-	-	-	1	-
IVSII - 1 /Cd-5 (n=1)	-	-	-	-	1	-
No Mutation (n=4)	-	-	1	-	3	-

Discussion

Beta thalassaemia is a major health burden in Pakistan. Despite preventive measures, around 5000 children with transfusion dependent TI or TM are annually added to the registry. No permanent cure is available, except stem cell transplantation, which is unaffordable due to high cost. The majority of patients with transfusion-dependent thalassaemia have no choice but to have regular blood transfusions and iron chelation therapy. Haemoglobin F augmentation through HU therapy is another ray of hope to avoid blood transfusions and associated complications. It is thus important to predict phenotype from genotype as; different patients have different clinical severity and response to treatment to HU depending upon their genetic makeup. The present study showed that mean age at diagnosis in Thalassemia major patients was 8 months and those of Thalassemia Intermedia was more than 3 years. Consanguinity was seen in 95% cases majority being 1st cousins. In a study conducted by Khan et al in district Bannu to find consanguinity ratio in Beta - Thalassemia major patients, consanguinity was reported in 74% parents while 26% were unrelated.[10] Regarding genetic mutations, Fr 8-9 and IVS1-5 were the most common mutations seen in different studies (Table 6). Similar finding are reported by Ali et al.[11] Another study conducted by Usman et al in 2009 reported that IVS-1-5 (G→C), Fr 8/9 (+G), Fr 41/42 (-TTCT), IVS-1-1(G→T) and Del 619 comprised 90 % of Beta Thalassemia mutations in Pakistani population.[12] In a study conducted by Tariq et al, it was reported that out of the 13 mutations tested, three mutations accounted for 71% of the total, and these included, IVS1-5, Fr 8-9 and del 619 bp.[13] Our study shows similar result.

The population of Pakistan is mainly divided into Punjabis and Pathans in the North and Balochis and Sindhis in South. Apart from these, there are other groups such as Urdu speaking, Memons, Gujrati, Saraiki, Kashmisri, Pothohari etc. Frequency of mutations may be different in different ethnic groups. Khan et al reported that four most common mutations, IVSI-5 (G+C) (37.7%), codons 8/9

(+G) (21.1%), the 619 bp deletion (12.4%), and IVSI-1 (G+T) (9.5%), collectively comprise 80.7 % of mutations. They also reported that in the four provinces of Pakistan, IVSI-5 (G-C) mutation was more prevalent in Sindh and Balochistan, which share borders with India in the south and Iran in the southwest, while Fr 8-9 mutation was more common in the Punjab and the Khyber Pakhtoon Khawa Province, sharing borders with India in the Northeast and Afghanistan, respectively. They further highlighted that 619 bp deletion was high (46%) in Gujratis and Memons residing in the Province of Sindh, neighboring the Indian Gujrat.[14]

Table 5: Different types of mutations in Thalassemia Major and Intermedia patients(n 100)

Mutations	Thalassemia Major (n=88)	Thalassemia Intermedia (n=12)
Fr 8-9 (n=23)	22	1
IVSI-5 (n=17)	15	2
Fr 41-42 (n=10)	10	0
del 619 (n=10)	8	2
Fr-16 (n=6)	5	1
Cd-30 (n=4)	4	0
Fr 8-9 / del619 (n=3)	3	0
Fr 8-9/IVSI-5 (n=5)	4	1
IVSI – 1 (n=3)	2	1
Fr 41-42/IVSI-5 (n=2)	2	0
Cap + 1 (n=2)	2	0
Cap + 1 / IVS11-1 (n=1)	0	1
Cd-30/Fr 1 (n=1)	1	0
Fr 16 / Fr 8-9 (n=1)	0	1
Fr 41-42 / Fr 8-9 (n=1)	1	0
Fr 41-42 / IVSI-1 (n=1)	1	0
Fr 8-9 /Cd-5 (n=1)	1	0
Fr 8-9/ cap+1 (n=2)	1	1
IVSI-5 /IVSII-1 (n=1)	1	0
IVSII - 1 /Cd-5 (n=1)	0	1
IVSII-1 (n=1)	1	0
Uncharacterized (n=4)	4	0

We found that Fr 8-9 was the most common mutation in Punjabis and Pathans followed by IVS 1-5. Ansari et al found that IVS1-5 was the most common mutation in Sindis, Blochis and Punjabis followed by Fr 8-9, whereas in Pathans Fr 8-9 was the commonest mutation followed by IVS1-5. [6] Similarly a study done by Akhtar et al to look for spectrum of Beta thalassaemia mutations, reported predominance of FR 8-9 and IVSI-5 mutation in Punjabis and Pathans and CAP+1 as the commonest silent mutation.[15] Same results have been observed for the Indian population as shown by the studies of Garewal G et al [16] and Varawalla NY et al [17].

A study done by Baig et al to look for spectrum of beta-thalassemia mutations in various regions of Punjab and Islamabad, reported that distribution of mutations in Punjab and the Capital territory was different from the overall pattern in Pakistan. They reported that three most common mutations; IVS-I-5 (G-C), codons 8/9, (+G) and codons 41/42 (-TTCT) constitute 86.8% of mutations. They further highlighted that IVS-I-5 was the most common mutation seen in 39.0% cases in all regions being highest in South Punjab (45.0%) i.e. D.G. Khan, Bahawalpur and Multan region, whereas codons 8/9 (+G) was the second most frequent mutation seen in 37.3% cases but was number one in the patients from Rawalpindi-Islamabad (34.9%). The deletion of TTCT between codons 41 and 42 of the beta-globin gene was the third most common mutation (10.6%) in their study. The rest of 14 beta thalassemia mutations were less common or rare and comprised only13.9 %.[18]

Taking into consideration all the studies, the primary and secondary panels of primers of ethnic specific mutations can be devised. With reference to uncharacterized mutation, samples should be sent for genetic sequencing.

A study conducted to analyze spectrum of β-thalassaemia mutations in India, Pakistan and Sri Lanka showed that IVSI-5 (G>C) was the most common β-thalassaemia mutation in all three countries, with different frequencies, i.e. 64.6% in Sri Lanka to 56.3% in India and 36.5% in Pakistan. The second most common mutation in India was a 619-bp deletion (9.2%), in Pakistan Fr 8-9 (31.2%), and in Sri Lanka IVS I-1 (G>A) (17.5%). In Pakistan, the frequency of Fr 8-9 (31.2%) was close to that of IVSI-5 (G>C) (36.5%). This study also highlighted the frequency of mutations in different ethnic groups.

Table 6: Frequency of mutations seen in different studies from Pakistan							
Type of mutation	**Khan et al 1998 (14)**	**Usman et al 2009 (120**	**Akhtar et al 2012 (15)**	**Ali et al 2015 (11)**	**Baig et al 2006 (18)**	**Ansari et al 2011 (6)**	**Present Study 2017**
IVS 1-5	37.7	44.4	24.5	36	38.9	40.89	21%
Fr 8-9	21.1	14.6	35.5	40	37.3	15.7	29.5%
Fr 41-42	-	17.5	14.8	4	10.6		12%
Fr 16	-		1.6	2			7%
Cd5	3.1		6.6	8	1.3	2.16	1%
Cd15	3.1		07	8	1.8		-
Cd30	-		3.9	1	0.7	8.02	4.5%
IVS II-1	0.7			1	0.8		2.5%
619 bp del	12.4	0.6	1.6		1.9	11.11	6.5%
IVS 1-1 G-T	9.5	7.5	1.9		1.9	8.17	3.5%
Cap+ 1	1.2		1.7				3.5%
Uncharacterized			0.8				2%

They reported that data from Pakistan showed a significant variability in the distribution of β-thalassaemia mutations in four provinces. It was reported that, unlike any region of India, IVSI-5 (G>C) was the most common mutation in Sindh and Baluchistan, whereas, Fr 8/9 (+G) was the most common β-thalassaemia allele in both Punjab (38.6%) and Khyber Pakhtunkwha (47.7%), though it was only the fourth most common allele across India, suggesting that its origin may have been effected by mutations of adjoining regions of neighboring countries, such as Afghanistan.[19] These findings are comparable to our study, where Fr 8-9 was most common mutation observed in Punjabis and Pathans. In our study, Cap + 1 mutation was seen in 5 cases (2 homozygous and 3 heterozygous cases). Karim et al reported Cap+1 mutation in 5% in targeted thalassemic families (having patients with beta-thalassemia intermedia) while its frequency was observed 2% in total thalassemic genes in Pakistani population.[20]

In our study, number of patients of Thalassemia intermedia was low, i.e. 12 patients and among them IVSI-5 and del 619 were most common mutations. In a study conducted by J Khan et al, IVS-I-5 (50.0%) was the most frequent mutation in Beta-TI followed by Fr 8-9 (9.5%), codons 41/42 (3.4%), and Cap + 1 (3.1%). Homozygous Beta $^{+}$ allele, co-inheritance of alpha gene deletion and co-inheritance of *Xmnl1* polymorphism all contribute to reduced severity of disease in Thalassemia intermedia.[21] Response to HU therapy has also been reported as more favorable in the presence of *Xmnl1* polymorphism.[11] However, limitation of our study was that we did not evaluate for *Xmnl1* polymorphism and alpha gene deletion. We also observed, though some patients presented after two years but they were started regular transfusions without knowing their genotype (Thalassemia major or Intermedia) and were treated as Thalassemia major cases. It is thus suggested to evaluate the genetic mutation of all cases of beta thalassemia major and intermedia first, and put them on transfusion therapy accordingly. As TI patients, not only require less frequent transfusions, they can withstand low hemoglobin level as compared to TM patients. And respond better to HU than Thalassemia major patients. If we know the genotype of these patients of Thalassemia intermedia, and they are managed with less transfusions, HU therapy and good iron chelation we can significantly improve their quality of life.

Conclusion

This study adds to the pre-existing data in Pakistan. We can device primary and secondary panels of primers of genetic mutations for a specific population. Molecular characterization of Thalassemia major and intermedia patients is very essential so that we can set a trigger hemoglobin accordingly before putting them on regular transfusion. Less frequent transfusion, iron chelation and

HU therapy will significantly reduce serum ferritin, liver and spleen size of this group of patients and thus significantly improve their quality of life.

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