ORIGINAL ARTICLE

CHARACTERIZATION OF BETA THALASSAEMIA MUTATIONS IN PATIENTS HAVING BORDERLINE HAEMOGLOBIN A2 LEVELS

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Background: The occurrence of a single beta thalassaemia allele is frequently related with microcytic hypochromic red blood cells and a rise in HbA2 levels. In some beta thalassaemia carriers, the outcome of this allele or its collaboration with other acquired or genetic defects may result in normal or borderline Haemoglobin bA2 levels. Objective was to establish the importance of molecular analysis in borderline HbA2 individuals and its significance in a population screening program. **Methods:** It was a cross-sectional study conducted over a period of six months, from July-December 2023. All 123 individuals with borderline HbA2 levels between (3–3.9%) diagnosed by High-performance liquid chromatography (HPLC)/Capillary Zone Electrophoresis underwent molecular testing using multiplex amplification refractory mutation system-Polymerase Chain Reaction (ARMS-PCR) to detect common beta thalassaemia mutations: Fr8-9, IVS1-5, Fr41-42, Cd15, Cd5, IVS1-1, IVS1-1, Cd30, Cd30, Fr16, IVSII-1, Del619, and CAP+1 in the Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi .Statistical tests were applied to compare Red Blood Cell indices and Haemoglobin A2 values among beta thalassaemia carriers and non-carriers. **Results:** Among those tested, 47.1% (n=58) were found to carry Beta thalassaemia mutations. The most prevalent mutations were IVS1-5 (n=19,15.4%) and Fr8-9 (n=19,15.4%) followed by Fr41-42 (n=08,6.5%). Subjects with mutations exhibited significantly lower mean corpuscular volume and mean corpuscular haemoglobin compared to those without mutations (*p*-value= <0.001). Beta thalassaemia mutations were seen more frequently when HbA2 was in range of 3.5-3.9% (n=37,63.8%), as compared to HbA2 that was 3-3.4% (n=21,36.2%) and this difference was found to be significant (*p*-value= <0.001). The CAP+1 mutation was associated (n=02,1.6%) with normal mean MCV and MCH compared to other identified mutations. **Conclusions**: It is concluded that molecular study for the common beta thalassaemia mutations in Pakistani population plays a pivotal role in confirmation of borderline HbA2 thalassaemia carriers, specifically in areas with a high prevalence of the disease. Molecular testing for beta thalassaemia should be offered to all individuals with borderline HbA2 with values especially between 3.4–3.9% and having microcytic hypochromic indices.

Keywords: Borderline HbA2; Silent carriers; Beta Thalassaemia mutation; Red Cell Indices; Molecular analysis

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INTRODUCTION

The occurrence of a single beta thalassaemia allele is frequently related with microcytic hypochromic red blood cells and a rise in HbA2 levels. In some beta thalassaemia carriers, the outcome of this allele or its collaboration with other acquired or genetic defects may result in normal or borderline Haemoglobin bA2 levels.[1] Borderline HbA2 levels refers to values between (3.0–3.9%).[2] Those beta thalassaemia carriers having borderline HbA2 levels may be missed on haemoglobin studies and may only be diagnosed after the birth of diseased child. Hence, these borderline cases are vulnerable for having offspring with beta thalassaemia if their spouse is also a beta-thalassemia carrier.[3] Red blood cell indices and HbA2 levels may be normal in silent beta thalassemia carriers and their status can only be confirmed by DNA analysis.

Borderline HbA2 levels are not rare in countries like Thailand[4] China[5] Italy[6,7] and Greece[8] with a high prevalence of beta-thalassaemia. People with borderline HbA2 levels also have also been reported in Middle Eastern populations[9,10] and India[11]. People with borderline HbA2levels have been reported in 31 different countries worldwide. Migration and inter-marriages between individuals from different genetic backgrounds can lead to the transmission of globin gene defects (like beta-thalassaemia mutations) to populations where these disorders were previously less common or unknown.[12,13]

Local literature shows prevalence of beta thalassaemia trait in Pakistan is 6–7% and the actual numbers may be higher.[14] The present situation of beta-thalassaemia in Pakistan warrants the recognition of individuals with borderline HbA2 levels in our population. Presently, there is lack of information on how to deal with cases having borderline HbA2 levels and information regarding different factors affecting HbA2 levels remains mainly unexplored.[15] Hence, this study was designed to identify silent beta thalassaemia carriers having borderline HbA2 levels by molecular analysis.

MATERIAL AND METHODS

This cross-sectional study was carried out at Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi, over a period of six months, i.e., from July to December 2023 after getting approval from Institutional Review Board under IRB-No: IRB/24/2698. The sample size was 123 and it was calculated by WHO calculator and sampling was done by using non-probability consecutive technique. Individuals with border line HbA2 levels (3.0–3.9%) who were referred for molecular studies at our Institute were interviewed and detailed clinical history and examination findings were recorded. A 3 ml of blood was collected in Ethylene Diamine Tetra Acetic Acid (EDTA) vacutainer from each individual after verbal and written informed consent whereas in children consent was taken from their parents. Our study included 123 individuals above 1 year of age, either gender, and having borderline HbA2 levels already determined by High-performance liquid chromatography (HPLC)/ Capillary Zone Electrophoresis method, while patients with bicytopenia, pancytopenia, other haemoglobinopathies, raised HbA2 levels more than 4%, having recent transfusion history within one month period and low ferritin levels were excluded from the study. The HbA2 cut-off for diagnosis of heterozygous beta-thalassaemia was taken as HbA2 ≥4% and hence these 123 subjects were labelled as borderline HbA2 individuals [3.0–3.9%].

Three milliliters of blood were obtained from the study population by using EDTA vacutainers for subsequent investigation. RBC parameters were assessed using automated Sysmex XP100 Haematology analyzer. The blood samples were processed immediately. DNA extraction from blood samples was performed by mini kit method, as per manufactures protocol with subsequent quantification of extracted DNA samples performed by using a nano-drop spectrophotometer. The target DNA was amplified using primers specific to beta-chain mutations to identify both homozygous and heterozygous states in individuals to identify common, uncommon, and rare mutations prevalent in Pakistan including Fr8-9, IVS1-5, Fr41-42, Cd15, Cd5, IVS1-1, IVS1-1, Cd30, Cd30, Fr16, IVSII-1, Del619(bp), and CAP+1. Multiplex amplification refractory mutation system-Polymerase Chain Reaction (ARMS-PCR) was sequentially conducted. Identification of primary mutations involved multiplex PCR assays labelled as Allelic Discrimination assays (AD1, AD2, and AD3) each employing a distinct set of primers including a control primer. After confirming primary mutations, specific PCR amplification of the DNA fragment was carried out, and the resulting product was visualized on 6% Polyacrylamide Gel Electrophoresis (PAGE) using a 1.0 kb DNA marker.

The data was analyzed on Statistical package of social sciences version 26. Mean & Standard Deviation was calculated for quantitative variables like age, Hb, MCV, MCH, MCHC, RDW, HbA, HbF and HbA2 while frequency and percentages were calculated for qualitive variables like gender, carriers and non-carriers. Tests for statistical significance were t-test (for quantitative) and chi-square test (for qualitative variables). *p*-value of less than 0.05 was considered significant.

RESULTS

A total of 123 individuals were enrolled in our research study. There were 73 (59.3%) males and 50 (40.7%) females with a mean age of 22.0±13.5 years. Out of 123 participants, seven individuals had history of disease of beta thalassaemia major in close family.

We characterized borderline HbA2 into two groups, one group with HbA2 in a range of 3–3.4% and other in a range of 3.5–3.9%. Molecular analysis of these borderline individuals confirmed 58 out of total 123 individuals to be confirmed beta thalassaemia carriers. The comparison of the both groups of borderline HbA2 levels showed a highest number of individuals to be carriers in the group of HbA2 3.5–3.9% vs 3.0–3.4% that is n=37,63.8% vs n=21,36.2% respectively with *p*-value of <0.001. Beta thalassaemia mutations were seen more frequently in HbA2 range of 3.5–3.9%. The non-carriers counted were 65 out of the total 123 individuals having borderline HbA2 levels but with no beta thalassaemia mutation detected by PCR. These were in highest number in the borderline HbA2 range of 3-3.4% with not a single individual seen in the HbA2 range of 3.5–3.9%. The association between these two HbA2 groups and beta thalassaemia carrier status was statistically significant (*p* = <0.001) Table-1. DNA analysis confirmed that 58 out of 123 individuals were beta thalassaemia carriers. Seven different mutations (IVS1-1, CAP+1, Fr41-42, IVS1-5, Cd-30, Cd-15, and Fr8-9) were identified (Figure 1). The most common mutation identified was IVS1-5 (n=20,16.3%), followed by Fr8-9 (n=1, 25%). The frequency of carriers at different HbA2 levels is shown in Figure-1. The red cell parameters of each individual with or without beta thalassaemia mutation are shown in Table 2. The red cell parameters including Hb, MCV, MCH, MCHC among the carrier's vs non-carriers' individuals were found to be highly significant (*p*=<0.001), RDW (*p*=0.03) and only RBC count was found not to be significant (*p*=0.44). Comparison of the red cell parameters

in carriers having beta thalassaemia mutations showed a significantly lower Hb, MCV, MCH and MCHC value (*p*-value = <0.001) while higher RBC, RDW-SD values (*p*-value= 0.09 and 0.3 respectively). The tests were highly significant as shown in Table-3.

Table-1: Comparison of HbA2 groups among carrier and non-carriers.

HbA2 Groups	Carrier status n (%)		Total	*p*-value
	Carrier	Non- carrier		
3.0–3.4%	21 36.2%	65 100%	86 69.9%	<0.001
3.5–3.9%	37 63.8%	0 0	37 63.8%	<0.001
Total	58 100%	65 100%	123 100%	

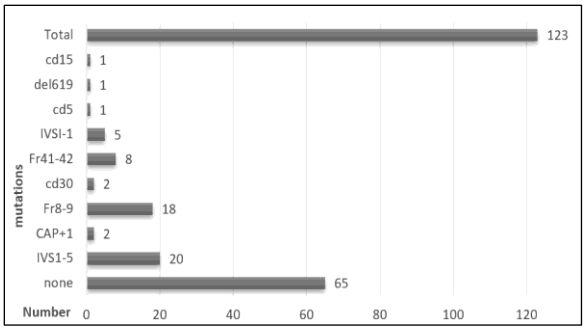

Figure-1: Percentage frequency of beta thalassemia mutations in carriers.

Table-2: Comparison of red cell indices of individuals with carrier status of beta thalassemia

RBC indices	Carrier status	n	Mean	SD	t-test	*p*-value
Haemoglobin	Non-Carrier	65	12.7	2.70	6.75	<0.001
	Carrier	58	9.8	2.08	6.84	<0.001
RBC count	Non-Carrier	65	4.65	1.01	-0.42	0.671
	Carrier	58	4.74	1.37	-0.41	0.676
MCV	Non-Carrier	65	84.4	10.8	8.99	<0.001
	Carrier	58	66.4	11.3	8.97	<0.001
MCH	Non-carrier	65	28.2	4.15	8.71	<0.001
	Carrier	58	20.4	5.72	8.56	<0.001
MCHC	Non-carrier	65	33.3	1.82	7.64	<0.001
	Carrier	58	28.7	4.45	7.33	<0.001
RDW-SD	Non-carrier	65	44.3	13.1	-1.61	0.109
	Carrier	58	47.9	11.2	-1.63	0.105

Table-3: Cross-tab of PCR mutations with in red cell indices of individuals

Red cell indices		PCR mutations, n (%)											*p*-value
		None	IVS1-5	Fr8-9	Fr41-42	IVS1-1	CAP+1	Cd 30	Cd5	Cd15	Del619	Total	
Hb	Low (<11g/dl)	24 36.9%	19 95%	18 100%	6 75%	5 100%	0	2 100%	1 100%	0	1 100%	76 61.8%	
	normal (11-16.5g/dl)	41 63.1%	1 05%	0	2 25%	0	2 100%	0	0	1 100%	0	47 38.2%	<0.001
RBC	Low ($3.5x10^6$/µL	9 13.8%	5 25%	7 38.9%	1 12.5%	0	0	1 50%	0	0	0	23 18.7%	
	Normal ($3.5-5.5x10^6$/µL	40 61.5%	7 35%	2 11.1%	3 37.5%	3 60%	1 50%	1 50%	1 100%	1 100%	0	58 47.2%	0.096
	High >$5.5x10^6$/µL	16 24.6%	8 40%	9 50%	4 50%	2 40%	1 50%	0	0	0	1 100%	42 34.5%	
MCV	low <80fL	10 15.4%	18 90%	15 83.3%	7 87.5%	4 80%	0	2 100%	1 100%	1 100%	1 100%	61 49.6%	
	Normal 80-98fL	50 76.9%	2 10%	3 16.7%	1 12.5%	0	2 100%	0	0	0	0	57 46.3%	<0.001
	High >98fL	5 7.7%	0	0	0	0	0	0	0	0	0	5 4.1%	
MCH	Low 26.5pg	11 16.9%	19 95%	16 88.9%	7 87.5%	4 80%	0	2 100%	1 100%	1 100%	1 100%	62 50.4%	
	Normal 26.5-33.5pg	49 75.4%	1 5%	2 100%	1 12.5%	0	2 100%	0	0	0	0	56 45.5%	<0.001
	High >33.5pg	5 7.7%	0	0	0	0	0	0	0	0	0	5 4.1%	
MCHC	Low <32g/dl	11 16.9%	18 90%	16 88.9%	7 87.5%	4 80%	1 100%	1 100%	1 100%	1 100%	1 100%	61 99.6%	
	Normal 32-36g/dl	49 75.4%	2 100%	2 100%	2 11.1%	1 12.5%	1 20%	0	0	0	0	57 46.3%	
	High >36g/dl	5 7.7%	0	0	0	0	0	0	0	0	0	5 4.1%	<0.001
RDW-SD	Normal 35-56fL	40 61.5%	10 50%	6 35.3%	4 50%	2 40%	1 50%	0	0	1 100%	0	64 52.5%	
	High >56fL	25 38.5%	10 50%	11 64.7%	4 50%	3 60%	1 50%	2 100%	1 100%	0	1 100%	58 47.5%	0.36

DISCUSSION

Haemoglobinopathies represent unique genetic disorders due to the potential to determine carrier status in the majority (approximately 90%) of cases through haematological findings alone.[14] Utility of red blood cell parameters and HbA2 quantification in the diagnosis of beta thalassaemia trait has proved to be effective for disease prevention and is widely applied globally as a carrier screening test.[16] However, challenges such as the lack of standardized optimal HbA2 cut-offs internationally, insufficient local studies defining normal ranges, and the diverse distribution of HbA2 in the Pakistani population suggest that relying solely on red cell parameters and HbA2 may not be sufficient particularly in silent carriers of beta thalassaemia include individuals with hypochromic microcytic RBC parameters but having normal HbA2 levels. These silent carriers account for approximately 2.5% of all beta thalassaemia carriers in Pakistan.[17] Our study underscores the importance of molecular examination in identifying these silent carriers who may be missed by conventional methods.

The study participants primarily included individuals from families with identified patients of thalassaemia and those with family history of haemoglobinopathies. This selection bias could account for the notably increase proportion of beta thalassaemia carriers detected among individuals with borderline A2 levels (47.1%), in contrast to findings from comparable researches in India (32%),[18] Malaysia (30%),[19] and Thailand (5.7%)[20].

Prior research indicates that the IVS1-5 and Fr8-9 mutations are frequently found among beta thalassaemia carriers in Pakistan, with a prevalence ranging from 16.3–14.6%, and it is distributed across all major ethnic groups in our country[14] which are similar to the results of our study of borderline beta thalassaemia carriers. In our study population, 2 (1.6%) individuals with CAP+1 mutation were identified. This mutation involves a point change in the promoter region, leading to a β+ mutation and is commonly observed in the Indo-Asian region. It is classified as a silent mutation, where individuals heterozygous for this mutation typically exhibit nearly normal RBC indices and HbA2 levels, often resulting in it being overlooked during screening tests. When combined with other beta thalassaemia mutations, CAP+1 can contribute to the development of beta thalassaemia intermedia.[21,22] Hence, molecular analysis is crucial for its diagnosis. In our study, individuals affected by the CAP+1 mutation exhibited normal mean MCV and MCH values compared to those with other identified mutations, displaying low MCV and MCH.

Besides IVS1-5 and Fr8-9 other identified mutations included, Fr41-42, IVS1-1 CAP+1, Cd-30, Cd-15, Cd-5, and Del 619 in descending order of frequency. These mutations are similar with that typically found in carriers in Pakistan, where the most prevalent mutations are IVS1-5, Fr 8-9, Del 619, Fr 41-42, and IVS1-1 in descending order[14] only in contrast with one mutation of Del 619 not found to be very common in our study. Study done by Colah R *et al* and Rija T *et al* has shown an opposite trend with CAP+1 being the most common mutations found in borderline A2 cases along with IVS1-5.[23,24] This mutation spectrum varies in other Asian nations; for example, in Malaysia, the most common genetic mutations among silent carriers are Cd19, IVS1-1, and Poly A mutations, with the CAP+1 mutation present in only 2.7% of borderline cases.[25]

The discovery of beta^0 mutations in our research was expected, given that recent studies have indicated their presence in individuals with borderline A2, although at a low frequency.[26] Additionally, in alpha or delta globin gene mutations coinheritance may have led to reduced levels of HbA2 in these individuals with β^0 mutations. It's worth noting that KLF1 mutations can also influence borderline HbA2 levels,[27] although our study did not explore this aspect. We ensured that confounding factors such as iron deficiency[28] and megaloblastic anemia[15] were omitted from our study population.

CONCLUSION

In countries like Pakistan with a high prevalence of thalassaemia, affordable molecular tests like ARMS-PCR are crucial for identifying silent beta thalassaemia carriers in individuals having borderline HbA2 levels. This is especially important for couples where both partners might be carriers, as it allows for preventing the birth of children with thalassaemia major. The detection of these silent carriers will contribute to effective prevention of thalassaemia, once genetic counselling and extended family screening is employed.

Limitations: Further research with large sample size with distribution from different parts of the country is needed to determine the ideal HbA2 cut-off for maximizing carrier identification using this cost-effective method.

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AUTHORS' CONTRIBUTION

AN, MB, HSM: Conceptualization of the study design, literature search, write-up. RM, AK, AK, NS, RI: Data collection, data analysis, data interpretation, write-up, proof reading, review.

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