6 Characterization of β-thalassaemia mutations

Introduction:

This chapter describes study of β-thalassaemia mutations in all ethnic groups in Pakistan. In addition, data on the molecular pathology of thalassaemia intermedia is also presented.

Subjects:

A total of 712 unrelated individuals were studied. These included 184 β-thalassaemia heterozygotes, 519 β-thalassaemia homozygotes and 9 cases of Hb-S/β-thalassaemia who were also receiving transfusions. The heterozygotes were identified from individuals referred to the department of haematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, for screening of β-thalassaemia. Their diagnosis was made by red cell indices and HbA_2 estimation. The patients with β-thalassaemia major and Hb-S/β-thalassaemia included in the study were receiving treatment at Fatimid Foundation Thalassaemia Treatment Centres, Karachi, Lahore, and Peshawar, Hussaini Blood Bank, Karachi and Pakistan Thalassaemia Welfare Society Thalassaemia Treatment Centre, Rawalpindi. The patients were diagnosed at various government and private laboratories.

At the time of sampling each individual or his/her parents were interviewed. Information including age, ethnic group, parental consanguinity and transfusion history was recorded. From each individual 3-5 ml of blood was collected in EDTA and kept refrigerated until DNA was extracted.

Thalassaemia intermedia:

Patients of β-thalassaemia who had a mild phenotype, characterized by late start or infrequent requirement of transfusions, were further investigated for the type of β-thalassaemia mutation, Xmn-I polymorphism and coincidence of α-thalassaemia ($-\alpha^{3.7}$ and $-\alpha^{4.2}$). Xmn-I polymorphism was also investigated in 39 randomly selected patients of severe β-

thalassaemia major and 58 apparently normal individuals (Controls).

Results:

Distribution of ethnic groups:

The subject's diagnosis and ethnic group is shown in Table: 6.1.

Table: 6.1. Distribution of the subjects according to ethnic group and diagnosis.

Ethnic Group:	Thalassaemia trait:	Thalassaemia major:	Hb-S/ Thalassaemia:	Total Subjects:	Total mutant chromosomes:
Punjabi:	117	139	-	256	395
Pathan:	57	78	4	139	221
Sindhi:	3	129	1	133	263
Baluchi:	6	83	1	90	174
Mohajirs:	1	90	3	94	187
TOTAL:	184	519	9	712	1240

Age distribution:

Subjects with β-thalassaemia major ranged in age from 3 months to 35 years (Fig: 6.1). Their distribution according to age was as follows: <1 year 6%, 1-2 years 9%, 2-5 years 33%, 5-10 years 34%, and >15 years 18%. The ages of patients with Hb-S/β-thalassaemia ranged from 4-17 years (Mean 9 years).

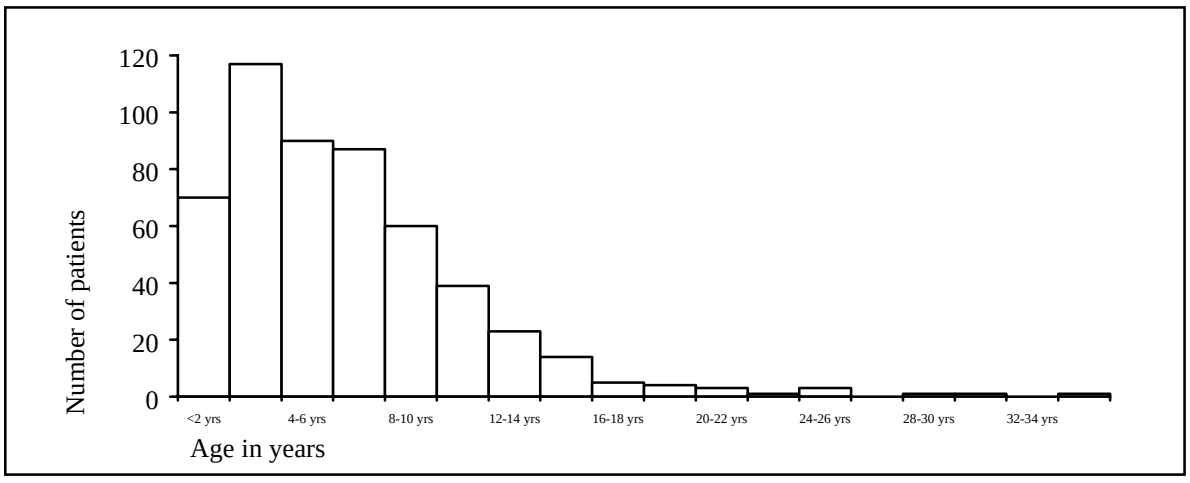

Fig: 6.1. Age distribution of 519 patients of thalassaemia major in whom mutation analysis was done.

Mutation analysis:

Each heterozygote of β-thalassaemia represented one mutant allele and homozygotes represented two alleles. In 184 heterozygotes, 519 cases of β-thalassaemia major and 9 cases of Hb-S/β-thalassaemia 1240 mutant alleles were identified.

Screening by ARMS:

The ARMS method identified 1218/1240 (98.2 %) alleles. Bright and clear bands showed a positive result (Fig: 6.2a). For most of the mutations negatives were clear negative but a faint false positive band was seen in some (Fig: 6.2b).

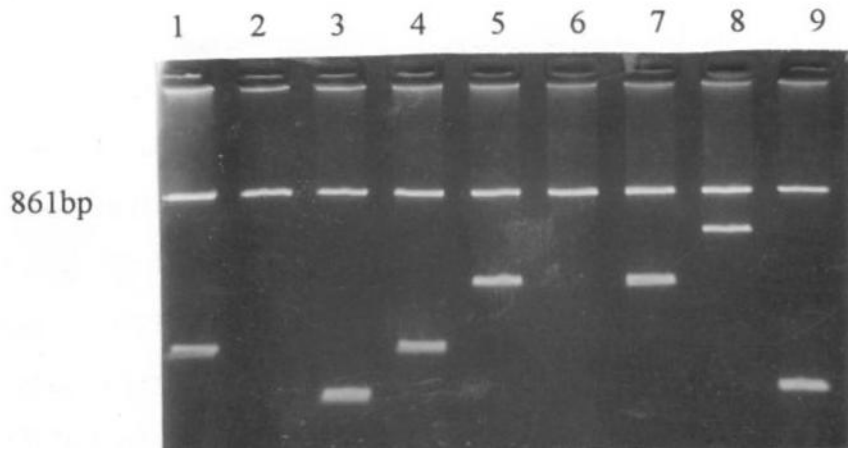

Fig: 6.2a. Ethidium bromide stained agarose gel electrophoresis of ARMS amplified products. All lanes show 861bp control bands. Lane 1 and 4 show bands of IVSI-5, lane 3 is Cd5, lane 5 and 7 are Fr 41-42, lane 8 is IVSII-1 and lane 9 is Fr 8-9.

False positive bands were always seen with Hb-E mutant and Cd 15 (G-A) normal primers. Similarly, faint positive bands, although of much less intensity, were also given by Fr 8-9 (+G) mutant, Cap+1 (A-C) mutant, and IVSI-1 (G-T) normal primers. The false positive bands could be made less intense by (a) reducing the amount of Taq polymerase from Advanced Biotechnologies, UK (b) using Taq from Perkin Elmer UK, (c) increasing the annealing temperature to 67^{o} C instead of the 65^{o} C (d) comparing the intensity of 861 bp control band and the mutant/normal band: the presence of a brighter control band (861 bp) suggested a false positive result. Comparing the bands for known positive or negative samples also helped to identify false positive bands. The false positive band shown by Hb-E

mutant primer was made even fainter by redesigning the primer with a (G/G) mismatch instead of the (G/A) mismatch at position -3 relative to the 3' end of the primer (Table: 6.3).

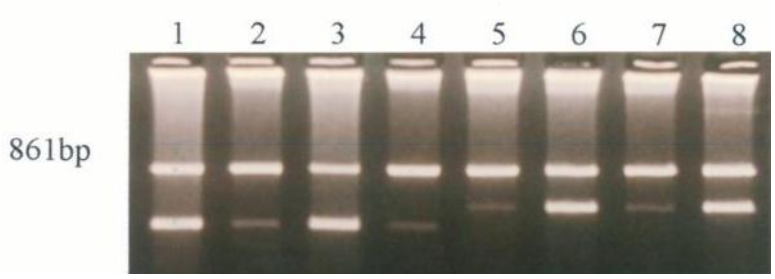

Fig: 6.2b. Agarose gel electrophoresis of ARMS amplified products for Cd15 (lanes 1 and 3) and Cap+1 (lanes 6 and 8). False positive bands for the same mutations can be seen in the lanes 2, 4, 5 and 7. 861 bp control fragments are visible in all lanes.

Twenty two alleles were not characterized by the ARMS method. Subsequent investigations (DGGE and sequencing) suggested that 8 of these were false negatives and analysis of the records showed that the negative result was a consequence of human errors. In five cases testing by ARMS had been omitted due to a clerical error and in 3 the absence of 861 bp control band, indicating failure of PCR, had been overlooked. These eight samples gave a clear positive result when subsequently tested by the respective ARMS primers i.e. Cd 15 (G-A): 3, Fr 16 (-C): 2, Cap+1 (A-C): 2, and Cd 30 (G-A): 1). This left 14/1240 (1.1%) uncharacterized mutations.

Denaturing Gradient Gel Electrophoresis (DGGE):

The β-globin gene was tested by DGGE in overlapping regions (Fig 3.1). The primers used covered the gene except the major portion of IVSII (IVSII-30 to IVSII-588), the last 14 codons, the termination site and the polyadenylation site.

Fragments I and II:

This region contains most of the promoter region sequences and the first few codons of Exon-1. The majority of mutations including the C-T polymorphism in the 2nd codon was identified by DGGE of this region (Fig: 6.3). The fast moving bands in a particular lane represented homoduplexes containing the mutation while the slower bands resulted from heteroduplex formation between the mutant and the normal sequences. Single bands were seen when the

test sample was homozygous for either the mutation/polymorphism or the normal sequence.

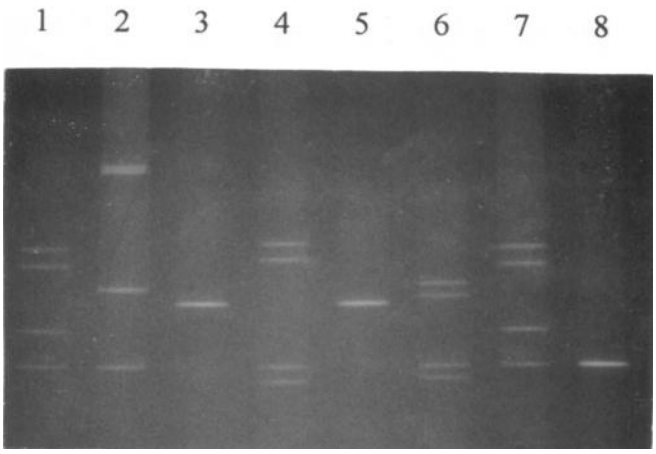

Fig: 6.3. DGGE of the amplified fragment-I after ethidium bromide staining. Lane 1 and 7 show heterozygous polymorphism and lane 8 is homozygous polymorphism at Cd2, lane 2 is heterozygous Cd15, lane 3 and 5 are homozygous Fr16, lane 4 is heterozygous Cd5 and lane 6 is heterozygous Fr 8-9.

DGGE of fragment-I revealed seven mutant alleles. Some samples were also homozygous or heterozygous for the polymorphism at the Cd2. Careful analysis of the patterns produced by the polymorphism was required to distinguish it from a mutation. The problem was overcome by including known samples with or without Cd2 polymorphism. The fragment-II was amplified by a set of primers in which the GC clamp was shifted to the downstream primer as compared to the GC clamp used with the upstream primer that amplified the fragment-I. This resulted in identification of three mutantions, all shown by subsequent sequencing to have the -88 (C-T) mutation. In fact, close proximity of -88 (C-T) to the GC clamp in fragment-I prevents identification by using this fragment.

Fragment-III:

Fragment-III was large (424bp) and encompassed promoter sequences, Exon-I and most of the IVS-I. It was found to be useful for identification of mutations located in the splice junction of Exon-I and the IVS-I. DGGE of this fragment resulted in identification of two mutations. Subsequent sequencing revealed them to be Cd 30 (G-A), and IVSI-1 (G-A).

Fragments IV and V:

Fragments IV and V were useful for identifying mutations in Exon-II. DGGE of this fragment identified one mutation which on subsequent sequencing showed Cd 39 (C-T) which is a Mediterranean mutation.

Fragment VI:

This fragment spans the terminal part of IVSII and the major portion of Exon-III. This region contains several thalassaemia mutations and a C-T polymorphism at position IVSII-666 (Ghanem et al, 1992). DGGE of this fragment did not reveal any mutant allele. However, the polymorphism was seen on 7 out of 22 chromosomes.

Genomic sequencing:

Genomic sequencing elucidated the precise nature of the mutations identified by DGGE. The results of sequencing on single stranded as well as the double stranded templates were comparable but the double stranded protocol had the advantage of being quicker.

The results are summarised in Table: 6.2. New mutations identified by sequencing include Cd 39 (C-T), IVSI-1 (G-A) and -88 (C-T). A Pathan patient with severe transfusion dependent thalassaemia was found to be homozygous for a novel mutation in the Exon-III of β-globin gene, namely a 17 bp deletion (-TGC/AGG/CTG/CCT/ATC/AG) involving codons 126 to 131 (Fig: 6.4). The deletion was located between two copies of a CAG sequence present in Cd 125/126 and Cd 131. It was not possible to ascertain whether the breakpoint was before or following the CAG sequence in Cd 125/126. However, the Cd 125 was left intact. The deletion caused a frame-shift leading to a premature stop signal (TAA) at the new Cd 133. This deletion was not detected by DGGE because the sample had failed to amplify with the primers for the fragment VI as the downstream primer for this fragment is complementary to Cd 125-132 (Primer DG11 in Table: 3.5), deleted in this sample.

In four subjects with thalassaemia major and two with thalassaemia intermedia only one β-thalassaemia allele was identified by ARMS and DGGE. Sequencing of the entire β-globin gene including 110 bases upstream to the Cap site and 10 bases beyond the poly-A site in all six samples could not identify the second mutant allele.

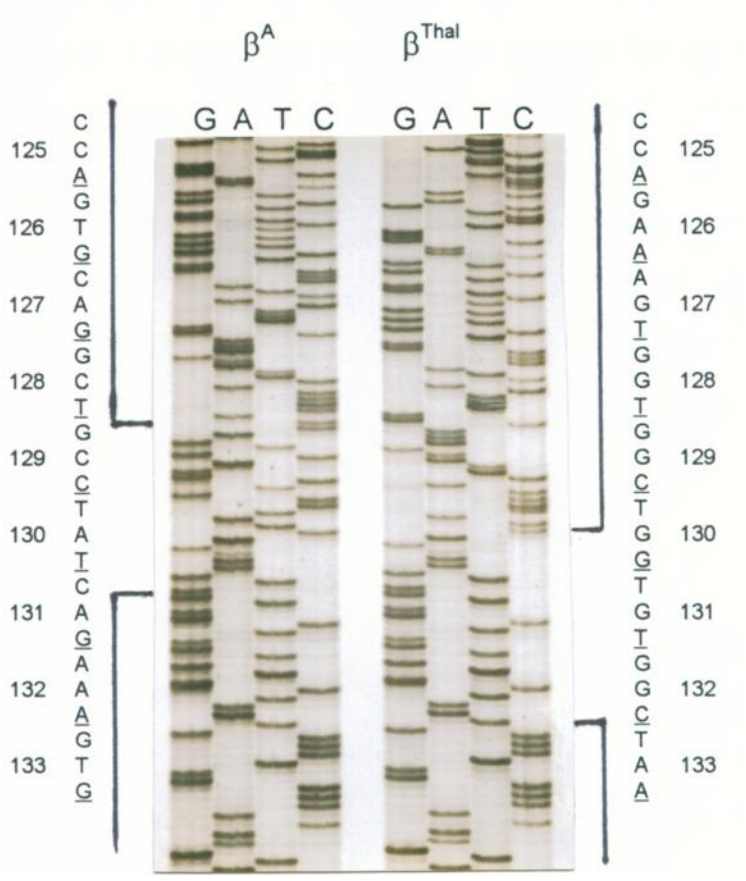

Fig: 6.4. DNA sequencing gel showing the non coding strand of exon-III containing the 17bp deletion. Sequence of the normal DNA is also shown.

Table: 6.2. Mutations characterized by genomic sequencing.

Mutations:	**Number of alleles:**	**Mutations:**	**Number of alleles:**
Cd 15 (G-A)	3	IVSI-1 (G-A)	2
Fr 16 (-C)	2	Cd 39 (C-T)	1
Cd 30 (G-A)	1	Fr 125-131 (-17 bp)	2
-88 (C-T)	3	Cap+1 (A-C)	2

Spectrum of β-thalassaemia mutations:

A total of 1225 β-thalassaemia alleles were characterized, 19 different β-thalassaemia mutations being identified (Table: 6.3). In addition, 9 alleles of Hb-S in combination with

different β-thalassaemia mutations were identified. The five most frequent mutations were called common mutations. The mutations that were not common but had a frequency of 1% or more were called uncommon and those with less than 1% frequency were called rare mutations. The common mutations, IVSI-5 (G-C) (37%), Fr 8-9 (+G) (25.6%), Del 619bp (7%), Fr 41-42 (-TTCT) (6.6%), and IVSI-1 (G-T) (5%) accounted for 81% of the alleles (Table: 6.3). Six uncommon mutations formed 15% of the total and another nine (4%) were categorized as rare mutations. Six subjects with transfusion dependent anaemia had only one β-thalassaemia mutation and the other allele remained unknown. Three of these had IVSI-5 (G-C), two had Fr 8-9 (+G), and one had Fr 41-42 (-TTCT).

Punjabi:

The total of 393 alleles from Punjabi subjects included 16 different mutations (Table: 6.3). The common mutations Fr 8-9 (+G), IVSI-5 (G-C), Fr 41-42 (-TTCT), IVSI-1 (G-T) and Cd 30 (G-C) constituted 82% of the alleles. Cd 30 (G-C) replaced del 619 in the common group of mutations. The uncommon mutations formed 15.3% and rare mutations were 2.5% of the total.

Pathan:

Fr 8-9 (+G) comprised 48% of the alleles identified. The other common mutations were IVSI-5 (G-C), Fr 41-42 (-TTCT), Cd 5 (-CT), and Cd 15 (G-A) (Table: 6.3). Cd 5 (-CT) and Cd 15 (G-A) replaced IVSI-1 (G-T) and Del 619 in the common group. Two β^+ mutations Cap+1 (A-C) and -88 (C-T) comprised 4.5% of the alleles. Compound heterozygotes of Hb-S and β-thalassaemia formed 4/82 (5%) of the subjects with transfusion dependent haemoglobin disorder in Pathans. A novel 17bp deletion that involved Cd 126-131 was found in a transfusion dependent Pathan child with consanguineous parents. Two Pathan patients with apparent thalassaemia major only one β-thalassaemia allele was identified.

Sindhi:

The common mutations including IVSI-5 (G-C), del 619, IVSI-1 (G-T), Fr 8-9 (+G) and Cd 30 (G-C) comprised 88% of the alleles (Table: 6.3). Del 619 was found at a frequency of 13.6% and only one allele of IVSI minus 25 was seen. In two Sindhi patients with apparent transfusion dependent thalassaemia only one β-thalassaemia allele was identified.

Baluchi:

The mutation pattern amongst Baluchis was comparatively less heterogenous. IVSI-5 (G-C) accounted for 75% of the alleles (Table: 6.3). The other common mutations included Fr 8-9 (+G), Cd 15 (G-A) and Fr 16 (-C). There was one unidentified allele in a patient with apparent β-thalassaemia major.

Mohajir:

The pattern of mutations in Mohajirs was fairly heterogeneous (Table: 6.3). The common mutations including IVSI-5 (G-C), del 619bp, Fr 8-9 (+G), Fr 41-42 (-TTCT) and Hb-E accounted for 80% of the alleles. There were 10 (10.8%) compound heterozygotes with Hb-E/β-thalassaemia and 3 (3.2%) with Hb-S/β-thalassaemia.

Parental consanguinity and inheritance of mutations:

Both β-thalassaemia alleles were identified in 513 homozygotes. The relationship between parental consanguinity and inheritance of mutations in these patients is summarized in Table: 6.4. On the whole 315 (61.4%) were offspring of first cousin marriages, 89 (17.3%) had more distant related parents (relationship not beyond second cousin) and 109 (21.2%) had totally unrelated parents. Forty three percent of subjects whose parents were unrelated had inherited the same mutation from both the parents. It increased to 58.4% for those with more more distantly related parents, and to 88.3% for the subjects whose parents were first cousins. The pattern was generally similar in all ethnic groups (Table: 6.4). However, amongst the Baluchis, where IVSI-5 (G-C) was found at a frequency of 75%, there was no difference in the frequency of inheriting the same mutation whether the parents were consanguineous or not. In 9 Hb-S/β-thalassaemia patients 5 had unrelated parents and 4 had consanguineous parents (1^{st} cousins).

Table: 6.3. β-thalassaemia mutations and Hb-S disorder in the ethnic groups of Pakistan.

All ethnic groups:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
IVSI-5 (G-C)	457 (36.9%)	Cd 15 (G-A)	51 (4.1%)	Cd 30 (G-A)	11 (0.9%)
Fr 8-9 (+G)	317 (25.6%)	Cd 30 (G-C)	43 (3.5%)	IVSII-1 (G-A)	10 (0.8%)
Del 619	85 (6.9%)	Cd 5 (-CT)	31 (2.5%)	Hb-S	9 (0.7%)
Fr 41-42 (-TTCT)	82 (6.6%)	Fr 16 (-C)	29 (2.3%)	-88 (C-T)	3 (0.3%)
IVSI-1 (G-T)	65 (5.2%)	Cap +1 (A-C)	20 (1.6%)	IVSI-1 (G-A)	2 (0.2%)
		Hb-E	13 (1.1%)	Fr 47-48 (+ATCT)	2 (0.2%)
				Fr126-131 (-17bp)	2 (0.2%)
				Cd 39 (C-T)	1 (0.1%)
				IVSI minus 25	1 (0.1%)
				Unknown	6 (0.5%)
Total 1240 (100%)	1006 (81.1%)		187 (15.1%)		47 (3.8%)
Punjabi:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
Fr 8-9 (+G)	146 (37.2%)	Del 619	14 (3.6%)	Cd 30 (G-A)	3 (0.8%)
IVSI-5 (G-C)	107 (27.2%)	Cd 15 (G-A)	14 (3.6%)	Hb-E	3 (0.8%)
Fr 41-42 (-TTCT)	36 (9.2%)	Cd 5 (-CT)	11 (2.8%)	Fr 47-48 (+ATCT)	2 (0.5%)
IVSI-1 (G-T)	19 (4.8%)	Cap +1 (A-C)	9 (2.3%)	IVSI-1 (G-A)	1 (0.3%)
Cd 30 (G-C)	15 (3.8%)	Fr 16 (-C)	6 (1.5%)	-88 (C-T)	1 (0.3%)
		IVSII-1 (G-A)	6 (1.5%)		
Total 393 (100%)	323 (82.2%)		60 (15.3%)		10 (2.5%)
Pathan:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
Fr 8-9 (+G)	105 (47.7%)	Fr 16 (-C)	8 (3.6%)	Cd 30 (G-A)	2 (0.9%)
IVSI-5 (G-C)	27 (12.3%)	Cap +1 (A-C)	8 (3.6%)	Fr126-131 (-17 bp)	2 (0.9%)
Fr 41-42 (-TTCT)	18 (8.2%)	IVSI-1 (G-T)	4 (1.8%)	-88 (C-T)	2 (0.9%)
Cd 5 (-CT)	17 (7.7%)	Del 619	4 (1.8%)	IVSII-1 (G-A)	1 (0.5%)
Cd 15 (G-A)	14 (6.4%)	Hb-S	4 (1.8%)	Cd 30 (G-C)	1 (0.5%)
				Cd 39 (C-T)	1 (0.5%)
				Unknown	2 (0.9%)
Total 220 (100%)	181 (82.3%)		28 (12.7%)		11 (5.0%)

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Table: 6.3 β-thalassaemia mutations and Hb-S disorder in the ethnic groups of Pakistan. (Continued from previous page).

Sindhi:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
IVSI-5 (G-C)	114 (43.2%)	Fr41-42 (-TTCT)	16 (6.1%)	Hb-S	1 (0.4%)
Del 619	36 (13.6%)	Fr 16 (-C)	6 (2.3%)	IVSI minus 25	1 (0.4%)
IVSI-1 (G-T)	33 (12.5%)	Cd 15 (G-A)	5 (1.9%)	IVSI-1 (G-A)	1 (0.4%)
Fr 8-9 (+G)	29 (11.0%)			Unknown:	2 (0.8%)
Cd 30 (G-C)	20 (7.6%)				
Total 264 (100%)	232 (87.8%)		27 (10.2%)		5 (1.9%)
Baluchi:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
IVSI-5 (G-C)	132 (75.4%)	Fr 16 (-C)	6 (3.4%)	Fr 41-42 (-TTCT)	1 (0.6%)
Fr 8-9 (+G)	14 (8.0%)	Cd 30 (G-C)	3 (1.7%)	Cd 5 (-CT)	1 (0.6%)
Cd 15 (G-A)	9 (5.1%)	Cd 30 (G-A)	2 (1.1%)	IVSII-1 (G-A)	1 (0.6%)
		Del 619	2 (1.1%)	Hb-S	1 (0.6%)
		IVSI-1 (G-T)	2 (1.1%)	Unknown	1 (0.6%)
Total 175 (100%)	155 (88.6%)		15 (8.6%)		5 (2.9%)
Mohajir:					
Common mutations:		**Uncommon mutations:**		**Rare mutations:**	
IVSI-5 (G-C)	77 (41.0%)	Cd 15 (G-A)	9 (4.8%)	Unknown	1 (0.5%)
Del 619	29 (15.4%)	IVSI-1 (G-T)	7 (3.7%)		
Fr 8-9 (+G)	23 (12.2%)	Cd 30 (G-C)	4 (2.1%)		
Fr 41-42 (-TTCT)	11 (5.9%)	Cd 30 (G-A)	4 (2.1%)		
Hb-E	10 (5.3%)	Fr 16 (-C)	3 (1.6%)		
		Cap +1 (A-C)	3 (1.6%)		
		Hb-S	3 (1.6%)		
		Cd 5 (-CT)	2 (1.6%)		
		IVSII-1 (G-A)	2 (1.1%)		
Total 188 (100%)	150 (79.8%)		37 (19.7%)		1 (0.5%)

Table: 6.4. Relationship between parental consanguinity and inheritance of thalassaemia mutations in patients where both of the β-thalassaemia genes were identified.

Ethnic Groups (n):	**1st cousins**			**Related but not 1st cousins**			**Unrelated**		
	Same mutation	**Different mutations**	**Total**	**Same mutation**	**Different mutations**	**Total**	**Same mutation**	**Different mutations**	**Total**
Punjabi (137)	81 (83.5%)	16 (16.5%)	97	15 (71.4%)	6 (28.6%)	21	10 (52.6%)	9 (47.40%)	19
Pathan (75)	42 (93.3%)	3 (6.7%)	45	4 (30.8%)	9 (69.2%)	13	7 (41.2%)	10 (58.8%)	17
Sindhi (128)	77 (90.6%)	8 (9.4%)	85	10 (55.6%)	8 (44.4%)	18	7 (28.0%)	18 (72.0%)	25
Baluchi (83)	54 (87.1%)	8 (12.9%)	62	11 (84.6%)	2 (15.4%)	13	7 (87.5%)	1 (12.5%)	8
Mohajir (90)	24 (92.3%)	2 (7.7%)	26	12 (50.0%)	12 (50.0%)	24	16 (40.0%)	24 (60.0%)	40
All groups (513)	278 (88.3%)	37 (11.7%)	315	52 (58.4%)	37 (41.6%)	89	47 (43.1%)	62 (56.9%)	109

Thalassaemia Intermedia (TI):

In the 519 patients of β-thalassaemia on transfusions 39 (7.5%) had a mild phenotype characterised by a late start and less frequent requirement of blood transfusions. The Xmn-I polymorphism, type of mutations, and coincidence of α-thalassaemia are presented in Table: 6.5. In 24/39 (61.5%) patients a definite cause for mildness of the disease was identified. Xmn-I +/+ genotype was found in 14 patients, β^+-mutations with or without coincident α-thalassaemia were found in 12 patients, and coincident α-thalassaemia was found in 2 patients. Two patients had unidentified mutations in combination with IVSI-5. In 14 patients Xmn-I -/+ genotype was found. However, two such patients had Cap+1 and a suspected unknown mutation outside the β-globin gene. In the remaining 12 patients Xmn-I -/+ alone or a suspected coincidental α-thalassaemia was the likely cause of thalassaemia intermedia.

Age distribution:

The ages of TI patients ranged from 3-35 years with mean age of 12 years (Fig: 6.5). The average age at first transfusion ranged from 3-26 years (mean 7 years) (Fig: 6.6). Mean age at first transfusion when examined in relation to the underlying cause of thalassaemia intermedia (Table: 6.6), it was a maximum of 10 years in patients who either had confirmed coincidence of α-thalassaemia or in whom it was suspected. It was 6 years in patients with Xmn-I +/+ genotype and 3 years in patients with a β^+-mutation.

Table: 6.5. Molecular basis of Thalassaemia Intermedia in the ethnic groups of Pakistan.

Sr. No	Age: (yrs)	First Trans[#]	β-thalassaemia Mutations	Xmn-I genotype	α-Thal genotype*	Probable cause of Thalassaemia Intermedia
Punjabi:						
1	3 yrs	None	IVSI-5 (G-C)/ IVSI-5 (G-C)	+/+	αα/αα	Xmn-I +/+
2.	5 yrs	3 yrs	IVSI-5 (G-C)/ IVSI-5 (G-C)	+/+	αα/αα	Xmn-I +/+
3.	12 yrs	4½ yrs	IVSI-5 (G-C)/ IVSI-5 (G-C)	+/+	αα/αα	Xmn-I +/+
4.	12 yrs	3½ yrs	IVSI-1 (G-T)/ IVSI-1 (G-T)	+/+	αα/αα	Xmn-I +/+
5.	5½ yrs	3 yrs	IVSI-1 (G-T)/ IVSI-1 (G-T)	+/+	αα/αα	Xmn-I +/+
6.	7 yrs	5½ yrs	IVSI-1 (G-T)/ IVSI-1 (G-T)	+/+	αα/αα	Xmn-I +/+
7.	6 yrs	5 yrs	IVSII-1 (G-A)/IVSII-1 (G-A)	+/+	αα/αα	Xmn-I +/+
8.	8 yrs	5 yrs	IVSII-1 (G-A)/IVSII-1 (G-A)	+/+	αα/αα	Xmn-I +/+
9.	8 yrs	4½ yrs	Cd 30 (G-C)/Cd 30 (G-C)	+/+	αα/αα	Xmn-I +/+
10.	13 yrs	3½ yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
11.	5½ yrs	2 yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
12	6 yrs	3 yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
13.	13 yrs	5 yrs	Fr 8-9 (+G)/Cap+1 (A-C)	-/-	$-\alpha^{3.7}\alpha/\alpha\alpha$	β^+ thal mutation & $-\alpha^{3.7}$
14.	10 yrs	None	Fr 8-9 (+G)/ IVSII-1 (G-A)	-/+	?	Xmn-I -/+, Coincident α-thal ??
15.	7 yrs	3 yrs	IVSI-5 (G-C)/Fr 8-9 (+G)	-/-	$-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$	Coincident α-thalassaemia
16.	20 yrs	15 yrs	Fr 47-48 (-ATCT)/Fr 47-48	-/-	$-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$	Coincident α-thalassaemia
Pathan:						
17.	35 yrs	24 yrs	IVSI-1 (G-T)/IVSI-1 (G-T)	+/+	αα/αα	Xmn-I +/+ & α-thal (nd??)[$]
18.	5 yrs	2½ yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/+	αα/αα	Xmn-I -/+, β^+ thal mutation
19.	15 yrs	3 yrs	IVSI-5 (G-C)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
20.	5 yrs	2½ yrs	Cd 5 (-CT)/Cap+1 (A-C)	-/-	αα/αα	β^+ thal mutation
21.	23 yrs	17 yrs	Fr 8-9 (+G)/-88 (C-T)	-/-	αα/αα	β^+ thal & α-thal (nd??)[$]
Sindhi:						
22.	18 yrs	3½ yrs	IVSI-5 (G-C)/Cd 30 (G-C)	+/+	?	Xmn-I +/+
23.	13 yrs	5 yrs	IVSI-1 (G-T)/del 619 bp	-/+	?	Xmn-I -/+,Coincident α-thala ??
24.	15 yrs	5 yrs	IVSI-5 (G-C)/Cd 30 (G-C)	-/+	?	Xmn-I -/+,Coincident α-thala ??
25.	16 yrs	9 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	-/-	?	Coincident α-thala ??
26.	15 yrs	12 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	-/+	?	Xmn-I -/+,Coincident α-thala ??
Baluchi:						
27.	16 yrs	7 yrs	IVSI-5 (G-C)/IVSII-1 (G-A)	-/+	?	Xmn-I -/+,Coincident α-thala ??
28.	11 yrs	10 yrs	IVSI-5 (G-C)/?	-/+	?	Unknown mutation ??
Mohajir:						
29.	9 yrs	5 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	+/+	?	Xmn-I +/+
30.	30 yrs	9 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	+/+	?	Xmn-I +/+
31.	17 yrs	6 yrs	IVSII-1 (G-A)/IVSII-1 (G-A)	+/+	?	Xmn-I +/+
32.	26 yrs	12 yrs	IVSI-5 (G-C)/Hb-E	-/+	?	Xmn-I -/+, β^+-mut & α-thal ??
33.	21 yrs	18 yrs	IVSI-5 (G-C)/Hb-E	-/+	?	Xmn-I -/+, β^+-mut & α-thal ??
34.	15 yrs	9 yrs	IVSI-5 (G-C)/Hb-E	-/+	?	Xmn-I -/+, β^+-mut & α-thal ??
35.	7 yrs	None	Cd 15 (G-A)/Cap+1 (A-C)	-/-	?	Xmn-I -/+, β^+-mut & α-thal ??
36.	21 yrs	5 yrs	IVSI-5 (G-C)/del 619 bp	-/+	?	Xmn-I -/+, Coincident α-thal ??
37.	31 yrs	26 yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	-/+	?	Xmn-I -/+, Coincident α-thal ??
38.	6 yrs	5½ yrs	IVSI-5 (G-C)/IVSI-5 (G-C)	-/+	?	Xmn-I -/+, Coincident α-thal ??
39.	14 yrs	5 yrs	IVSI-5 (G-C)/?	-/+	?	Unknown mutation ??

[#] Age at first transfusion; * α-thalassaemia screening included $-\alpha^{3.7}$ and $-\alpha^{4.2}$ kb deletions; [$]α-thal (nd): non-deletional α-thalassaemia.

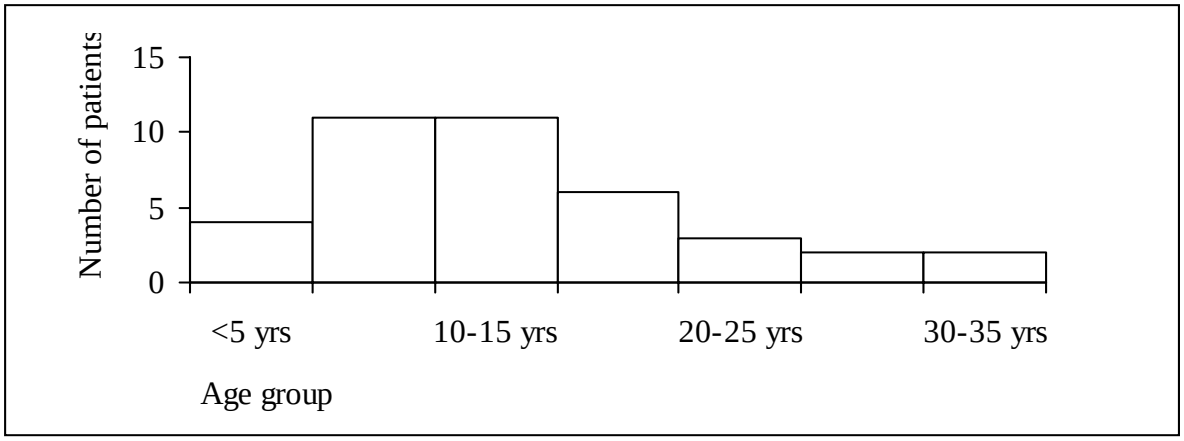

Fig: 6.5. Age distribution of 39 patients of thalassaemia Intermedia.

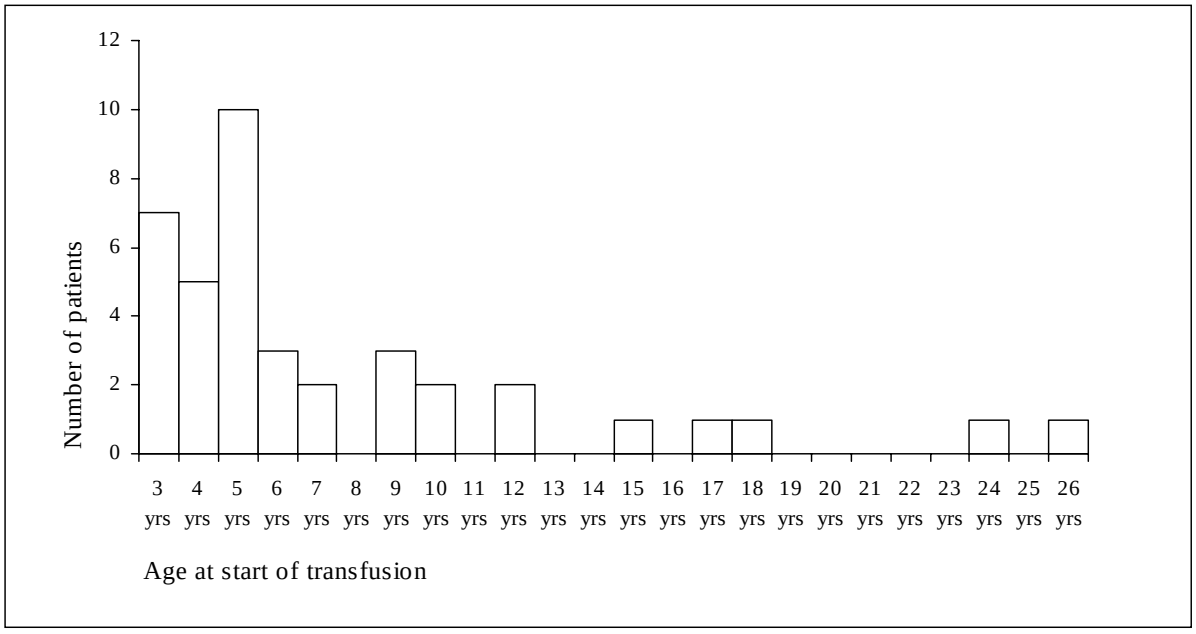

Fig: 6.6. Age distribution of 39 thalassaemia intermedia patients at the start of transfusion.

Table: 6.6. Average age at 1st transfusion and the time of examination in relation to the underlying cause of thalassaemia intermedia.

Cause of Thalassaemia Intermedia:	**n:**	**Mean age:**	
		At 1st transfusion:	**At examination:**
Xmn-I +/+ genotype	14	6 years	13 years
β^+-mutation	6	3 years	8 years
β^+-mutation and suspected coincident α-thal	6	11¼ years	18 years
Unidentified thalassaemia mutation	2	7½ years	12½ years
Confirmed coincident α-thalassaemia	2	9½ years	13½ years
Suspected coincident α-thalassaemia	9	9½ years	16 years
Total	39	7 years	14 years

Xmn-I genotype:

Xmn-I +/+ genotype accounted for thalassaemia intermedia in 14/39 (36%) patients. It was also the commonest cause for the mild phenotype in Punjabi patients where 9/16 (56%) had +/+ genotype. Xmn-I +/+ genotype was relatively uncommon in the other ethnic groups. A comparison of the Xmn-I +/+ genotype between patients of thalassaemia intermedia, severe β-thalassaemia major and normal individuals showed significant differences (Table: 6.7). There was a marked difference between thalassaemia intermedia and thalassaemia major (p=0.0005). The difference was less marked between thalassaemia intermedia and normal individuals (p=0.046) and between normal individuals and thalassaemia major (p=0.024). Xmn-I -/+ genotype was observed in 13/39 (33.3%) patients of thalassaemia intermedia. However, 9/39 (23%) patients of thalassaemia major also had Xmn-I -/+ genotype (Table: 6.8).

Correlation between the mutations and the Xmn-I genotypes in patients of thalassaemia intermedia (Table: 6.5) and thalassaemia major (Table: 6.8) showed that a linkage between some mutations and the '+' site was present (Table: 6.9). IVSI-5 (G-C), for example, was definitely linked to the '+' site in 12/33 alleles in thalassaemia intermedia and 1/25 alleles in thalassaemia major. Its linkage was suspected in 9/33 alleles in thalassaemia intermedia and 3/25 alleles in thalassaemia major patients. IVSI-1 (G-T), IVSII-1 (G-A) and Cd 30 (G-C) were other mutations that showed linkage to the "+" site.

Table: 6.7. A comparison of the Xmn-I genotypes in patients of thalassaemia intermedia, thalassaemia major and normal individuals.

Xmn-I genotype:	**Thal Intermedia:**	**Thal Major :**	**Normal:**
-/-	12 (30.8%)	30 (76.9%)	30 (51.7%)
-/+	13 (33.3%)	9 (23.1%)	20 (34.5%)
+/+	14 (35.9%)	None	8 (13.8%)
Total:	39 (100%)	39 (100%)	58 (100%)

Table: 6.8. β-thalassaemia and Xmn-1genotypes in patients of thalassaemia major.

Mutation:	**Xmn-1 genotype:**	**Mutation:**	**Xmn-1 genotype:**	**Mutation:**	**Xmn-1 genotype:**
IVSI-5/IVSI-5	-/-	IVSI-5/Fr 8-9	-/-	Fr 8-9/IVSII-1	-/+
Fr 41-42/Fr 41-42	-/-	Fr 8-9/Hb-E	-/-	IVSI-5/Hb-E	-/+
Fr 41-42/Fr 41-42	-/-	Fr 8-9/Fr 8-9	-/-	IVSI-5/IVSII-1	-/+
IVSI-5/IVSI-5	-/-	Fr 8-9/Fr 8-9	-/-	IVSI-5/Cap+1	-/-
Fr 41-42/Cd 5	-/-	Fr 8-9/IVSI-5	-/-	IVSI-1/del 619	-/+
IVSI-5/IVSI-5	-/-	Cd 15/Cd15	-/-	Cd 5/Cap+1	-/-
Del 619/Cd 5	-/-	IVSI-5/IVSI-5	-/-	Fr 8-9/Cap+1	-/-
Fr 41-42/Fr 41-42	-/-	IVSI-5/IVSI-5	-/-	Fr 8-9/Fr 8-9	-/-
IVSI-5/IVSI-5	-/+	IVSI-5/Cd 15	-/+	IVSI-5/Cap+1	-/-
Fr 8-9/Fr 8-9	-/-	IVSI-5/IVSI-5	-/-	Cd 15/Cd 15	-/+
Fr 8-9/Fr 8-9	-/-	Fr 8-9/Fr 41-42	-/+	Fr 41-42/Fr 41-42	-/-
Fr 41-42/Cd 15	-/+	IVSI-5/IVSI-5	-/-	Fr 8-9/Fr 41-42	-/-
Fr 8-9/Fr 8-9	-/-	Fr 8-9/Fr 8-9	-/-	IVSI-5/IVSI-5	-/-

Table: 6.9. Linkage between Xmn-I "+" site and β-thalassaemia mutations in patients of thalassaemia major and thalassaemia intermedia.

Mutation:	**Thalassaemia major:**				**Thalassaemia Intermedia:**			
	-	**+**	**+?***	**Total:**	**-**	**+**	**+?***	**Total:**
IVSI-5 (G-C)	21	1	3	25	12	12	9	33
Fr 8-9 (+G)	20	-	1	21	3	-	1	4
Fr 41-42 (-TTCT)	10	-	2	12	-	-	-	-
IVSI-1 (G-T)	-	-	1	1	-	8	1	9
IVSII-1 (G-A)	-	-	2	2	-	6	2	8
Cd 15 (G-A)	3	1	2	6	1	-	-	1
Cd 30 (G-C)	-	-	-	-	-	3	1	4
Cap +1 (A-C)	4	-	-	4	7	-	1	8
Cd 5 (-CT)	3	-	-	3	1	-	-	1
Hb-E	1	-	1	2	-	-	3	3
Del 619	1	-	1	2	1	-	1	2
Others	-	-	-	-	3	-	2	5
Total:	63	2	13	78	28	29	21	78

* Suspected "+"

Type of β-thalassaemia mutations:

Table: 6.10 gives a comparison between the frequency of mutations in thalassaemia major and thalassaemia intermedia patients. Significant differences in the frequency of Fr 8-9, IVSI-1 (G-T), Cap+1 and IVSII-1 were observed between the two groups. Similarly, differences in the frequency of Fr 8-9, Del 619, IVSI-1 (G-T) and Cd 5 were also observed between the younger (<3 years) and the older (≥3 years) patients of thalassaemia major.

There were 12/39 (31%) patients who had mild β-thalassaemia mutations in combination with another β^o or β^+-mutation. These included Cap+1 (8), Hb-E (3) and -88 (1). Out of 519 cases of homozygous β-thalassaemia 17 had Cap+1 mutation in combination with another severe β-thalassaemia mutation. However, their transfusion dependency was variable. Nine patients started transfusions during the first year of life while 3 received their first transfusion at or around three years of age and five received the first transfusion at an average age of 10 years. In the later group of five patients one had a coincident α-thalassaemia ($-\alpha^{3.7}\alpha/\alpha\alpha$) while in the other four although α-thalassaemia could not be shown due to technical reasons, it was suspected because no apparent cause for the mild phenotype was seen.

There were 10/519 (1.9%) patients who had Hb-E in combination with a β-thalassaemia mutation. Three of these patients had thalassaemia intermedia (mean age at first transfusion 13 years) while all others had severe thalassaemia phenotype (mean age at first transfusion 1½ years). In the former three patients coincident α-thalassaemia was suspected.

In two patients with thalassaemia intermedia only one β-thalassaemia allele (IVSI-5) was identified and the other allele could not be found in the β-globin gene.

Co-incident α-thalassaemia:

Effect of α-thalassaemia could not be investigated in samples where DNA was extracted without the use of Proteinase-K, as the quality of DNA was not good for PCR based determination of α-thalassaemia. In 22 patients, in whom it was possible to test for α-thalassaemia deletions, 2 had $-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$ genotype. One patient of thalassaemia intermedia, who also had the Cap+1 (mild β^+-mutation), had $-\alpha^{3.7}\alpha/\alpha\alpha$ genotype. The phenotype of this patient was less severe as compared to the other patients with the Cap+1 mutation.

Screening for α-thalassaemia in 100 randomly selected patients of thalassaemia major found five patients with $-\alpha^{3.7}\alpha/\alpha\alpha$ genotype (Chapter 4). All five had severe β-thalassaemia mutations but none had an intermedia phenotype. In 13 patients coincident α-thalassaemia (deletional or non-deletional) was suspected.

Table: 6.10. Comparison of β-thalassaemia mutations in patients with thalassaemia major and thalassaemia intermedia.

Mutation:	**All patients:**	**Thalassaemia major (TM):**			**Thalassaemia Intermedia (TI):**	***p* value:**	
		< 3 years:	**≥ 3 years:**	**All:**		**TM: < 3 years versus ≥ 3 years:**	**TI vs TM:**
IVSI-5	413 (39.8%)	97 (36.7%)	283 (40.7%)	380 (39.6%)	33 (42.3%)	-	-
Fr 8-9	236 (22.7%)	87 (33.0%)	146 (21.0%)	233 (24.3%)	3 (3.8%)	0.003	0.0003
Del 619	80 (7.7%)	12 (4.5%)	66 (9.5%)	78 (8.1%)	2 (2.6%)	0.01	-
Fr 41-42	67 (6.5%)	15 (5.7%)	52 (7.5%)	67 (7.0%)	-	-	-
IVSI-1 (G-T)	57 (5.5%)	5 (1.9%)	43 (6.2%)	48 (5.0%)	9 (11.5%)	0.009	0.025
Cd 15	40 (3.9%)	13 (4.9%)	25 (3.6%)	38 (4.0%)	2 (2.6%)	-	-
Cd 30 (G-C)	39 (3.8%)	6 (2.3%)	29 (4.2%)	35 (3.6%)	4 (5.1%)	-	-
Fr 16	26 (2.5%)	10 (3.8%)	16 (2.3%)	26 (2.7%)	-	-	-
Cd 5	18 (1.7%)	11 (4.2%)	6 (0.9%)	17 (1.8%)	1 (1.3%)	< 0.0001	-
Cap+1	17 (1.6%)	4 (1.5%)	5 (0.7%)	9 (0.9%)	8 (10.3%)	-	<0.0001
Hb-E	11 (1.1%)	1 (0.4%)	7 (1.0%)	8 (0.8%)	3 (3.8%)	-	-
IVSII-1	10 (1.0%)	1 (0.4%)	1 (0.1%)	2 (0.2%)	8 (10.3%)		<0.0001
Cd 30 (G-A)	9 (0.9%)	2 (0.8%)	7 (1.0%)	9 (0.9%)	-	-	-
-88	3 (0.3%)	-	2 (0.3%)	2 (0.2%)	1 (1.3%)	-	-
IVSI-1 (G-A)	2 (0.2%)	-	2 (0.3%)	2 (0.2%)	-	-	-
Fr 47-48	2 (0.2%)	-	-	-	2 (2.6%)	-	-
Cd 126-132	2 (0.2%)	-	2 (0.3%)	2 (0.2%)	-	-	-
Unknown	6 (0.6%)	-	4 (0.9%)	4 (0.4%)	2 (2.6%)	-	-
Total:	1038 (100%)	264 (100%)	696 (100%)	960 (100%)	78 (100%)	-	-

Discussion:

This study provides a basis for characterizing β-thalassaemia mutations in Pakistan. In the subsequent sections the choice of method and the screening strategy for mutation detection will be discussed.

Choice of method for mutation analysis:

Three different methods were used: ARMS, DGGE and genomic sequencing. Most alleles were identified by the ARMS method, which is quick, sensitive and specific for identifying known β-thalassaemia mutations. False positive results may be overcome by modifying the procedure e.g. redesigning primers, and reducing the amount of Taq polymerase. Comparison with known positive or negative samples and careful comparison of the amplification of control and mutant/normal sequences can further reduce the risk of error. In ARMS PCR the mutant/normal sequences are smaller and are preferentially amplified relative to the 861bp control fragment so a true positive band is always brighter than the control and a false positive band is less bright.

Carry-over from one sample to another leading to false positive results is avoided by careful procedures: using separate pipettes for pre and post amplification steps, avoiding splashes, and using positive displacement pipettes (Kwok and Higuchi 1989). Use of uracil DNA glycosylase to control carry over contamination in PCR can also be useful (Longo et al, 1990). Occasionally, extraneous DNA may contaminate PCR reagents whose presence can be excluded by using reagent blanks i.e. PCR run without adding DNA.

Denaturing Gradient Gel Electrophoresis (DGGE) provides information about the presence of a mutation but it gives little information about its nature (Myers et al, 1987). DGGE is extremely useful as the first line investigation for locating unknown sequence changes in the genome (Ghanem et al, 1992). To use DGGE alone for characterizing mutations requires each batch of samples to include appropriate controls for all the mutations and polymorphisms being investigated (Cai and Kan 1990). However, DGGE is labour intensive and less sensitive than ARMS, is unable to identify mutations very close to the primer with the GC clamp and polymorphisms can cause difficulty in interpreting results. The polymorphism present in the second codon is particularly important, as many β-thalassaemia

mutations are located in its vicinity and the same mutation gives different results in the presence or absence of this polymorphism.

Direct sequencing on PCR-amplified DNA is the standard method for identifying unknown sequences (Engelke et al, 1988; Rao 1994). A single stranded DNA template is used in most conventional sequencing protocols. The template may be prepared by asymmetric PCR (Varawalla et al, 1991b) or by magnetic particle separators (Hultman et al, 1989; Thein and Hinton 1994), but this is time consuming. In this study sequencing of double stranded and single stranded DNA templates gave comparable results. The later has the advantage of being simple and quick.

Spectrum of thalassaemia mutations:

Most studies of molecular genetics of thalassaemia on the Indian Subcontinent have been done on selected individuals settled in the Western countries (Kazazian et al 1990; Varawalla et al, 1991). These studies are inadequate because of the lack of representation of the ethnic groups. The present study of over 1200 mutant alleles provides a comprehensive picture of β-thalassaemia mutations in Pakistan.

The overall spectrum of mutations in Pakistan is fairly heterogenous and 19 different mutations were identified. The five common mutations, IVSI-5 (G-C) (37%), Fr 8-9 (+G) (25%), del 619bp (7%), Fr 41-42 (-TTCT) (7%), and IVSI-1 (G-T) (5%) account for 81% of the alleles. In each ethnic group four or five common mutations account for over 80% of the alleles. The spectrum of mutations is heterogenous in all of the ethnic groups except in Baluchis where IVSI-5 alone comprises 75% of the alleles.

Geographical and ethnic differences in the prevalence of mutations:

The spectrum of mutations in almost all ethnic groups in this study is fairly heterogenous. However, the spectrum in particular is more heterogenous in the Punjabis and the Pathans that mainly reside in the northern parts of the country. Several factors e.g. time since appearance of a mutation, population migrations, and random genetic drift might have contributed to the genetic heterogeneity (Bodmer and Cavalli-Sforza 1976).

The effect of time since appearance of a mutation:

IVSI-5 (G-C) and Fr 8-9 (+G) are consistently the most common mutations through out Pakistan. Several studies have shown that IVSI-5 (G-C) is the most prevalent mutation on the Indian subcontinent (Varawalla et al, 1991a), Burma (Brown et al, 1992), Iran and the UAE (Quaife et al, 1994). The wide distribution of IVSI-5 (G-C) indicates that it is probably the oldest and the first mutation to appear in this region. IVSI-5 (G-C) is also associated with several haplotypes as compared to other mutations (Varawalla et al, 1992). This suggests that sufficient time has elapsed since its first appearance to allow several meiotic recombination events creating more than one haplotype. Alternatively, IVSI-5 (G-C), like Cd39 (C-T) in the Mediterranean (Pirastu et al, 1987), might have arisen at more than one occasion, each time on a new haplotype. Fr 8-9 (+G) is also widely distributed in the northern region of Pakistan and could have been one of the first mutations to appear in this region. It appears that once the most common mutations had established themselves in a state of balanced polymorphism, widespread propagation of the subsequently developing or arriving mutations was prevented by the already "established" gene frequencies of the common mutations (Modell and Berdoukas 1984).

Effect of population migration:

New mutations may be brought in to an area through migration of populations. It is likely that three typical Mediterranean mutations (Cd39 (C-T), IVSI-1 (G-A), and IVSII-1 (G-A) observed in some subjects from the northern Pakistan arrived through population migrations as invaders from Central Asia, Iran, Turkey and Greece have all had their influence on this area (Halliday 1994). Hb-E amongst the Mohajirs, many of whom migrated to Pakistan from the province of Bihar in the eastern part of India, is also the result of population migration.

Previous studies found the 619 bp deletion in over 50% of β-thalassaemia alleles in Sindhis (Thein et al, 1984, Varawalla et al, 1991a), but in this study it accounted for only 14% of the alleles in Sindhis. The higher prevalence in previous reports was probably due to selected sampling. Del 619 was also seen quite frequently in Mohajirs who also include people of Gujrati descent among whom the 619bp deletion is common (Varawalla et al, 1991a).

In this study Baluchis were found to have the least heterogeneous pattern of mutations and

IVSI-5 (G-C) accounted for 75% of the alleles. The history of the Baluchis dates back to 700 BC when a tribe called Baluch migrated from Iran to the present Baluchistan (Bokhari 1975). The high frequency of IVSI-5 in Baluchis may be due to (a) founder effect (b) relative isolation (c) relatively small numbers and (d) few marriages outside the tribe preventing entry of new mutations.

Novel β-thalassaemia mutation:

Most β-thalassaemia mutations are well characterized and the chance of finding new mutation appears small. In this study only one out of 712 individuals (1240 β-thalassaemia chromosomes) investigated showed a new mutation. The 17bp deletion in Exon-III of β-globin gene was located between two copies of a CAG sequence present in Cd 125/126 and Cd 131. Sequence characteristics like a reiterated nucleotide sequence of two to eight base pairs (CAG in this case) separated by a few nucleotides appear to be involved in causation of nearly all small deletions (Bunn and Forget 1986).

Uncharacterized thalassaemia mutations:

In this study six subjects with apparent transfusion dependent thalassaemia were found to have only one mutation in the β-globin gene. All of these patients were heterozygous for a typical β-thalassaemia mutation, the second mutation could not be identified in the β-globin gene. In these patients there may be an unidentified mutation in another gene located elsewhere in the genome which is important in β-globin gene expression (Kazazian et al, 1990) or the mutation could be in the Locus Control Region (LCR) of the β-gene cluster (Cao et al, 1994). Co-inheritance of triplicate α-globin gene can also be a cause for transfusion dependency in these patients (Garewal et al, 1984). Lastly, these patients might have some other haematological abnormality e.g. congenital dyserythropoietic anaemia, pure red cell aplasia, or congenital sideroblastic anaemia which caused transfusion dependency and heterozygous β-thalassaemia could just be an incidental finding.

Screening strategy for prenatal diagnosis:

The objective of studying thalassaemia mutations is to carryout its prenatal diagnosis. Knowledge of the frequency of mutations in the target population is necessary for identifying

the mutation(s) in at risk couples rapidly and efficiently. This study provides a comprehensive picture of the common, uncommon and rare β-thalassaemia mutations in the major ethnic groups of Pakistan. There is considerable heterogeneity within each ethnic group and the frequency of various mutations varies considerably between ethnic groups. However, in all groups up to five mutations account for over 80% of the alleles. Therefore the appropriate strategy is to first screen for the five commonest mutations in the ethnic group of the couple. The vast majority will be assigned a mutation at this stage. In the second round, if required, uncommon mutations may be sought and the rare mutations examined at the end.

The results of this study show that 79% of thalassaemia major patients had consanguineous parents and 88% of couples who were first cousins had identical mutations. This frequency declined to 58% for the more distantly related parents and to 43% for the unrelated parents. This can be very helpful for rapid identification of mutations in the consanguineous at risk couples.

ARMS is the ideal method for mutation analysis in a Pakistani setting. It is quick, efficient and there are minimum chances of a false positive or negative result provided necessary precautions are taken. DGGE can be used when an unknown mutation is encountered. In a very small proportion of couples who might have uncharacterized mutations Restriction Fragment Length Polymorphism (RFLP) can be used as a backup support for prenatal diagnosis (Varawalla et al, 1992).

Thalassaemia Intermedia:

Thalassaemia intermedia is a form of homozygous thalassaemia in which affected children can survive without transfusions or with only intermittent transfusions at least in the first few years of life (Lukens 1993). The main phenotypic determinant in a typical case of β-thalassaemia major is the degree of globin chain imbalance (Weatherall et al, 1989). Any factor that tends to ameliorate the chain imbalance would also influence the clinical outcome. At least three well defined molecular mechanisms including mild β-thalassaemia mutations (Huisman 1990; Meloni et al, 1992), coincidence of α-thalassaemia (Wainscoat et al, 1983), and enhanced production of γ-globin chains (Thein et al, 1987) can lead to a thalassaemia

intermedia phenotype. The modifying factors may be present independently or in combination in one patient (Ratip et al, 1997). Coincident α-thalassaemia reduces the chain imbalance by lowering the α-chain output, β^+-mutations act by minimally reducing the output of β-globin gene, and increased production of γ-chains results in improvement of the imbalance between α and non-α chains (Cao et al, 1994).

Diagnosis of thalassaemia intermedia is mostly retrospective. However knowledge about its molecular basis can help in prospective diagnosis at an early stage (Cao et al, 1994). The information about thalassaemia intermedia in subjects from the Indian subcontinent is scanty (Thein et al, 1988). This study initiates an analysis of the molecular basis of thalassaemia intermedia in Pakistani patients. The results indicate that the clinical outcome may be determined by the underlying molecular basis. The least severe clinical picture was associated with coincident α-thalassaemia ($-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$). Coincidence of single gene deletion ($-\alpha^{3.7}\alpha/\alpha\alpha$) had no effect on the phenotype unless it was associated with a mild mutation (Gringras et al, 1994). β^+-mutations alone had minimal effect on the phenotype and Xmn-I +/+ genotype caused phenotypic effects that were in between the other two mechanisms. The results also suggest that the molecular basis of thalassaemia intermedia and therefore the clinical picture also vary considerably in the ethnic groups. In Punjabis, for example, Xmn-I +/+ genotype and in Pathans mild β-thalassaemia mutations like Cap+1 are important causes. In Sindhis, Baluchis and Mohajirs, coincident α-thalassaemia may be an important factor.

Studies in Sickle cell disease (SS) and β-thalassaemia heterozygotes have shown that A-T polymorphism at position -158 relative to $^G\gamma$ Cap site (recognized by Xmn-I) is associated with 3-11 fold increase in production per $^G\gamma$-globin gene (Gilman and Huisman 1985). The Xmn-I +/+ genotype acts by increasing the output of $^G\gamma$-globin gene in patients of homozygous β-thalassaemia. The beneficial effect of Xmn-I -/+ genotype, however, is not well defined. Winichagoon et al, (1993) have shown that -/+ genotype does not affect the phenotype. Whereas a study from Azerbaijan suggests that even Xmn-I -/+ genotype may alter the phenotype (Dr. M. Petrou personal communication). The patients of homozygous β-thalassaemia with Xmn-I +/+ genotype usually have a thalassaemia intermedia phenotype because excessive γ-chains combine with free α-chains and results in increased production of

Hb-F and lessening of dyserythropoiesis. The prevalence of Xmn-I polymorphism appears to vary between different populations. In the Mediterranean, for example, a +/+ geneotype is very uncommon (Gringras et al, 1994, Ratip et al, 1997). On the contrary it is quite frequent in the Asian Indians (Thein et al, 1987; present study) and the Southeast Asians (Winichagoon et al, 1993).

The study of linkage between Xmn-I "+" site and β-thalassaemia mutations is not large enough to draw firm conclusions. But the results give some indication that at least four mutations i.e. IVSI-5 (G-C), IVSI-1 (G-T), IVSII-1 (G-A) and Cd 30 (G-C) are more frequently associated with a "+" site than the other mutations. β-thalassaemia mutations are thought to have arisen in a relatively recent past on globin gene frameworks that are common in each population. The frequent finding of the same mutation linked to a "-" as well as a "+" site may be explained by recurrent mutations on different backgrounds, or multiple meiotic recombination events (Antonarakis et al, 1985). Multiple origin for IVSI-5 (G-C) is also supported by frequent finding of this mutation on several different globin gene haplotypes (Varawalla et al, 1992).

The frequency of β-thalassaemia mutations in patients of thalassaemia intermedia were different from thalassaemia major. In the former group milder mutations were over represented. There are also differences in the frequency of mutations between younger and older patients of thalassaemia major. Severe β-thalassaemia mutations are likely to be associated with more sever disease and therefore eliminated in the early years of life. Converse is true for the mild mutations. This highlights the difficulty in obtaining the true population frequencies of thalassaemia mutations. Representation form all age groups can minimize the selection bias due to age.