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Significance of Molecular Analysis in a Population Screening Program for Identification of Silent Beta Thalassemia Carriers in a Country with High Disease Prevalence

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Abstract

Objective: The objective of this study is to establish the importance of molecular analysis in such individuals and its significance in a population screening program.

Methodology: This descriptive cross-sectional study was conducted by the Punjab Thalassemia Prevention Project during a period of 1 year from July 2020 to June 2021. A total of 67 individuals suspected to be silent Beta Thalassemia carriers were tested by the multiplex amplification refractory mutation system-Polymerase Chain Reaction to identify underlying mutations. Independent samples T-test was used to compare the RBC indices and HbA2 values of subjects with and without underlying mutations taking P value <0.05 as statistically significant.

Results: Of the individuals tested, 73% (n=49) had underlying Beta Thalassemia mutations. CAP+1 (n=30, 61%) was the most common mutation identified followed by IVS1-5 (n=12, 25%). Subjects with a mutation displayed a significantly lower mean corpuscular volume and mean corpuscular haemoglobin than those without mutation (P = 0.002 and 0.003 respectively). The mean HbA2 in the subjects with mutation was 3.4% vs mean of 3.2% for those without an underlying mutation (P = 0.011).The mean MCV and MCH of CAP+1 mutation was higher when compared to the other mutations identified.

Conclusions: Molecular analysis should be offered to all those individuals who have borderline HbA2, carrier spouse and/or suggestive family history for identification of silent carriers and effective disease prevention.

Key words: Silent carriers, Silent carriers, Beta Thalassaemia Major, Beta Thalassaemia Major, Molecular testing, Molecular testing, CAP 1 mutation.

Introduction

Hematological parameters including red blood cell (RBC) indices followed by Hemoglobin A_2 (HbA_2) quantification are the most commonly used screening tests conducted worldwide for Beta Thalassemia carrier identification and disease prevention. However, sole reliance on them can lead to misdiagnosis in individuals with HbA_2 in the borderline zone (atypical or silent Beta Thalassemia Carriers). Beta Thalassemia is the most prevalent hemoglobin disorder in Pakistan, with approximately 9-10 million carriers and more than 50,000 children suffering from this disease.[1] There is an average increase of 5000 new Thalassemia children in Pakistan annually.[2] Optimum treatment, consisting of regular blood transfusions, iron chelation and management of complications, can be afforded by only a few. Therefore, to curb the significant burden of this disease on the health and transfusion services of our resource constrained country, prevention is the most efficient and cost effective approach. This is implemented by pre-marital carrier screening especially in index families (cascade screening) and pre-natal diagnosis coupled with termination of affected Thalassemia major fetus.[3]

An elevated HbA_2 value confirms that an individual is carrier for Beta Thalassemia (Thalassemia Minor)[4] and this is an effective screening method that is being used in Thalassemia prevention programs worldwide.[5] However, there is inconsistency in defining the exact HbA_2 cut-off above which an individual can be labelled as a carrier and different laboratories use different values ranging from 3.5-4.0% to define this lower limit.[6] Individuals who lie within the gray zone, i.e. just above the normal HbA_2 range but below the typical carrier range are the ones

Authorship Contribution: [1]Conceived and planned the idea of the study, [2]drafting the work or revising it critically for important intellectual content, [4]final approval of the version to be published, [3]Collecting the data, [5]Data Analysis

Funding Source: none
Conflict of Interest: none
Received: Aug 26, 2022
Accepted: Dec 11, 2022

that can be misdiagnosed if solely HbA_2 quantification is used for diagnostic purpose. Furthermore, individuals with silent Beta Thalassemia mutations may have normal red blood cell indices and HbA_2 and only DNA analysis can confirm their carrier status. In cases where the partner is also a Beta Thalassemia Carrier, such misdiagnosis may risk the birth of an affected Thalassemia Major child. Therefore, in the above mentioned scenarios molecular analysis plays a significant role in confirmation of carrier status and Thalassemia prevention.

Many factors influence HbA_2 level and thus affect carrier detection. Factors that lower HbA_2 levels include concomitant iron deficiency, co-inherited mutations in alpha or delta globin gene[7] and silent mutations of Beta Thalassemia (e.g. mild β^+ mutations, CAP+1 and promoter region mutations).[8,9] On the other hand, megaloblastic anemia, Zidovudine therapy[10], alpha globin gene triplication and KLF mutations may spuriously raise HbA_2 levels resulting in a false positive result.[11]

Punjab Thalassemia Prevention Project (PTPP) is a government sponsored program which provides free of cost carrier screening and pre-natal diagnostic services in all the nine divisions of Punjab, the most populous province of Pakistan.[12] It caters to a diverse socio-cultural and ethnic population. Carrier screening is done by complete blood count (CBC) followed by HbA_2 quantification using High Performance Liquid Chromatography (HPLC) and/or Capillary Zone electrophoresis (CZE). DNA analysis is performed whenever there is diagnostic confusion. This study was conducted to determine the frequency of silent Beta Thalassemia carriers and the mutations identified especially in the borderline A_2 range. This will help reflect upon the significance of DNA analysis in such patients and the importance of DNA support in a Thalassemia population screening program.

Methodology

This descriptive cross-sectional study was conducted by the Punjab Thalassemia Prevention Project during a period of 1 year from July 2020 to June 2021, after ethical approval from the institutional review board. The samples processed at PTPP include referrals from health professionals, walk in individuals and samples taken as part of cascade screening of index families and screening camps from general population. After initial screening with history, CBC and HPLC, subjects with suspected silent Beta Thalassemia mutations (i.e. those with microcytic hypochromic RBC indices, Borderline $A_2$3.1-3.9%[13], carrier spouse and/or strong family history of Thalassemia, anemia and silent mutations) were sent for DNA analysis. Individuals with bicytopenia, pancytopenia and/or other hemoglobin variants were excluded from the study. Individuals with iron deficiency and megaloblastic anemia were also excluded from the study. Informed consent was taken from all study participants.

Three milliliter of blood was collected from the subjects and their parents in EDTA vacutainers for analysis. RBC indices were analyzed on Sysmex XP100 Hematology analyzer. The BIO-RAD Variant II HPLC (BioRad Corp, USA) beta-thalassemia short program and/or Sebia Capillary 2 Flex-Piercing (Capillary Zone) Electrophoresis was used for HbA_2 quantification in accordance with the manufacturer's instructions. Multiplex amplification refractory mutation system-Polymerase Chain Reaction (MARMS-PCR) testing was done step wise to detect the common, uncommon and rare mutations prevalent in Pakistan. These include Fr8-9(+G), IVS1-5(G-C), Fr41-42(-TCTT), Cd15(G-A), Cd5(-CT), IVS1-1(G-T), IVS1-1(G-A), Cd30(G-C), Cd30(G-A), Fr16(-C), IVSII-1(G-A), Del619(bp) and CAP+1 (A-C). Deoxyribonucleic acid (DNA) of blood samples was extracted by using the mini kit method (Macherey Nagel Kit). The extracted DNA samples were quantified by a nano-drop spectrophotometer. Primary mutations were identified by dividing ARMS–PCR into three multiplex PCR labelled as Allelic discrimination assays (AD1, AD2 and AD3) each including a different set of primers along with a control primer. After confirmation of primary mutations, a second panel of specific PCR was performed to amplify the specific DNA fragment. Amplified product was run on 2% agarose gel electrophoresis along with DNA marker of 1.0 kb and visualized through Gel Documentation system (Bio-Rad).

Data were recorded and analyzed in Statistical Package for Social Sciences (SPSS) Program version 23.0. The frequencies of carriers and the mutations identified were reported as number and percentages. Chi-square test was performed to determine the association between different HbA_2 groups (3-3.4% & 3.5-3.9%) with the

presence of Beta Thalassemia mutation. Independent samples T-test was used to compare the RBC indices and HbA_2 values of subjects with and without underlying mutations. P-value <0.05 was regarded as statistically significant.

Results

A total of 67 individuals were shortlisted for the study analysis in accordance with the study criteria. Among these, 40 (60%) were males and 27 (40%) were females. The mean age of the subjects was 28.1 years. Seventeen individuals had an immediate family member suffering from Thalassemia Major.

DNA analysis confirmed that 49 out of 67 (73%) individuals were Beta Thalassemia carriers. Six different mutations (CAP+1, IVS1-5, Cd-30, Cd-15, Cd-5 and Fr8-9) were identified (Figure 1). The most common mutation identified was CAP+1 (n=30, 61% of the carriers), followed by IVS1-5 (n=12, 25% of the carriers). The frequency of carriers at different HbA_2 levels is shown in Figure 2.

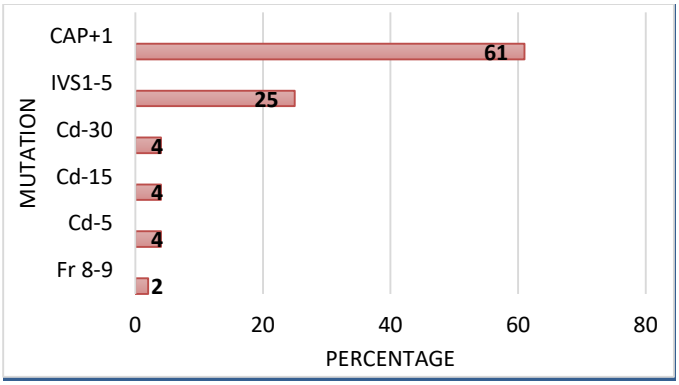

Figure 1. Frequency of Different Mutations identified in Beta Thalassemia Carriers

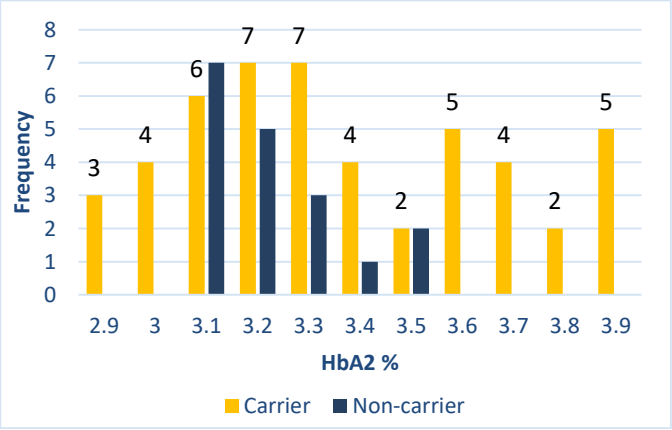

Figure 2. Frequency of Carriers at Different HbA2 levels.

When categorized into two groups of HbA_2, Beta Thalassemia mutations were identified more frequently in HbA_2 range of 3.5-3.9% (90%), as compared to that of 3-3.4% (63.6%). The association between these two HbA_2 groups and carrier status was statistically significant (p = 0.03) (Table I).

Table I: Association between HbA_2 groups and Carrier Status. (p = 0.03)

HbA_2 Groups	Carrier Status		Total	p-value
	Carrier	Non Carrier		
3.0 - 3.4%	28 (63.6%)	16 (36.4%)	44 (100.0%)	0.030
3.5 - 3.9%	18 (90.0%)	2 (10.0%)	20 (100.0%)	
Total	46 (71.9%)	18 (28.1%)	64 (100.0%)	

Independent sample t-test was used to compare the RBC indices and HbA_2 values of subjects with and without underlying Beta Thalassemia mutations (Table II). Subjects with a mutation displayed a significantly lower MCV and MCH value (p- value = 0.002 and 0.003 respectively). The mean HbA_2 in the subjects with mutation was 3.4% vs mean of 3.2% for those without an underlying mutation (p = 0.011).

When comparing the RBC indices of CAP+1 mutation with the other mutations identified, the mean MCV was 77.7fL vs. 61.0fL and mean MCH was 23.9pg vs. 18.1pg respectively.

Table II: Comparison of RBC indices and HbA2 values of subjects with and without Beta Thalassemia mutations.

	Mutation Groups	n	Mean	SD	t-test	p-value
RBC Count	With Mutation	49	5.57	0.87	1.97	0.054
	Without Mutation	18	5.20	0.57		
Hb	With Mutation	49	11.95	1.84	-1.79	0.078
	Without Mutation	18	12.82	1.52		
MCV	With Mutation	49	71.23	10.46	-3.30	0.002
	Without Mutation	18	78.63	7.07		
MCH	With Mutation	49	21.69	3.69	-3.11	0.003
	Without Mutation	18	24.82	3.55		
MCHC	With Mutation	49	30.49	2.30	-1.61	0.113
	Without Mutation	18	31.48	2.06		
HBA2	With Mutation	49	3.37	0.31	2.63	0.011
	Without Mutation	18	3.22	0.13		

Discussion

Hemoglobinopathies are unique genetic disorders because it is possible in majority (approx. 90%) of cases to determine the carrier status of individuals by hematological findings only.[14] The use of RBC indices and HbA_2 quantification in Thalassemia screening programs have proven to be a cost effective method in disease prevention and is the most widely used carrier screening test worldwide.[15] However, the lack of international standardization of an optimum HbA_2 cut off, lack of local studies to define the normal range and extreme distribution of HbA_2in Pakistani population as well as possibility of missing out atypical carriers indicate that sole reliance on hematological parameters cannot be made. This is true especially in populations with high disease prevalence.[6] Individuals with microcytic hypochromic RBC indices but normal A_2 and serum ferritin; those with completely normal RBC indices and HbA_2; and those with HbA_2 in the borderline range, can all be silent carriers for Beta Thalassemia. These silent carriers constitute approximately 2.5% of all the Beta Thalassemia carriers in Pakistan.[14] We conducted this study to emphasize on the importance of DNA analysis in these silent carriers which can be missed in a routine screening program.

The individuals tested in the study were mainly those belonging to families with index cases of Thalassemia (cascade screening) and/or those with significant family history. This selection bias can explain the very high percentage of Beta Thalassemia carriers identified in our borderline A_2 individuals (73%) when compared to similar studies in India (32%)[16], Malaysia (30%)[17] and Thailand (5.7%).[18]

Previous studies show that CAP+1 (A-C) is the most common mutation identified in silent carriers in Pakistan with a frequency ranging from 1.5-2% and it is present in all major ethnic groups in our country.[19] The most common mutation identified in our study population was also CAP+1 (61%). This is a point mutation in the promoter region, causing a β^+ mutation and is prevalent in the Indo-Asian region.[20] It is a silent mutation and heterozygotes typically have almost normal RBC indices and HbA_2levels, therefore it is often missed in screening tests.[21] The combination of CAP+1 and other Beta Thalassemia mutations can result in Beta Thalassemia Intermedia. Therefore molecular analysis is essential for its diagnosis. The mean MCV and MCH of CAP+1 mutation affected individuals in our study were higher when compared to those with other mutations identified, and five of them had completely normal RBC indices, which could have been missed in initial screening.

Apart from CAP+1 other mutations identified included IVS1-5, Cd-30, Cd-15, Cd-5 and Fr8-9 in order of decreasing frequency. This spectrum is different from that of the typical carriers in Pakistan, where the commonest mutations are IVS1-5 (G-C), Fr 8-9, Del 619bp, Fr 41-42 and IVS1-1 (G-T) in order of decreasing prevalence.[14]. Studies in India have demonstrated a similar pattern with CAP+1 and IVS1-5 to be the two most common mutations identified in Borderline A_2 cases.[22] The spectrum is different in other Asian countries, e.g. in Malaysia where the commonest mutations identified in silent carriers are CD19, IVS1-1 and Poly A mutation while CAP+1 mutation was present in only 2.7% of the borderline cases.[17]

The identification of β^0 mutations in our study doesn't come as a surprise, as recent studies have also shown that β^0 mutations may be present in Borderline A_2 individuals albeit in a low percentage.[23] Furthermore, possible co-inherited alpha or delta globin gene mutations might have caused the lowering of HbA_2 in these individuals with β^0 mutations. KLF1 mutations can also contribute to borderline HbA_2 in non-carriers.[24] These were not investigated in our study. Confounding factors like iron deficiency[25] and megaloblastic anemia had already been excluded in our study population.

Conclusion

Molecular analysis has a significant role in the identification of silent Beta Thalassemia Carriers, which is essential for Thalassemia prevention especially in countries with high disease burden like Pakistan. The identification of silent carriers is especially important if the spouse is also a carrier to prevent the birth of Thalassemia Major affected fetus. ARMS-PCR is a relatively inexpensive method that can be utilized to identify carriers in the borderline A_2 range especially in resource constrained countries. Further studies with a larger cohort needs to be conducted to identify the optimum cutoff of HbA_2 required to incorporate the maximum number of carriers in our population. Also, the impact of coexisting factors and genetic mutations need

to be investigated especially in β^0 mutations leading to borderline A_2.

Acknowledgements: The authors acknowledge the efforts of the technical staff of Hematology and DNA Laboratories, PTPP, who have done their utmost efforts to maintain high quality and standardization of sample testing despite limited resources.

Disclosure Statement: The abstract of this study was presented in Haemcon 2022 (24th PSH Annual Conference dated 5th-6th February 2022) and won the 3rd position in Young Investigator Award.

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