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Molecular genetics of β-thalassaemia in Pakistan: a basis for prenatal diagnosis

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Summary. Thalassaemia is the most common inherited disorder in Pakistan and there are very inadequate treatment facilities for over 4000 homozygotes born each year. Prevention of these disorders therefore forms an essential part of the management of this enormous health problem. We have characterized 1216 β-thalassaemia alleles from the five major ethnic groups of Pakistan. The complete spectrum comprised 19 different mutations. There are important ethnic and regional differences in the prevalence of mutations. The five most common mutations, IVSI-5 (G-C) (37·3%), Fr 8–9 (+G) (25·9%), del 619 (7·0%), Fr 41–42 (−TTCT) (6·7%) and IVSI-1 (G-T) (5·4%), constitute 82·3% of the total. Fr 8–9 (+G) is the most common mutation in Northern Pakistan (41·3%), whereas IVSI-5 (G-C) is the most frequent mutation in Southern Pakistan (52·2%). Six subjects with transfusion-dependent thalassaemia major showed only a single mutant allele. One subject with transfusion-dependent thalassaemia major showed a novel 17 bp deletion involving Cd126–131. Our findings provide a comprehensive basis for carrying out prenatal diagnosis of thalassaemia in a geographical area where it is found in high frequency.

Keywords: thalassaemia, mutations, ARMS, DGGE, prenatal diagnosis.

Pakistan has a population of 120 million people. The annual rate of population growth is 3% and almost 40% of the population is below 15 years of age. There are four major ethnic groups: Punjabis and Pathans living in the North, and Sindhis and Baluchis living in the South. In addition, Mohajirs, a large number of immigrants from various parts of Northern India who migrated after the partition of the subcontinent, are settled mainly in the South. Each ethnic group is subdivided into Casts or 'Biradris' of people ranging from a few thousand to millions. There is a very strong tendency for people to marry within their ethnic group, and particularly their 'Biradri'. Another very common custom is marriage between close relatives, especially first cousins (Shami & Zahida, 1981; Darr & Modell, 1988). This peculiar situation results in an unusually high frequency of autosomal recessive disorders.

Infant mortality is falling as the major communicable diseases are coming under control and, consequently, inherited disorders are becoming a recognized problem. β-Thalassaemia is the most common inherited disorder in Pakistan and studies on its carrier frequency have shown an average rate of 5% (Khattak & Saleem, 1992). It is estimated that over 4000 thalassaemic children are born in Pakistan each year, and the available health facilities are inadequate to cope with the number of sick children. The essential part of the approach to this problem is therefore a community-based preventive programme including carrier screening, genetic counselling, and the offer of prenatal diagnosis (Cao, 1987). Prenatal diagnosis will depend on knowledge of the local mutations causing thalassaemia (Kazazian *et al*, 1990), but to date there has been no systematic study of the molecular genetics of thalassaemia in relation to ethnic groups in Pakistan. The only information available is derived from a limited number of Pakistani subjects residing in the U.K. (Varawalla *et al*, 1991a, b). In this study we have investigated a large number of mutant alleles causing β-thalassaemia in the five major ethnic groups of Pakistan.

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MATERIALS AND METHODS

Subjects

The material of this study is derived from a total of 703 unrelated subjects including 184 β-thalassaemia minor cases and 519 cases of transfusion-dependent thalassaemia major. The thalassaemia major patients ranged from 3

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months to adult. Representation by ethnic group was as follows: Punjabi 256, Pathan 135, Sindhi 132, Baluchi 89, and immigrants from various parts of India (Mohajirs) 91. The subjects were identified by the Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi, by the Fatimid Foundation's Thalassaemia Treatment Centres at Peshawar, Lahore, Karachi, and by the Hussaini Blood Bank, Karachi.

Mutation analysis

Amplification refractory mutation system (ARMS). The samples were first tested by the method of Amplification Refractory Mutation System (Newton *et al*, 1989; Kwok *et al*, 1990) for the 15 known mutations previously reported in Pakistani and Indian subjects (Varawalla *et al*, 1991a, b). The list and the sequence of the primers used is given in Table I. Subjects with thalassaemia major found to be positive for any mutation were then tested for the presence of the normal allele. Those found to have the normal allele (double heterozygotes) were further tested for other mutations. All of the homozygotes and double heterozygotes were counted as representing two thalassaemic alleles.

PCR was carried out on a 25 μl reaction mixture containing 5 pmol of each primer, 0·3 units of Taq polymerase (Advanced Biotechnologies, U.K.), 30 μM of each dNTP (Boehringer Manheim), 10 mmol Tris HCl (pH 8·3), 50 mmol KCl, 1·5 mmol $MgCl_2$, 100 μg/ml gelatin and 0·3–0·5 μg of genomic DNA. The thermal cycling consisted of 25 cycles of denaturation at 94°C for 1 min, primer annealing at 65°C for 1 min and DNA extension reaction at 72°C for 1·5 min. In the final cycle, the extension reaction was prolonged to 3 min. The PCR products were electrophoresed in 4% NuSieve Agarose gel (Flowgen, U.K.), stained in ethidium bromide and photographed under 302 nm UV light.

Denaturing gradient gel electrophoresis. The samples that did not reveal any mutation by ARMS were subjected to Denaturing Gradient Gel Electrophoresis (DGGE) (Myers *et al*, 1987). Four overlapping regions of the β-globin gene were amplified as described by Cai & Kan (1990). PCR was carried out on a 50 μl reaction mixture containing 10 pmol of each primer, 1·0 unit of Taq polymerase (Perkin Elmer, U.K.), 200 μM of each dNTP (Boehringer Manheim), 10 mmol Tris HCl (pH 8·3), 50 mmol KCl, 1·5 mmol $MgCl_2$, 100 μg/ml gelatin and 0·3–0·5 μg of genomic DNA. The thermal cycling regimen consisted of initial denaturation for 5 min at 95°C and then 40 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and DNA extension

Table I. List of the mutations screened and the ARMS primers used for their identification.

Mutation	Primer	Used with	Fragment size
IVSI-5 (G-C)	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG	3	285
IVSI-5*	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC	3	285
Fr 8–9 (+G)	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC	3	215
Fr 8–9*	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT	3	214
IVSI-1 (G-T)	TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA	3	281
IVSI-1*	GAT GAA GTT GGT GGT GAG GCC CTG GGT AGG	4	450
Fr 41–42 (−TTCT)	GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT	3	439
Fr 41–42*	GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA	3	443
Del 619 bp	CAA TGT ATC ATG CCT CTT TGC ACC	2	242
Cd 15 (G-A)	TGA GGA GAA GTC TGC CGT TAC TGC CCA GTA	4	500
Cd 15*	TGA GGA GAA GTC TGC CGT TAC TGC CCA GTG	4	500
Cd 5 (−CT)	ACA GGG CAG TAA CGG CAG ACT TCT CCG CGA	3	205
Cd 5*	ACA GGG CAG TAA CGG CAG ACT TCT CCG CAG	3	207
Cd 30 (G-C)	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACG	3	280
Cd 30 (G-A)	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACT	3	280
Cd 30*	TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC	3	280
Fr 16 (−C)	TCA CCA CCA ACT TCA TCC ACG TTC ACG TTC	3	238
IVSII-1 (G-A)	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAT	3	239
IVSII-1*	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAC	3	634
Cd 26 (G-T)(Hb-E)	TAA CCT TGA TAC CAA CCT GCC CAG GGC GTT	3	278
Cap +1 (A-C)	ATA AGT CAG GGC AGA GCC ATC TAT TGG TTC	4	567
Fr 47–48 (+ATCT)	ATA ACA GCA TCA GGA GTG GAC AGA TAG ATC	3	467
1VS1–25 (25b del)	CTC TGG GTC CAA GGG TAG ACC ACC AGC ATA	3	354
1. Control	CAA TGT ATC ATG CCT CTT TGC ACC		
2. Control	GAG TCA AGG CTG AGA GAT GCA GGA		
3. Common	ACC TCA CCC TGT GGA GCC AC		
4. Common	CCC CTT CCT ATG ACA TGA ACT TAA		

* Primers for the normal alleles.

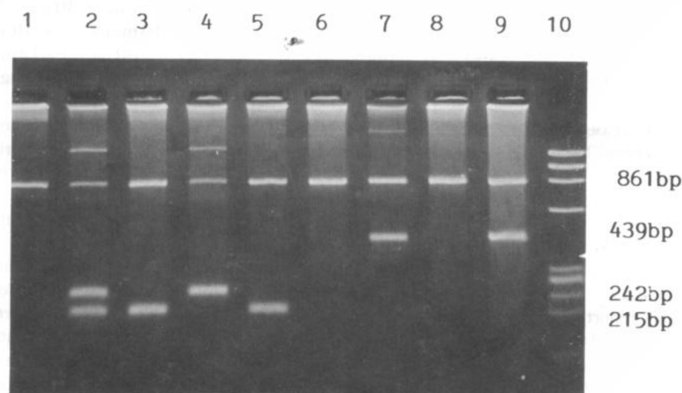

Fig 1. Ethidium bromide stained agarose gel electrophoresis of ARMS PCR products. All lanes show 861 bp amplified products of PCR internal control. Lanes 2, 3 and 5 show 215 bp fragment of Fr 8–9 mutation. The sample in lane 2 also has a 242 bp fragment representing del 619. Lanes 7 and 9 show 439 bp fragment of Fr 41–42 mutation. Lane 10 contains Hae III digest of ØX174.

reaction at 70°C for 1 min. In the final cycle, the extension reaction was prolonged to 3 min. At the end of thermal cycling 10 μl of the amplified product was run on 2% agarose gel to determine the quality of amplification.

DGGE was carried out on 7% polyacrylamide gel containing 32% formamide and 5·6 M urea as denaturants. Varying degrees of gradients ranging from 25% to 80% were prepared by a gradient mixer. The gels were run at 60°C overnight at 40 V.

Genomic sequencing. The region of the β-globin gene shown by DGGE to have a mutation was amplified by a set of primers flanking the mutation. 5 μl of the amplified product was incubated at 37°C for 15 min with 10 units of Exonuclease-I and 2 units of Shrimp Alkaline Phosphatase (United States Biochemical). The Exonuclease-I removes residual single-stranded primers and any extraneous single-stranded DNA produced during PCR. The Shrimp Alkaline Phosphatase removes the remaining dNTPs from the PCR mixture which would interfere with labelling reactions of sequencing. The enzymes were inactivated by heating at 80°C for 15 min.

DNA sequencing was done by the dideoxy chain termination method (Sanger *et al*, 1977). The Sequenase Version 2.0 DNA sequencing kit (United States Biochemical) was used. The enzyme-treated double-stranded amplified product was denatured at 100°C for 2 min with 10 pmol of an internal sequencing primer. The mixture was cooled by placing on ice for 5 min. Labelling was done using 5 μCi ^{35}S-dATP (Amersham, U.K.) and 2 units of Sequenase Version 2.0. The labelled DNA was run on 6% polyacrylamide gel containing 7 M urea at a constant power of 50 W for 2–3 h. The results were read after autoradiography for 48 h.

RESULTS

A total of 1222 mutant alleles were investigated. It was possible to characterize a β-thalassaemia mutation in 1216 alleles. The results of ARMS, DGGE, and genomic sequencing are shown in Figs 1, 2 and 3, respectively. Six transfusion-dependent thalassaemia major cases revealed only one mutation. It was not possible to demonstrate the second mutation even after sequencing the entire β-globin gene and its immediate 5′ and 3′ flanking regions. The 1216 mutant alleles comprised of 19 different mutations. The results of mutations in different ethnic groups is presented in Table II. The five most common mutations, i.e. IVSI-5 (G-C), Fr 8–9 (+G), IVSI-1 (G-T), del 619 bp, and Fr 41–42 (−TTCT) accounted for 82·3% of the alleles. Many regional and ethnic differences in the distribution of mutations were noted (Fig 4). Fr 8–9 (+G) was found to be the most common mutation in Northern Pakistan. The two major ethnic groups of this region, i.e. Pathans and Punjabis, had Fr 8–9 (+G) in 49·1% and 37·2% of the alleles respectively. IVS1-5 (G-C) was found to be the predominant mutation in Southern Pakistan. Sindhis, the major ethnic group of Southern Pakistan, had IVSI-5 (G-C) in 43·9% and Baluchis had IVS1-5 (G-C) in 76·2% of the alleles. Del 619 bp was

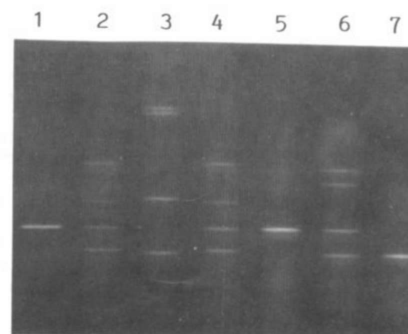

Fig 2. Ethidium bromide stained DGGE of a 286 bp fragment of β-globin gene extending from position −123 to Cd24. Samples in lanes 1 and 5 are homozygous for the normal sequence. Lanes 2 and 4 are heterozygous for a polymorphism at Cd2. Lane 3 is heterozygous for Cap+1 mutation. Lane 6 is heterozygous for Cd15 and lane 7 is homozygous for polymorphism at Cd2.

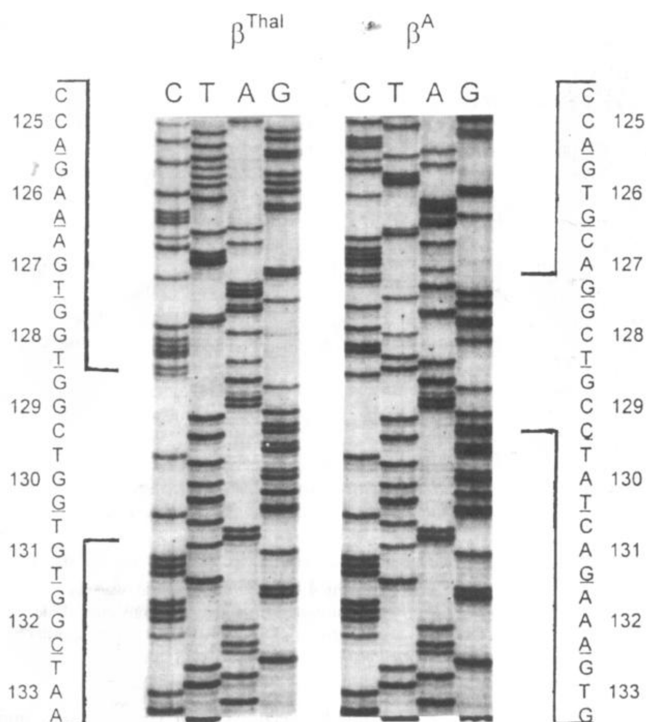

Fig 3. DNA sequencing gel showing the non-coding strand of exon III containing the 17 bp deletion. Sequence of the normal DNA is also shown.

Table II. Prevalence of β-thalassaemia mutations in the major ethnic groups of Pakistan.

Mutation	Punjabi	Pathan	Sindhi	Baluchi	Mohajir	All
Common mutations						
IVSI-5 (G-C)	107 (27·2%)	27 (12·9%)	114 (43·9%)	131 (76·2%)	75 (41·4%)	454 (37·3%)
Fr 8–9 (+G)	146 (37·2%)	103 (49·1%)	29 (11·2%)	14 (8·1%)	23 (12·7%)	315 (25·9%)
Del 619 bp	14 (3·6%)	4 (1·9%)	36 (13·9%)	2 (1·2%)	29 (16·0%)	85 (7·0%)
Fr 41–42 (−TTCT)	36 (9·2%)	18 (8·6%)	16 (6·2%)	1 (0·6%)	11 (6·1%)	82 (6·7%)
IVSI-1 (G-T)	19 (4·8%)	4 (1·9%)	33 (12·7%)	2 (1·2%)	7 (3·9%)	65 (5.4%)
Uncommon mutations						
Cd 15 (G-A)	14 (3·6%)	13 (6·2%)	5 (1·9%)	9 (5·2%)	8 (4·4%)	49 (4·0%)
Cd 30 (G-C)	15 (3·8%)	1 (0·5%)	19 (7·3%)	3 (1·7%)	4 (2·2%)	42 (3·5%)
Cd 5 (−CT)	11 (2·8%)	16 (7·6%)	0 (0·0%)	1 (0·6%)	2 (1·1%)	30 (2·5%)
Fr 16 (−C)	6 (1·5%)	8 (3·8%)	6 (2·3%)	6 (3·5%)	3 (1·7%)	29 (2·4%)
Cap+1 (A-C)	9 (2·3%)	8 (3·8%)	0 (0·0%)	0 (0·0%)	3 (1·7%)	20 (1·6%)
Hb-E	3 (0·8%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	10 (5·5%)	13 (1·1%)
Cd 30 (G-A)	3 (0·8%)	2 (1·0%)	0 (0·0%)	2 (1·2%)	4 (2·2%)	11 (0·9%)
IVSII-1 (G-A)	6 (1·5%)	1 (0·5%)	0 (0·0%)	1 (0·6%)	2 (1·1%)	10 (0·8%)
Rare mutations						
−88 (C-T)	1 (0·3%)	2 (1·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	3 (0·3%)
IVSI-1 (G-A)	1 (0·3%)	0 (0·0%)	1 (0·4%)	0 (0·0%)	0 (0·0%)	2 (0·2%)
Fr 47–48 (+ATCT)	2 (0·5%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	2 (0·2%)
Fr 126–131 (−17 bp)	0 (0·0%)	2 (1·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	2 (0·2%)
Cd 39 (C-T)	0 (0·0%)	1 (0·5%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	1 (0·1%)
IVSI minus 25	0 (0·0%)	0 (0·0%)	1 (0·4%)	0 (0·0%)	0 (0·0%)	1 (0·1%)
Total	393 (100%)	210 (100%)	260 (100%)	172 (100%)	181 (100%)	1216 (100%)

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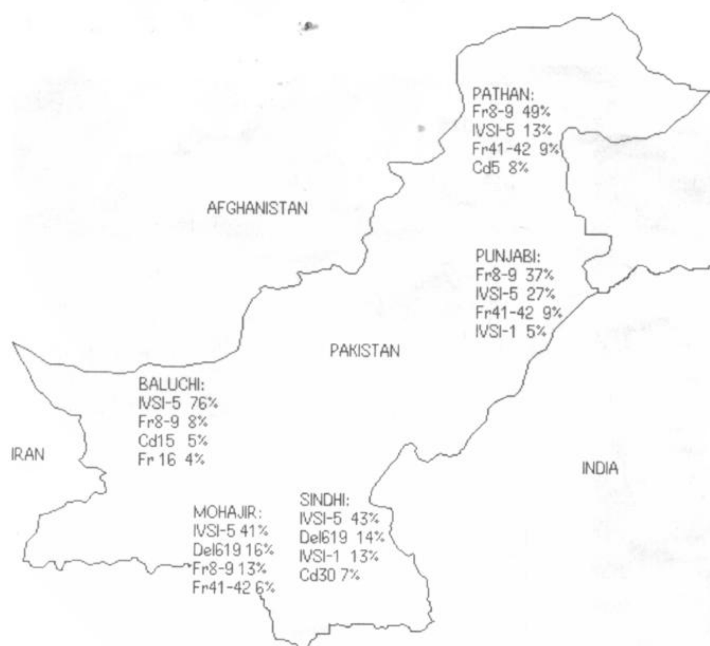

Fig 4. Distribution of β-thalassaemia mutations in different regions and ethnic groups of Pakistan.

seen predominantly in immigrants from India (16·0%) and in native Sindhis (13·9%). Cd5 (−CT), Cd15 (G-A) and Cap+1 (A-C) were seen predominantly in Northern Pakistanis. Fr47/48 (+ATCT), a mutation previously found in a Pakistani subject (Dr Mary Petrou, personal communication), was detected in a Punjabi subject who had thalassaemia intermedia phenotype. Three subjects had typical Mediterranean mutations including IVS1-1 (G-A) and Cd39 (C-T). A Pathan patient, who had consanguineous parents and had severe transfusion-dependent thalassaemia, was found to be homozygous for a novel β-thalassaemia mutation resulting in 17 bp deletion extending from Cd126 to Cd131 (-TGCAGCCTGCCTATCAG) (Fig 3).

The relationship between parental consanguinity and inheritance of mutations in the subjects with thalassaemia major, excluding the six subjects where only one mutant allele was identified, is presented in Table III. Out of the 513 subjects, 315 (61·4%) were offsprings of first cousin marriages. Another 89/513 (17·3%) were born to parents who were related but were not first cousins (relationship not beyond second cousins). In the consanguineous group 330/404 (81·7%) subjects had inherited the same mutation from both parents. The remainder were double heterozygotes for two different mutations. In the non-consanguineous group only 47/109 (43·1%) had inherited the same mutation from both parents.

Table III. Relationship between consanguinity and inheritance pattern of thalassaemia mutations in individuals with two copies of β-thalassaemia genes.

	First cousins			Related but not first cousins			Unrelated		
Ethnic groups (n)	Same mutation	Different mutation	Total	Same mutation	Different mutation	Total	Same mutation	Different mutation	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

DISCUSSION

The results of thalassaemia prevention programmes in the Mediterranean (Cao, 1987) have provided a new incentive to overcome the problem of thalassaemia in countries such as Pakistan. Experience in the Mediterranean has shown that preventive programmes for thalassaemia based on heterozygote carrier detection and genetic counselling alone are not effective in reducing the incidence of new births of children with thalassaemia major (Barrai & Vullo, 1980). Prenatal diagnosis of thalassaemia has given a new dimension to thalassaemia prevention. In order to carry out prenatal diagnosis it is most important to have a comprehensive knowledge of the prevalent mutations (Kazazian *et al*, 1990). The molecular genetics of thalassaemia has been studied extensively in subjects from the Indian subcontinent (Kazazian *et al*, 1984; Thein *et al*, 1988; Varawalla *et al*, 1991a, b); however, most of this work has been done on families settled in the West. There has been no well-planned study on the ethnic groups in Pakistan. This study gives a comprehensive picture of the molecular genetics of β-thalassaemia in Pakistan. The spectrum of mutations in all of the ethnic groups studied is heterogenous, and we have found 19 different mutations. An important reason for heterogeneity of the mutations appears to be the geographic location of Pakistan, particularly Northern Pakistan, which has been the gateway for most invasions of the Indian subcontinent. There is a strong possibility that many of the mutations were brought to this region by population migrations.

The results of this study partly conform with a previous study on immigrant Pakistanis who migrated to U.K. from some areas of Northern Pakistan (Varawalla *et al*, 1991a, b); however, we have found a much wider spectrum of mutations. In addition, del 619, previously reported to be present in 56% of Sindhis (Thein *et al*, 1984; Varawalla *et al*, 1991a), represented only 13·9% of alleles from the native Sindhi subjects. Previous studies showing a high prevalence of del 619 were probably carried out on selected groups of patients and were not a true representative of the Sindhi population. We have identified a novel 17 bp deletion in exon III of β-globin gene. The deletion is located between two copies of a CAG sequence present in Cd125/126 and Cd131. It is not possible to ascertain whether the actual breakpoint is before or following the CAG sequence in Cd 125/126. In either case Cd 125 is left intact. Sequence characteristics, such as a reiterated nucleotide sequence of two to eight base pairs (e.g. CAG in this case), separated by a few nucleotides, appear to be involved in almost all small deletions leading to haemoglobin disorders (Bunn & Forget, 1986). As a result of the deletion the reading frame is shifted and a premature stop signal (TAA) is encountered in the new Cd 133. Another interesting aspect of this study is the finding of six subjects who had transfusion-dependent thalassaemia and yet possessed only one thalassaemic allele. It is suspected that transfusion dependency in these apparently heterozygous subjects may in part be due to co-inheritance of triplicated α-globin genes (Garewal *et al*, 1994).

The relationship of mutation pattern and consanguinity shows that mutation analysis in consanguineous couples would be considerably easier, because there is over an 80% chance that both the parents in such couples would have the same mutation. It should be pointed out that the figures of parental consanguinity (61·4%) amongst the thalassaemic families is high because these families are selected and do not represent the overall level of consanguineous marriages in the population. In the Punjabi population, consanguinity has been reported to be around 40% (Shami & Zahida, 1981).

This study provides a comprehensive basis for prenatal diagnosis of thalassaemia in all ethnic groups in Pakistan, which has been introduced recently (Ahmed *et al*, 1994). The service has been offered primarily to parents with affected children. The preliminary results of the first 70 cases diagnosed prenatally at the Armed Forces Institute of Pathology (AFIP), Rawalpindi, indicate that this is technically feasible and is also accepted amongst these families (unpublished observation).

Studies in the U.K. (Petrou, 1994) have shown that British Pakistani couples have particular problems with late termination of pregnancy and only about 23% of couples accept prenatal diagnosis and selective abortion of affected fetuses in the second trimester of pregnancy (Modell *et al*, 1980; Petrou, 1994). However, when counselled in the first trimester of pregnancy, they often make use of prenatal diagnosis. Almost all prenatal diagnoses done at AFIP in Pakistan have been in the first trimester of pregnancy and >95% of the women with a fetus having thalassaemia major opted for termination of pregnancy. However, there is little awareness amongst the general population and health professionals about the role of prenatal diagnosis in the prevention of thalassaemia. Mediterranean programmes have shown that large-scale community-based thalassaemia prevention programmes can be implemented effectively if they are incorporated into a primary health-care programme (Angastiniotis *et al*, 1986; Cao, 1987). Since no primary health-care system exists in Pakistan, a different approach would be required for this purpose and this needs to be developed.

In a prospective study we will be evaluating the number of couples making use of prenatal diagnosis offered during pregnancy. Studies are also under way to determine suitable approaches for identifying at-risk couples prospectively, with a view to eventually offering them prenatal diagnosis.

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