ORIGINAL ARTICLE

MUTATIONAL ANALYSIS OF BETA THALASSAEMIA BY MULTIPLEX ARMS-PCR IN KHYBER PAKHTUNKHWA, PAKISTAN

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Background: Beta thalassaemia is one of the commonest genetic conditions in the world. More than 200 different mutations have been reported in the beta globin chain genes. Notably, regional and ethnic variations in most common mutations in beta-thalassaemia have been identified. It is therefore imperative that region- and ethnicity- specific commonest mutations be identified for cost-effective molecular diagnosis of β-thalassaemia mutations. The objective of this study was to determine the molecular mutations in β-globin chain gene in patients with thalassemia in Khyber Pakhtunkhwa (KP) using multiplex- Amplification Refractory Mutation System (ARMS) PCR. **Methods:** It was a cross sectional descriptive study. Blood samples from newly diagnosed β thalassemia patients was collected and used as source for DNA isolation. ARMS PCR was performed for detection of mutations in β-globin gene. SDS-PAGE was conducted for visualization of the amplicon. **Results:** Prominent mutations were Fr 8-9 (+G), CD 5 (-CT) and Fr 41-42 (-TTCT). Congenital marriages and lack of awareness are largest contributing factor for increasing the disease burden. Organomegaly being a serious clinical complication which contributes to morbidity was proportional to age and disease progression. Fr 8-9 (+G) & CD 5 (-CT) were the most frequent mutation prevalent among different ethnic groups residing in KP. **Conclusions:** Multiplex-ARMS PCR is capable of assessing for multiple mutations in a single tube. Regional- and ethnic- variations in the commonest mutations in KP are noted. Any mutational diagnostic strategy should consider costs and genetic variations in a particular setting.

Keywords: Beta Thalassemia; β-Thalassaemia; Mutation frequency; Allele distribution

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INTRODUCTION

β-Thalassaemia is one of the most devastating genetic disorders, prevalent in the world, historically associated with malarial endemic regions. It occurs due to mutation in β-gene of autosomal chromosome 11. Nearly 3% population of the world, i.e., almost 150 million people are carriers of beta-thalassemia gene mutations.[1,2] The lack of or insufficient production of β-globin peptides results in the clinical symptoms of β- thalassemia. More than 200 mutations in the beta-globin chain gene have been identified.[3] Homozygous mutations result in severe transfusion-dependent anaemia, ineffective erythropoiesis, and extra-medullary haematopoiesis. Carrier state is characterized by mild hypochromic microcytic anaemia with a compensatory relative increase in red cell count. Multiple strategies to prevent homozygous disease in progeny of carrier parents have been tested. One of these is pre-natal diagnosis using chorionic-villous sampling and genetic analysis of beta-globin chain gene.[4] Due to the large repertoire of mutations, it is not feasible to test for all the mutations. A sensible strategy is to test for the most common mutations prevalent in a population group. Furthermore, to reduce costs of testing, multiplexing strategy reduces the amount of reagents and consumables.[5] However, this method requires more optimization and interpretation can be challenging.

Almost all of the β –thalassemia mutations have already been specified in the population of Pakistan which reveals 21 β globin mutant gene types. Among these, only five-point mutations make about 86% of the all molecular variability's causing it in the country.[6] This renders the diagnosis at molecular level straight forward in most of the patients. Any cost-efficient and convenient detection system for these specified mutations can be used for identification of the mutant or defective alleles and prenatal diagnosis to avoid affected births. However, the regional and ethnic variations within Pakistan's provinces mean that these findings should be validated in the various population subgroups.

In this study, we intended to study mutational makeup of β-globin gene of KPK region. We employed Amplification-Refractory Mutation System (ARMS) PCR approach. We report Fr 8-9

(+G) to be the most frequent mutation in the targeted KP region followed by CD 5 (-CT) and Fr 41-42 (-TTCT).

MATERIAL AND METHODS

Sixty newly diagnosed Beta Thalassemia major patients from Hamza foundation and Fatamid foundation, Peshawar were designated for patient sample pool. Blood samples were collected from different individuals with informed consent. Molecular facilities available Institute of Basic Medical Sciences, Khyber Medical University Peshawar were used for DNA extraction. Patient blood was used for DNA isolation via DNA extraction kit (VIVANTIS® catalog no GF-BD-100).[7] DNA was extracted from peripheral blood lymphocytes. The buffers were reconstituted as per protocol. PCR for BT mutations/deletions was conducted via ARMS-PCR, for that 3 separate reaction mixtures were used. First multiplex contained primers (Invitrogen life technologies USA) for, Fr 8-9 (+G), IVSI-5 (G-C), Fr 41-42 (-TTCT), IVSI-1 (G-T) and Del 619 bp. Second multiplex contained primers for Fr 16(-C), Cd 5 (-CT), IVSI-1 (G-T), Cd30 (G-C), Cd 30 (G-A), and IVSII-1 (G-A). The 3rd multiplex contained primers for Cap +1 (A-C) and Cd 15 (G-A). These genotypes cover almost about 98% of beta thalassemia mutations or deletions in Pakistan. Table-1 shows the list of primers; mutation-specific ARMS primers used to diagnose the common β-thalassemia mutations in Khyber Pakhtunkhwa population.

PCR was performed with a reaction mixture of 25 µl which contained 5 pmol of prior specified set of primers, 0.3 units of Taq polymerase, 30 µM of each dNTP, 10 mmol Tris HCl (pH 8.3) 50 mmol KCl , 1.5 mmol $MgCl_2$, 00, 100 µg/ml gelatin and 0.3–0.5 µg of genomic DNA.

The standard PCR procedure (denaturation at 94 °C for 1 minute, primer annealing at 65 °C for 1 minute, and chain extension/ elongation reaction at 72 °C for 1.5 minute) was done with 25 cycles and finally (extension reaction for 3 minutes). The amplicon was electrophoresed on 6% polyacrylamide gel in 1X Trisborate-EDTA buffer for. 3-4µl of the prepared sample was loaded on the gel by using a long nose plastic tip. The gel was run at 150–200 volts for 15–45 minutes depending on the fragment size of the amplified products. The gel was placed in 0.1% silver nitrate solution for 15–20 minutes. The stain was discarded and the gel was washed with plenty of tap water. Fresh developing solution was prepared by adding 75µl formaldehyde to approximately 100 ml 1.5% NaOH which should be used within an hour. The gel was submerged completely in the developing solution. After incubating for 15–20 minutes the amplified product was visible on the gel as sharp bands. The background of the gel also became yellowish brown. The developing solution was discarded and the gel was washed with plenty of water when the DNA bands were clearly seen and visualized under a trans-illuminator. The evaluation of band patterns was performed by comparing it to the control. The following photograph shows the agarose gel electrophoresis pattern of ARMS-PCR for different mutations. For the cases with single mutation detection a second PCR reaction was run with set of primers containing a normal allele of the respective mutation for detection of normal (wild type) sequence. In case if the normal allele was also amplified in the reaction the DNA in question was assigned heterozygous. In case if normal counterpart was not detected DNA was labelled as homozygous for the respective mutation.[8]

RESULTS

Mutations were analysed in terms of gender and ethnic distribution. Most prominent homozygous mutation males were Fr 8-9 (+G) occurring 20% of the population while seven different heterozygous mutation combinations were observed. In terms of females, again Fr 8-9 (+G) was most frequent homozygous mutation with similar appearance frequency as in males. In contrasting manner Fr 41-42 (-TTCT) / CD 5 (-CT) was most frequent heterozygous mutation combination in females (Table-2). Ethnic influence revealed Fr 8-9(+G) and Cd-5(-CT) most frequent mutations in majority of the ethnic groups of KP province (Figure-2). Another interesting finding was correlation between Hb serum levels and the mutations (Figure-1 D). The patients whose blood Hb levels were lowest (3.1 mg/dl) had Fr 8-9 (+G) while patient with highest levels (10 mg/dl) was having FR 41 42 / CD 30 mutation. When assessed in comparative manner, unique mutations specific to minimum Hb production Fr 8-9 (+G)/Fr -16 (-C) heterozygous and CD 15(G-A) homozygous condition. Amplified ARMS-PCR product was ran on 6% acrylamide gel. Figure-3 depicts the representative electrophoresed gels for each of the mutant alleles detected. IVS 1-5(G-C) amplicon of size 238 bp, Fr 41-42 (-TTCT) amplicon was 439 bp long. Fr-16 (-C) exhibited band sized about 238 bp. Fr 8-9 (+G) amplified product was of size 215 bp in length. Cd-15(G-A) amplicon sized about 500 bp. Cd-5 (-CT) yielded 205 bp long amplicon. Cap +1 had amplicon of size 675 bp. Cd-30 (G-A) amplified product was 280 bp in length. Lanes marked M* depict the mutant genotype, C+ contains positive control, C- contains negative control and N* represents normal.

Table-1: List of primers used for detection of each mutation analysed.

Primer ID	Mutations	Primers	Product Size
AD-1	Fr 8-9(+G)	5'CCTTGCCCCACAGGGCAGTAACGGCACACC	215bp
	IVSI -5(G-C)	5'CTCCTTAAACCTGTCTTGTAACCTTGTTAG	285bp
	Fr 41-42(-TTCT)	5'GAGTGGACAGATCCCCAAAGGACTCAACCT	439bp
	IVSI -1(G-T)	5'TTAAACCTGTCTTGTAACCTTGATACGAAA	280bp
	Del 619bp	5'CAATGTATCATGCCTCTTTGCACC	242bp
AD-2	Cd 5(-CT)	5'ACAGGGCAGTAACGGCAGAACTTCTCCGCGA	205bp
	Fr 16 (-C)	5'TCACCACCAACTTCATCCACGTTCACGTTC	238bp
	IVSI -1 (G-T)	5'TTAAACCTGTCTTGTAACCTTGATACGAAA	280 bp
	Cd 30 (G-C)	5' TAAACCTGTCTTGTAACCTTGATACCTACG	280 bp
	Cd 30 (G-A)	5' TAAACCTGTCTTGTAACCTTGATACCTACT	280 bp
	IVSII-1 (G-A)	5'AAGAAAACATCAAGGGTCCCATAGACTGAT	634bp
AD-3	Cd 15 (G-A)	5'TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	500 bp
	Cap+1 (A-C)	5'AAAAGTCAGGGCAGAGCCATCTATTGGTTG	567 bp

Table-2: Gender wise mutation distribution frequency

S. No.	Mutations		Frequency
1	**Male Homozygous Mutations**	CD 30 (G-A)	1
		CD 15 (G-A)	1
		CD 5 (-CT)	7
		Fr 16 (-C)	1
		Fr 41-42 (-TTCT)	2
		Fr 8-9 (+G)	12
		IVS 1-5 (G-C)	3
2	**Male Heterozygous Mutations**	Cd- 15 (G-A) / Fr 8-9 (+G)	1
		Cd.5(-CT)/Cd-30(G-)	1
		Fr 41-42 (-TTCT) / Fr - 16(C)	1
		Fr 8-9 (+G) / Fr 16 (-C)	1
		Fr 8-9 (+G) / Fr 41-42 (-TTCT)	1
		Fr 8-9 (+G) / IVS 1-5 (G-C)	1
		IVS 1-5 (G-C) / Cap +1 (A-C)	1
3	**Female Homozygous mutations**	CD 15 (G-A)	3
		Fr 8-9 (+G)	12
		Fr 16 (-C)	1
4	**Female Heterozygous Mutations**	CD 5 (-CT) / Fr 8-9(+G)	2
		Fr 41-42 (-TTCT) / CD 5 (-CT)	3
		Fr 41-42 (-TTCT) / CD 30 (G-A)	1
		Fr 8-9 (+G) / Fr 16 (-C)	1
		Fr 8-9 (+G) / Fr 41-42 (-TTCT)	2
		Fr 8-9 (+G) / IVS 1-5 (G-C)	1

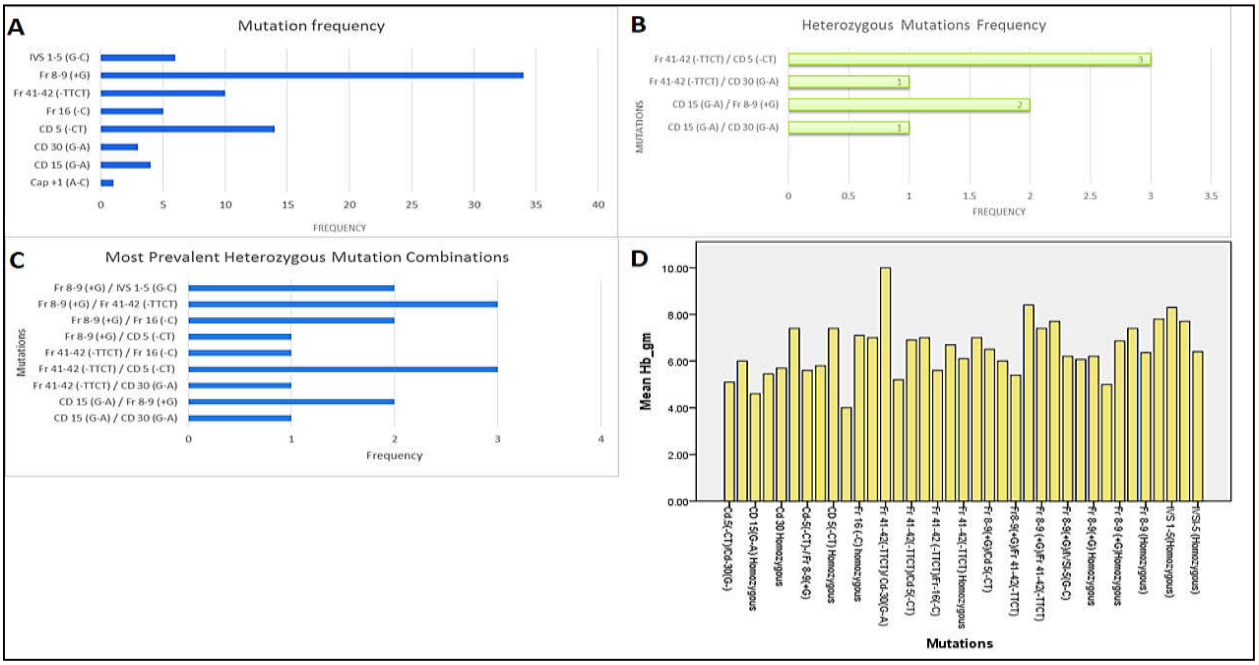

Figure-1: Mutational Analysis of Beta-Thalassemia genes in KP Pakistan: (A) Mutation frequency in sample population, (B) Frequency of heterozygous mutations in sample population. (C) Most prevalent heterozygous mutations in β thalassemia patients of KP, (D) Correlation of Hb levels with β-Thalassemia mutations in sample population.

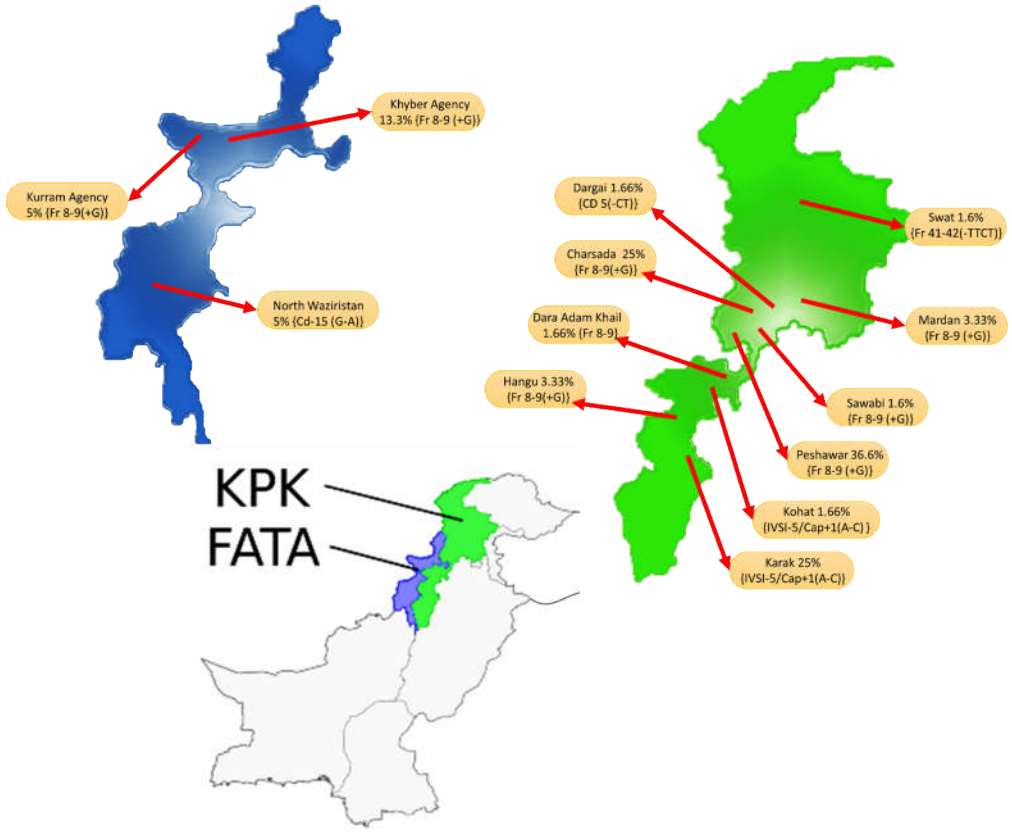

Figure-2: Geographical distribution of sample population and subsequent most frequently or the only detected Beta Thalassemia mutation in different regions of KP, Pakistan

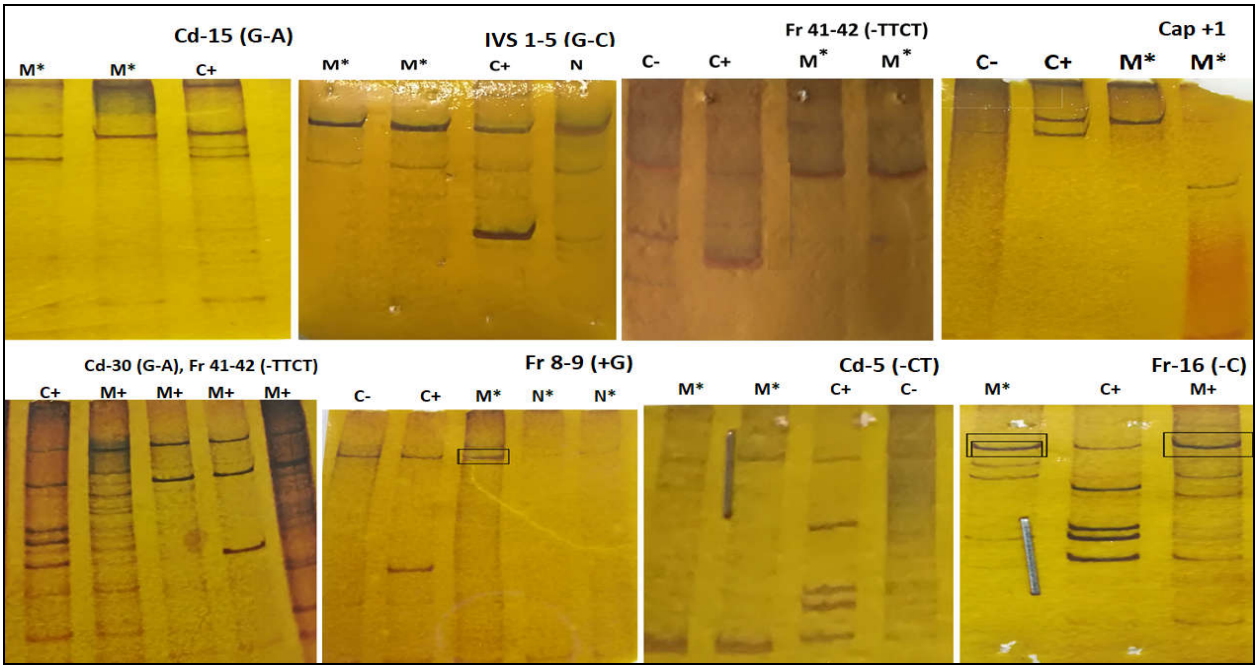

Figure-3: Gel depicting observed mutations. Lanes marked M* depict the mutant genotype, C+ contains positive control, C- contains negative control and N* represents normal.

DISCUSSION

β- Thalassemia is one of the commonest inheritable disorders in Pakistan. It causes reduced or absence of β-chain synthesis of Hb. This study was aimed at exploring β thalassemia mutations in Khyber Pakhtunkhwa population of Pakistan. Among the regionally prominent mutations reported, i.e., IVS-1-5 (G–C), Fr 8/9 (+G), Fr 41/42 (–TTCT), IVS-1-1 (G–T), IVS-II-1 (G–A), CAP+1, Cd 5 (–CT), Cd 16 and Cd 3 Cd 15 (G–A) (6-9). Mutational analysis revealed Fr 8-9 (+G) to be the most frequent or the only observed mutation in 13 ethnic groups while CD 5 (-CT) was the second most prevalent one. When considered in reference to geographic distribution, Fr 8-9 (+G) mutation was most common in most of the central regions of KP, i.e., Kurram, Khyber agencies in FATA, Peshawar Charsada, Mardan, Hangu, Swabi. While distant regions harbored different mutations, i.e., Cd-15 (G-A) in North Waziristan, ISV 1-5/Cap+1 (A-C) in Karak and Fr 41-42 (-TTCT) in Swat. These mutations are in accordance the reported mutations for South Asian north eastern region comprising of Pakistan.[10]

Ansari *et al* carried out a study on molecular epidemiology of beta Thalassemia gene mutation in various ethnic groups (Sindhi, Baloch, Pathan, Punjabi, Saraikee, Immigrant, Memon and Hazara) in Pakistan. A total of 648 samples were analysed by multiplex ARMS and (IVS 1–5, Fr 8-9, Deletion 619 bp, IVS 1-1, Fr 41- 42, Cd-30, Codon-5 and Codon-15) commons mutations were identified. Study result revealed that IVS 1–5 mutations was found in about 20% of Urdu speaking population and 76.9% in the Balochis. The second most prevalent variation was Fr 8-9 and detected about 31.3% in the Pathans and 47% found in Saraikee origin.[11] Khatak SA *et al* conducted a study on frequency of different type's mutations in beta thalassemia its correlation with hematological parameters. A total of 515 individuals enrolled in the study. The variations in Beta Gene were identified by ARMS-PCR. Study result shows that Fr 8-9 was the most common variation detected in 35.5% of patients followed by IVSI-5 in 24.5% and Fr 41-42 in 14.8% and IVSII-1 had the minimum frequency found in only 1 individual. Study concluded that Fr 8-9 is the most frequent beta thalassemia mutation with lowest red cell indices while CAP+1 mutation can present with normal red cell values.[12]

Shakeel Muhammad *et al* conducted a study on HBB gene mutation at Mardan, Pakistan. Five common mutations IVS-1-5, Fr 8/9, Cd 41/42, IVS-1-1, Cd 15 were found in the study. IVS-1-5 is most common mutation detected in Charsadah population.[13] In another study, Black *et al* reported most frequent mutations for Pakistani population were ISV 1-5 (G-C) 36.48%, Fr 8-9 (+G) 31.16%, Fr 41-42 (-TTCT) 7.11% and so on.[14] While our findings were in accordance with these results as we found Fr 8-9 (+G) 56.67% was most frequent mutation followed by CD 5 (-CT) in 25% of the samples. Fr 41-42 (-TTCT) was third most frequent mutation with appearing 16.667% of the population. Unlike the study by Black *et al*, ISV 1-5 (G-C) was observed in only 10% of the population. This difference could be attributed to the genotypic difference among the overall racial and ethnic makeup of Pakistani population which comprises of overwhelming share of Punjabis, Sindhis and Urdu speaking immigrants from northern Indian state of Utter Perdaish, along Pashtoons, Baluchis and other minority groups. All these groups harbor distinct genotypes and genetic makeup due to geographical separation. Regional studies have shown that ISV 1-5 (G-C) is more frequent in other neighboring countries, i.e., India 56.31%, Sri Lanka 64.60%, Iranian province of Baluchistan-Sistan 44.8%, UAE ~>50% and Bangladesh 56.25%.[14–16] These can be explained in terms of geographical separation and consequently differential genetic evolution of ethnicities of these separated regions.

In conclusion this mutational survey indicates Fr 8-9 (+G) to be the most frequent mutation in the targeted KP region followed by CD 5 (-CT) and Fr 41-42 (-TTCT). The variation in incidence of these mutations is dependent on ethnic diversity, migration, genetic factors, and other lifestyles. We, hence, recommend the mass screening, Prenatal Diagnostic Techniques, genetic counselling, transfusion programs and clinical management made available to these populations before adopting assisted reproductive and preimplantation technologies in Pakistan.

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AUTHORS' CONTRIBUTION

TJ: Performed research, analysed data and wrote manuscript. YMY: Conceptualized research analysed

data and wrote manuscript. IR: Analysed data and reviewed manuscript. AA: Recruited patients, analysed data and reviewed manuscript. SF: Recruited patients, analysed data, formatted and reviewed manuscript. SA: Supervised research, performed data analysis, reviewed manuscript. JA: Supervised research, performed data analysis.

REFERENCES

1. Aksoy M. Thalassaemia intermedia: a genetic study in 11 patients. J Med Genet 1970;7(1):47–51.
2. Weatherall D. Molecular biology at the bedside. Br Med J (Clin Res Ed) 1986;292(6534):1505–8.
3. Galanello R, Origa R. Beta-thalassemia. Orphanet J Rare Dis 2010;5:11.
4. Cousens NE, Gaff CL, Metcalfe SA, Delatycki MB. Carrier screening for Beta-thalassaemia: a review of international practice. Eur J Hum Genet 2010;18(10):1077–83.
5. Mishra KK, Patel P, Bhukhanvala DS, Shah A, Ghosh K. A multiplex ARMS PCR approach to detection of common β-globin gene mutations. Anal Biochem 2017;537:93–8.
6. Ahmed S, Petrou M, Saleem M. Molecular genetics of beta-thalassaemia in Pakistan: a basis for prenatal diagnosis. Br J Haematol 1996;94(3):476–82.
7. Baig SM, Azhar A, Hassan H, Baig JM, Aslam M, Din U, *et al*. Prenatal diagnosis of β-thalassemia in Southern Punjab, Pakistan. Prenat Diagn 2006;26(10):903–5.
8. Ahmed S, Saleem M, Sultana N, Raashid Y, Waqar A, Anwar M, *et al*. Prenatal diagnosis of beta-thalassaemia in Pakistan: experience in a Muslim country. Prenat Diagn 2000;20(5):378–83.
9. Hafeez M, Aslam M, Ali A, Rashid Y, Jafri H. Regional and ethnic distribution of beta thalassemia mutations and effect of consanguinity in patients referred for prenatal diagnosis. J Coll Physicians Surg Pak 2007;17(3):144–7.
10. Baig S, Azhar A, Hassan H, Baig J, Kiyani A, Hameed U, *et al*. Spectrum of beta-thalassemia mutations in various regions of Punjab and Islamabad, Pakistan: establishment of prenatal diagnosis. Haematologica 2006;91(3):ELT02.
11. Ansari SH, Shamsi TS, Ashraf M, Farzana T, Bohray M, Perveen K, *et al*. Molecular epidemiology of β-thalassemia in Pakistan: Far reaching implications. Indian J Hum Genet 2012;18(2):193–7.
12. Khattak SAK, Ahmed S, Anwar J, Ali N, Shaikh KH. Prevalence of various mutations in beta thalassaemia and its association with haematological parameters. J Pak Med Assoc 2012;62(1):40–3.
13. Shakeel M, Arif M, Rehman SU, Yaseen T. Investigation of molecular heterogeneity of β-thalassemia disorder in District Charsadda of Pakistan. Pak J Med Sci 2016;32(2):491–4.
14. Black M, Sinha S, Agarwal S, Colah R, Das R, Bellgard M, *et al*. A descriptive profile of β-thalassaemia mutations in India, Pakistan and Sri Lanka. J Community Genet 2010;1(3):149–57.
15. Rahim F, Abromand M. Spectrum of ß-Thalassemia mutations in various Ethnic Regions of Iran. Pak J Med Sci 2008;24(3):410–5.
16. Ibn Ayub M, Moosa MM, Sarwardi G, Khan W, Khan H, Yeasmin S. Mutation Analysis of the HBB Gene in Selected Bangladeshi β-Thalassemic Individuals: Presence of Rare Mutations. Genet Test Mol Biomarkers 2010;14(3):299–302.

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