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Unraveling the Genetic Landscape: Frequency of β-Globin Gene Mutations in Transfusion-Dependent Beta Thalassaemia Patients at an Urban Centre in Karachi

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Author`s Contribution

All authors contributed significantly to this work, participating in conception, study design, data acquisition, analysis, and interpretation. They were involved in drafting, revising, and critically reviewing the article, ultimately giving their approval for the version to be published.

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ABSTRACT

Objective: To identify the frequency of mutations in transfusion dependent beta thalassaemia patients presenting at a single urban thalassaemia Centre in Karachi.

Methodology: This single centre study was conducted at the Muhammadi Blood Bank and Diagnostic Centre and Baqai Medical University, Karachi. A total of 100 patients diagnosed with transfusion dependent beta thalassaemia were enrolled in this study. Amplification Refractory Mutation System - Polymerase Chain Reaction (ARMS-PCR) was used for a genetic analysis of 12 beta thalassaemia mutations, namely, Fr 8-9(+G), IVS I-5(G-C), Fr 41-42(-TCTT), IVS I-1(G-T), Del 619 bp, Cd-5 (-CT), Fr 16 (-C), Cd-15 (G-A,), Cd-30(G-C), Cd-30 (G-A), IVS II-1 (G-A), and Cap+ 1 (A-C).

Results: The Cd-5 (-CT) mutation was the most common β-thalassaemia mutation in our study population accounting for 37.4% mutated alleles, followed by Fr 8-9(+G) at 20.9%, Del 619 bp at 9.2% and Fr 16 (-C) at 8.6% cases. Out of 100 β-thalassaemia patients, 37% were identified as homozygous, while 63% were classified as compound heterozygous for thalassaemia mutations. A total of 10 different β-thalassaemia mutations were identified, distributed across 26 distinct genotypes. The most common genotype observed was compound heterozygous Fr 8-9(+G)/ Cd-5 (-CT), with a frequency of 25%. This was succeeded by homozygous Cd-5 (-CT), compound heterozygous Del619bp/Fr16(-C), and homozygous IVSI -1(G-T), which represented 19%, 6%, and 6% of cases, respectively.

Conclusion: Our study provides an extensive overview of mutational analysis of thalassaemia gene in transfusion dependent beta thalassaemia patients. The most frequently detected mutation was Cd-5 (-CT). We observed variation in the frequency of known mutations, emphasizing the intricate genetic makeup of thalassaemia disorder within our population.

Keywords: Beta- thalassaemia, Beta globin gene, Transfusion dependent thalassaemia, Homozygous and heterozygous mutations, genetic variations.

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Introduction

Thalassaemia's are the commonest single-gene disorders affecting α- and β- globin gene clusters and are group of anaemias resulting from inherited defects in the production of hemoglobin.[1] β-thalassaemia is caused by alterations in the β-globin gene and characterized by absent (β0-thalassaemia) or reduced (β+-thalassaemia) synthesis of β-globin chain of adult hemoglobin (HbA; α2β2).

The decrease in beta globin chain synthesis leads to reduced production of functional hemoglobin tetramers, leading to hypochromia and microcytosis. Moreover, the surplus unbound alpha-globin chains precipitate in

erythroid progenitors within the bone marrow and circulating red blood cells, triggering their premature destruction. This progresses into ineffective erythropoiesis and hemolytic anaemia.[2]

The β-globin gene (HBB) maps in the short arm of chromosome 11. Based on molecular analysis, the β-thalassaemia gene has exhibited a striking heterogeneity. So far, more than 400 β-thalassaemia mutations have been reported with only 40 β-thalassaemia alleles accounting for more than 90% of the β-thalassaemia mutations worldwide. [3,4]

The vast majority (approximately 200) of β-thalassaemia mutations are characterized as point mutations or small insertions or deletions involving one or two bases.[5] Nearly half of the β-thalassaemia alleles result from the introduction of premature termination codons, either because of single base substitution creating a stop codon or a change in the reading frame by insertion or deletion of a single to a few nucleotides.[6] β-Thalassaemia is rarely caused by deletions. Of these, only the 619-bp deletion at the 3′ end of the β-gene is a relatively common cause of β0-thalassaemia in Asian Indians.[7]

Determining the precise frequency of β-thalassaemia mutations is required for launching effective preventive health programs to combat this devastating disorder. Such programs include carrier detection screening, parental consultations to educate them regarding genetic risks, and prenatal testing.

These as a whole act as a cornerstone to manage or reduce the risk of this inherited disorder and also help in lowering the incidence of homozygous β-thalassaemia births in many regions worldwide.[8-10] In the current study, we aimed to determine the frequency of common beta thalassaemia mutations in transfusion dependent beta thalassaemia patients presenting at a thalassaemia Centre in Karachi.

Methodology

This single- Centre open label study was conducted between January2023 to January 2024. We recruited 100 known cases of transfusion dependent beta thalassaemia patients from Muhammadi blood bank and diagnostics Centre, Karachi. Written and informed consent was taken from all the study participants after explaining the details of the study. This study was approved by the Ethics Committee of Baqai Medical University, Karachi (Ref: BMU-EC/04-2022).

The blood samples from patients were collected in ethylenediaminetetraacetic acid (EDTA). DNA was extracted from peripheral blood lymphocytes using Quick-DNA™ Miniprep Plus Kit (Zymo Research) and were quantified by nano-drop spectrophotometer. Amplification Refractory Mutation System - Polymerase Chain Reaction (ARMS-PCR) was used for a genetic analysis of 12 beta thalassaemia mutations, namely, Fr 8-9(+G), IVS I-5(G-C), Fr 41-42(-TCTT), IVS I-1(G-T), Del 619 bp, Cd-5 (-CT), Fr 16 (-C), Cd-15 (G-A,), Cd-30(G-C), Cd-30 (G-A), IVS II-1 (G-A), and Cap+ 1 (A-C).

ARMS- PCR was carried in three separate reaction mixtures. The first reaction mixture included primers designed for amplification of Fr 8-9 (+G), IVSI-5 (G-C), Fr 41-42 (-TTCT), IVSI-1 (G-T) and Del 619bp. The second reaction mixture contained primers specific for Fr 16(-C), Cd 5 (-CT), IVSI-1 (G-T), Cd30 (G-C), Cd 30 (G-A), and IVSII-1 (G-A). Finally, the third reaction mixture contained primers specific for Cap +1 (A-C) and Cd 15 (G-A).

To further strengthen our results the detection of mutation in any of the panels was re-confirmed by a second PCR using allele specific primer. Table 1 shows the list of ARMS primers used to detect β-thalassaemia mutations.

A total of 50µl final PCR reaction volume was prepared by using 25 µl of DreamTaq Green PCR Master Mix (2X), 3 µl of Primer(panel), 4 µl of template DNA and 18 µl of H2Onuclease- free.

The PCR amplification process comprised of initial denaturation at 95^{O}C for 5 minutes followed by of 40 cycles of denaturation at 95^{O}C for 30seconds, primer annealing at 65^{O}C for 30 seconds, and DNA extension reaction at 72 ^{O}C for 1 minute and final extension at 72 ^{O}C for 10 minutes and finally a 04^{O}C as holding temperature.

The amplified product of PCR was resolved by agarose gel electrophoresis.10µL PCR product was loaded on a 1% agarose gel. The voltage conditions were set as :75 volts for 5 minutes, 90 volts for 5 minutes and 120 volts for 20 minutes. The gel was exposed to ultraviolet (UV) light to see the bands.

By using the DNA ladder (100bp or 50 bp DNA ladders) in the first lane as a guide, the size of the DNA in the sample lanes was inferred. The statistical analysis was performed by using Statistical Package for Social Sciences (SPSS) version 25.0. Data were compiled as frequency (%).

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Table I: Amplification refractory mutation system (ARMS) primer sequence for beta thalassaemia mutations

S.no	Mutation	5´-Primer sequences- 3´	Band Size	Reference
Panel-1	Fr 8-9(+G)	CCTTGCCCCACAGGGCAGTAACGGCACACC	215bp	[11,12]
	IVSI -5(G-C)	CTCCTTAAACCTGTCTTGTAACCTTGTTAG	285bp	
	Fr 41-42(-TTCT) IVSI -1(G-T)	GAGTGGACAGATCCCCAAAGGACTCAACCT	439bp	
	Del 619bp	TTAAACCTGTCTTGTAACCTTGATACGAAA	280bp	
		CAATGTATCATGCCTCTTTGCACC	242bp	
Panel-2	Cd 5(-CT)	ACAGGGCAGTAACGGCAGAACTTCTCCGCGA	205bp	
	Fr 16 (-C)	TCACCACCAACTTCATCCACGTTCACGTTC	238bp	
	IVSI -1 (G-T)	TTAAACCTGTCTTGTAACCTTGATACGAAA	280 bp	
	Cd 30 (G-C)	TAAACCTGTCTTGTAACCTTGATACCTACG	280 bp	
	Cd 30 (G-A)	TAAACCTGTCTTGTAACCTTGATACCTACT	280 bp	
	IVSII-1 (G-A)	AAGAAAACATCAAGGGTCCCATAGACTGAT	634bp	
Panel-3	Cd 15 (G-A)	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	500 bp	
	Cap+1 (A-C)	AAAAGTCAGGGCAGAGCCATCTATTGGTTG	567 bp	

Results

A summary of the beta thalassaemia mutations among the study participants (n=100) is shown in Figure 1(a,b). Eight mutations were identified as common including: Cd-5 (-CT) (HBB: c.17_18del) (37.4%); Fr 8-9(+G) (HBB: c.27 dup G) (20.9%); Del 619 bp(HBB:c.316-281_*209del619) (9.2%); Fr 16 (-C) (HBB: c.51del)(8.6%); IVS I-5(G-C)(HBB: c.92 + 5G > C) (6.7%); IVS I-1(G-T) (HBB: c.92+1G>T) (5.5%); IVS II-1 (G-A)(HBB: c.315+1G>A) (5.5%); Fr 41-42(-TCTT) (HBB: c.126_129del)(3.7%) accounting for a total of 97.5% of the β-thalassaemia alleles. The remaining two mutations were Cd-30(G-C) (HBB: c.92G>C) at 1.8% and Cd-30 (G-A) (HBB: c.92G>A) at 0.6%. However, Cd-15 (G-A) (HBB: c.48G>A) and Cap+1 (A-C) (HBB: c.-50A>C) mutations were not detected in any of the samples analyzed.

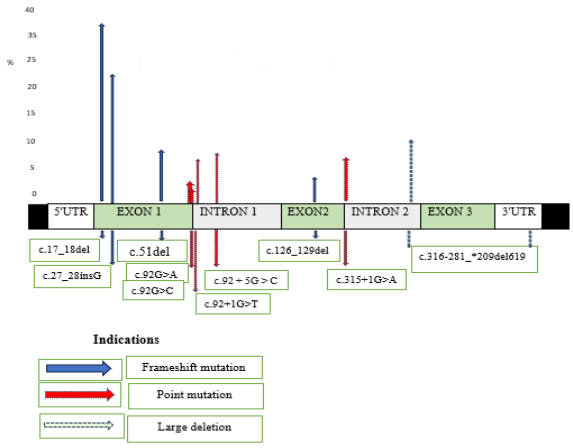

Fig: 1(a): Diagrammatic representation of distribution of β-globin gene mutations identified in study participants (n=100). HBB gene; https://www.ncbi.nlm.nih.gov/clinvar/RCV000029974/

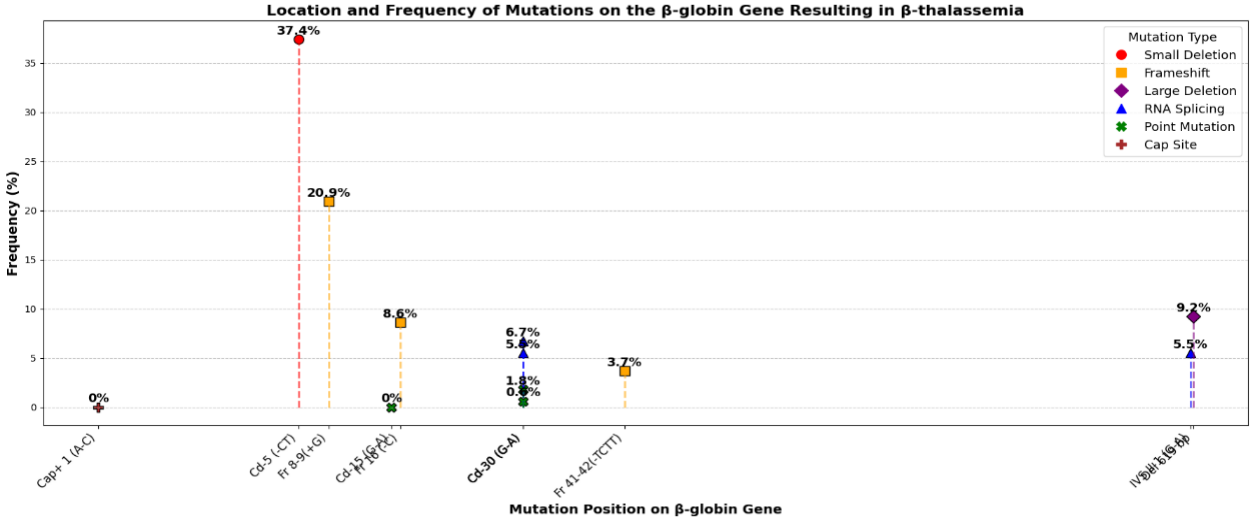

Fig. 1(b): A graphical illustration of location and frequency of mutations on the Beta globin gene in transfusion dependent beta thalassaemia patients (n=100).*Graph prepared by using Python: [https://www.python.org/].

The Cd-5 (-CT) mutation was the most common β-thalassaemia detected in our study participants. Gel electrophoresis image of beta thalassaemia mutations is shown in Figure 2.

A total of 10 different β-thalassaemia mutations arranged in 26 different genotypes were identified. The most common genotype observed was compound heterozygous Fr 8-9(+G)/ Cd-5 (-CT) with a frequency of 25%. This was followed by homozygous Cd-5 (-CT), compound heterozygous Del619bp/Fr16(-C), and homozygous IVSI -1(G-T) which accounted for 19%, 6%, and 6% of cases, respectively as shown in figure 3.

Among 100 β-thalassaemia patients, 37% were found to be homozygous and 63% were compound heterozygous. Homozygosity of the Cd 5(-CT) mutation was found in 51.4% patients (n=19/37), followed by IVSI-1(G-T) detected in 16.2% study participants (n=6/37) (**Table II**)

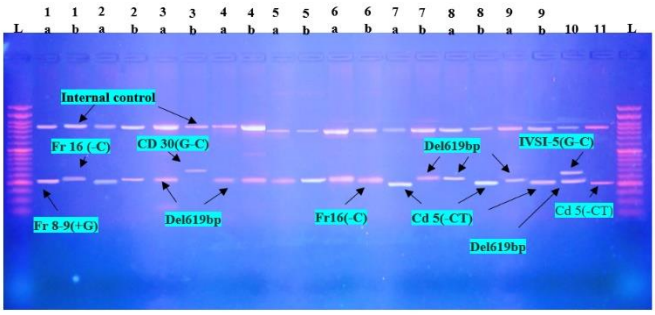

Fig. 2: A representative of agarose gel electrophoresis of PCR products utilizing ARMS for the detection of primary beta thalassaemia mutations. Lane L: 50-1500 bp DNA ladder. 861 bp fragment is an internal control. Lane 1-2: showing results of 2 patients who are compound heterozygous for Fr 8-9(+G) /Fr 16 (-C) (215bp/238bp) mutations; Lane 1a and 2a display Fr 8-9(+G) (215bp) mutation whereas,1b and 2b indicate Fr 16 (-C) (238bp) mutation. Lane 3: patient is compound heterozygous for Del619bp/CD 30(G-C) (242bp/280bp) mutation. Lane 4a -6a shows results of panel 1 of 3 patients having Del619bp (242bp) while, lane 4b-6b indicate results of panel 2 depicting Fr16(-C) (238bp) mutation. Lane 7-9: showing results of 3 patients who are compound heterozygous for Del619bp/Cd 5(-CT) (242bp/205bp) mutation; Lane 7a, 8b and 9b show Cd 5(-CT) (205bp) mutation. whereas 7b,8a, and 9a indicate Del619bp (242bp). Lane 10: Individual is compound heterozygous for Del619bp/IVSI-5(G-C) (242bp/285bp). Lane 11: showing result of patient homozygous for Cd 5(-CT) (205bp) mutation.

Among compound heterozygous cases(n=63), Fr 8-9(+G) /Cd 5(-CT) was found be most frequent genotype variation with estimated frequency of 39.7%(n=25/63) followed by Del619bp/Fr16(-C) i.e. 9.5% (6/63) (**Table III**).

Table II Frequency of homozygous mutations in patients with transfusion dependent β-thalassaemia major (n=37/100)

S.no.	Mutation	Type	N (%)
i.	Cd 5(-CT)	β^0 / β^0	19 (51.4)
ii.	IVSI-1(G-T)	β^0 / β^0	6 (16.2)
iii.	Fr16(-C)	β^0 / β^0	4 (10.8)
iv.	Del619bp	β^0 / β^0	3 (8.1)
v.	Fr8-9(+G)	β^0 / β^0	2 (5.4)
vi.	IVS11-1(G-A)	β^0 / β^0	1 (2.7)
vii.	IVSI-5(G-C)	B^+ / β^0	1 (2.7)
viii.	Fr41-42(-TTCT)	β^0 / β^0	1 (2.7)

Mutation types β+and β0 align with the information found on http://globin.cse.psu.edu/ [13]

Table III: Compound heterozygous mutations in beta-globin gene. (n=63/100)

S.no.	Mutation	Type	N (%)
i.	Fr 8-9(+G) /Cd 5(-CT)	β^0 / β^0	25 (39.7)
ii.	Del619bp/Fr16(-C)	β^0 / β^0	6 (9.5)
iii.	IVSI-5(G-C)/Cd 5(-CT)	β+ / β^0	5 (7.9)
iv.	Cd 5(-CT)/IVSII-1(G-A)	β^0 / β^0	4 (6.3)
v.	Cd 5(-CT) /Fr41-42(-TTCT)	β^0 / β^0	4 (6.3)
vi.	Del619bp/Cd 5(-CT)	β^0 / β^0	3 (4.8)
vii.	Fr 8-9(+G)/IVSII-1(G-A)	β^0 / β^0	3 (4.8)
viii.	Fr 8-9(+G) /Fr 16 (-C)	β^0 / β^0	2 (3.2)
ix.	IVSI-5(G-C)/Fr16(-C)	β+ / β^0	2 (3.2)
x.	Del619bp/CD 30(G-C)	β^0 / β^0	1 (1.6)
xi.	Fr 8-9(+G) /Cd 30 (G-C)	β^0 / β^0	1 (1.6)
xii.	Del619bp/IVSI-5(G-C)	β^0 / β+	1 (1.6)
xiii.	IVSI-5(G-C)/IVS1-1(G-T)	β+ / β^0	1 (1.6)
xiv.	Cd 5(-CT)/Cd30(G-A)	β^0 / β^0	1 (1.6)
xv.	IVSI-5(G-C)/IVSII-1(G-A)	β+ / β^0	1 (1.6)
xvi.	Fr (8-9) +G /IVSI-1(G-T)	β^0 / β^0	1 (1.6)
xvii.	Fr41-42(-TTCT)/Cd30(G-C)	β^0 / β^0	1 (1.6)
xviii.	Del619bp/IVS1-1(G-T)	β^0 / β^0	1 (1.6)

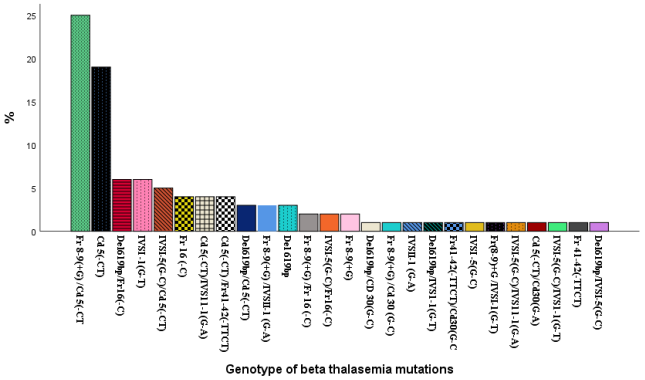

Fig. 3: The frequency of beta thalassaemia genotype variations in study participants

Discussion

Thalassaemia is documented as a significant global health concern. It is highly prevalent in Pakistan due to genetic, cultural, and socio-economic factors. Pakistan has a powerful historical background of invasions and trade interactions with neighboring countries, resulting in a unique blend of ethnicities leading to a significant genetic diversity.[14,15] This, augmented with a strong cultural belief of consanguineous marriages, has contributed to the high prevalence of autosomal recessive genetic disorders such as β-thalassaemia. In Pakistan, the inheritance rate of β-thalassaemia is between 5.0% and 7.0%, resulting in an approximately 5,000 to 9,000 new cases of thalassaemia major annually.[16,17] Therefore, this study was conducted to investigate and analyze the frequency of mutations in the β-globin gene in patients with beta thalassaemia syndrome.

In Pakistan, the most common thalassaemia mutations documented are: IVS I-5(G-C), Fr 8-9(+G), Del 619 bp, Fr 16 (-C), IVS I-5(G-C) , Cd-5 (-CT), IVS I-1(G-T), IVS II-1 (G-A), Fr 41-42(-TCTT), Cd-30(G-C), Cd-30 (G-A), CAP+1 and Cd 15 (G–A).[18] We observed eight mutations that accounted for 97.5% of the β-thalassaemia alleles rather than 5 -7 mutations reported in previous studies.[19,20] The Cd-5 (-CT) mutation was the most common β-thalassaemia mutation in our study population followed by Fr 8-9(+G), Del 619 bp, Fr 16 (-C). The most common genotype observed was compound heterozygous Fr 8-9(+G)/ Cd-5 (-CT) with a frequency of 25%. Mottar et al. in a study conducted in Karachi, Pakistan, reported that Fr 8-9(+G) and Del 619 bp were second most common mutation at 20% and third most common mutation at 18.4%, respectively. IVS I-5(G-C) was identified as the most frequent mutation, occurring in 32.4% of cases, which contrasts with our study results.[21] A similar spectrum of analysis was reported by Ansari et al. with Fr8-9 at 15.7%, and Del 619 at 11.1%, further supporting our findings. While, the IVS I-5(G-C) mutation was the most frequently detected mutation at 40.89%, and Cd-5 (-CT) was found at a rate of 2.16%. Furthermore, the frequencies of IVS I-1(G-T), Cd-30(G-C), Fr 41-42(-TCTT), and Fr 16 (-C) were 8.17%, 8.02%, 5.4%, and 0.92%, respectively. These findings did not align with our study results.[18]Moreover, Usman et al. conducted a study in Karachi to detect beta thalassaemia mutation, where IVS-I-5 (G→C) was found to be most common thalassaemia mutation with a frequency of 44.4%, followed by Fr 41/42 (-TTCT) at 17.5%, Fr 8/9 (+G) at 14.6%, IVS-1–1 (G→T) at 7.5% and Del 619 at 0.6%. which is inconsistent with our study results.[19] While Hashmi et al reported that IVSI-5, Fr8-9 and CD-30(G-C) were the three most common mutations found in 42.0%, 27.9%, and 22.2% of cases, respectively.[22]

A study conducted by Jalil et al. in Khyber Pakhtunkhwa, Pakistan also documented that Fr 8-9 (+G) (55.6%) and CD 5 (- CT) (25%) were the most prevalent mutations, supporting the results of our study.[11] Rehman et al carried out a study to investigate the frequencies of beta thalassaemia mutations in Bannu region. Fr 8-9 (+G) was seen as the most prevalent mutation, with a frequency of 42.5%, followed by Fr 41-42 (-TTCT) in 26% of cases. Moreover, IVS I-5(G-C) and CD 5 (-CT) were present in 19% and 12.5% cases, respectively. Codon 15 (G > A) mutations was not detected from any of the studied samples, consistent with our results.[23] In a study conducted by Khattak et al. the most frequent thalassaemia mutation was Fr 8-9 seen in 35.5% of patients. The IVSI-5 and Fr 41-42 were detected in 24.5% and 14.8% of cases.[24] The results of another study carried out in same region documented that the most common mutations in thalassemic patients were frameshift codons Fr 8-9, codons 41/42 (–TTCT), and IVS-I-5 (G>C) presented in 46.1%,30.8%, and 23.1% of cases. Codon 15 (G > A) were not detected in study samples consistent with our study results.[25] However, Saif et al., in a study conducted in the same province, reported a different sequence of mutations, with IVSI-5 at 52.2%, Fr8-9 at 22.6%, and CD41-42 at 18.6%.[26]

Baig et al also reported Fr 8-9(+G), the second most common mutation seen in 37.3% of cases as consistent with our study results, in a thalassaemia mutational analysis conducted in various region of Punjab and Islamabad. However, contrary to our findings IVS-I-5 (G→C) was the most common mutation, found in 39.0% of cases. The frequencies of other mutations were as following; Cd-5 (-CT) (1.3%), del 619 bp (1.9%), Fr 16 (-C) (1.0%), IVS I-1(G-T) (1.9%), IVS II-1 (G-A) (0.8%), Fr 41-42(-TCTT) (10.6%), Cd-30(G-C) (0.7%), Cd-30 (G-A) (0.1%) that stands in opposition to our study results.[27]

Hafeez et al in a study conducted in Lahore also reported a different sequence of mutations based on frequencies, where Fr 8-9(+G) was the most common with a frequency of 33.5%, IVS I-5(G-C) ranked second in 17.2%, and Fr41-42 was third with a frequency of 8%. IVS 1-1 was present in 5.2% cases which matches our study results.[28] Same results were seen in a study reported by Shahid et al in Faisalabad.[29] It seems that the frequency of thalassaemia mutations within the same region is not uniform, showing

notable variation across different areas. Despite being in close geographic proximity, people within the region display differing mutation rates, suggesting complex genetic diversity.

Moreover, the frequency of the IVS1-5(G-C) mutation was reported to be 5.5% by Sarookhani et al in a study in Iran. This result supports the finding of our study.[30] Colah et al reported that the CD 5(−CT) mutation was largely seen in Gujarat (India). It was the third most common mutation in this area, particularly predominant among the Prajapati caste group.[20]

The results of our study differed from those of previous studies conducted in other areas of Pakistan, indicating variability in the frequencies of known mutations. Karachi, being the financial pivot of the country, has appealed people from all areas of Pakistan, including immigrants from India following the Indo-Pak partition. This influx led to significant genetic heterogeneity, which is shown in the recognition of various common β-thalassaemia mutations with variable prevalence rate.

Conclusion

This study has successfully identified the frequency of mutations in transfusion dependent beta thalassaemia patients. It revealed the Cd 5(-CT) mutation, which was the most frequently identified mutation in our study that differs from those documented in previous studies. Moreover, we observed variability in the frequency of known thalassaemia mutations. These findings emphasize genetic diversity within our population and underscores the importance of region-specific data to handle this debilitating disease.

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