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ORIGINAL ARTICLE

Molecular Biomarkers for Prenatal Diagnosis of Beta-Thalassemia at Hyderabad Sindh

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ABSTRACT

Background: Thalassemia Is Disease Which Is Due To Defect In Hemoglobin Synthesis Resulting In Severe Anemia. The thalassemia disorder is genetic syndrome resulting from absence or markedly reduced production of one of the globin subunits of hemoglobin. In alpha (α)-thalassemia, production of alpha – subunit of globin is absent or decreased, while in the beta (β)-thalassemia, production of β – globin subunit is absent or reduced.

Aim: To find common mutations inside beta globin gene. To find new polymorphism inside beta-globin gene will help in future as part of prenatal diagnosis.

Methods: This study includes all cases of beta thalassemia minor diagnosed by normal prenatal testing while the mother's hemoglobin concentration is less than 27 pg and her total cell volume is less than 76 fl. Two EDTA tubes were filled with eight milliliters of blood after patients' samples were taken. ARMS PCR required 5 milliliters of EDTA sample, whereas HB electrophoresis and complete blood count (CBC) required 3 milliliters of EDTA tube. We used SPSS version 21 to evaluate all the data that was recorded using a self-made proforma.

Results: For the cases, the mean age was determined to be 26.83, whereas for the controls, it was 27.05.While the majority of patients had a history of thalassemia in their families (74%; 54/73), 27.4% (20/73) of the subjects did not have a consanguineous marriage, and 26.0% (19/73) did not have a thalassemia in their family history. The average hemoglobin (HB) level in the control group was 12.32 g/dl, but in the patients it was 10.10. The case group also had significantly lower MCV and MCH values than the control group. ARMS PCR revealed the following mutation frequencies: IVS-1-5 (42.5%) in 31 cases, Fr 8/9 (27%) in 20 cases, IVS-1-1 (11%) in 08 cases, Del 619 (5.5%) in 4 cases, CD-30 (5.5%) in 4 cases, CD-5 (5.5%) in 4 cases, and Fr 41/42 (3%), in two cases of selected Beta patients, as the fifth most common mutation.

Conclusion: Among the mutations detected in Hyderabad and the surrounding areas, the most common ones in beta thalassemia were IVS1-5 and Fr 8/9. Other variants were IVS1-1, Del-619, CD-30, CD-15, and fr 41/42. A sensitive molecular diagnostic and screening test for thalassemia can be used in our community with the ARMS PCR assay.

Keywords: ARMS PCR, Beta Thalassemia, Mutations

INTRODUCTION

The most common single gene disorders in the world population are hemoglobinopathies. Hemoglobinopathies are blood disorder which are inherited, results from defects in production of hemoglobin chain or produce mutated globin variants[1]. Different types of mutations on the genes that encode for alpha and beta chains of hemoglobin are responsible for haemoglobinopathies. On the chromosome 16, four alpha genes are located which encodes for alpha globin chain and on the chromosome 11 beta - gene present which encodes for beta globin chain[2]. Genetically Hemoglobin Disorders Are Divided into Thalassemia Syndromes and Other Abnormal Hemoglobin[3]. Thalassemia Is Disease Which Is Due To Defect In Hemoglobin Synthesis Resulting In Severe Anemia[4]. The thalassemia disorder is genetic syndrome resulting from absence or markedly reduced production of one of the globin subunits of hemoglobin. In alpha (α) thalassemia, production of alpha – subunit of globin is absent or decreased, while in the beta (β)-thalassemia, production of β – globin subunit is absent or reduced[5]. The tetrameric protein, which consists of two alpha and two beta globin chains, creates a pocket, which hold heme group for oxygen binding. In turn of their expression in the course of development two isolated gene –clusters of globin genes set on dissimilar chromosomal loci .Deficit production of alpha- globin chain of hemoglobin results in alpha thalassemia .Alpha thalassemia carrier or alpha thalassemia silent is said to be when individual contain a mutation in alpha –globin chain on a single chromosome (if one gene is involved) it is associated with mild anemia .While moderate to severe hemolytic anemia expressed by homozygote's or compound heterozygote's called as HBH disease . In adults, non-functional beta chain tetramers known as HbH (4 tetramers) are produced, but in the foetal stage, chain tetramers known as Hb Bart's (4 tetramers) are produced. Hb Barts Hydrops foetalis syndrome is the most severe form of alpha thalassemia, with no expression of alpha genes[6]. The most common monogenic disorders among beta haemoglobinopathies are beta thalassemia and sickle cell disease. About > 200 mutations in Hbb gene are causing beta thalassemia, This gene encodes beta subunit which is present in adult hemoglobin[7]. HBB gene which is present on chromosome 11, if point mutation or deletion on this gene results in beta-thalassemia. Further beta thalassemia is divided into two types, based on reduction of beta-globin (β+-thalassemia) or absence of beta-globin (β0-thalassemia)[8]. Beta - thalassemia is the most frequent inherited autosomal recessive disorder, which present with microcytic hypochromic anemia, pallor, massive splenomegaly and skeletal abnormalities such as bossing of frontal bone. Affected child fail to thrive and have short life expectancy without treatment[9].Within 6 month of diagnosis beta thalassemic patient used to die[10]. If thalassemia runs in the families it increases the risk of thalassemia. Mostly patient diagnosed at age of 6 month[11]. About 5% carriers of hemoglobin disorder are present in worldwide[1]. Decreased the fresh births of Thalassaemia major to about nil in developed countries due to successful preventive programs. However picture is unlike in developing and under developed countries[12]. Beta thalassemia is most common in Mediterranean countries, also in Southeast Asia, Middle East, India, Africa and the Central America. Due to decrease knowledge about thalassemia in Pakistanis people results increase consanguineous marriage, which leads to increase birth rate, poverty n decrease education level. Early marriages and consanguinity due to lack of awareness head to Pakistan towards increase ratio of children which depend on transfusion[13]. About 180 million inhabitants in Pakistan[14]. However, although a formal register is not yet available in Pakistan, around 5000-9000 children

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with β -thalassemia are born each year.. The prevalence of scarriers of beta thalassemia is around 5-7%, with the total population carriers rate are 9.8 million[15]. Roughly forty thousand cases of beta thalassemia syndrome are currently register, which are transfusions dependent and about 5000 affected children are born per year in Pakistan[14]. B-thalassemia puts a rising problem in Pakistan for health concern services, so it's impossible to provide all patients to blood transfusions and iron chelation therapy due to the lack of national resources. Bone marrow transplantation is tremendously costly & too expensive for most of the Pakistani patients. Prevention is a low-cost and effective way to deal with beta thalassemia in Pakistan[20]. For beta thalassemia, complete blood count with peripheral film and hb electrophorsis are considered baseline testing, whereas chorionic villous sample and the ARMS assay are the very extensively employed for molecular screening. Electrophoresis shows a decrease in haemoglobin A and a significant increase in foetal haemoglobin (HbF) for beta thalassemia. Beta thalassemia trait, on the other hand, is frequently asymptomatic and is discovered by chance when microcytic hypochromic anemia is present. If the value of Red Blood Cell indices (MCV, MCH, MCHC) are lower, a person is considered to be a thalassemia carrier, On CBC red blood cells are greater than 5 million/cm, and haemoglobin A2 is greater than 3.5 percent on Hb electrophoresis. However, a confirmed diagnosis can be difficult in some situations with borderline values, and in these cases a genetic molecular study to screen for any silent mutation like (CAP+1) is indicated for definite diagnosis.

Nonetheless, the patient's clinical history, family history of beta thalassemia, and any underlying conditions such as iron deficiency anemia or pregnancy must all be considered when making a definitive diagnosis[16]. The WHO has realized the importance of control of rising cases of haemoglobinopathies, specially β-thalassaemia, as a priority[13]. Basics for a successful prevention programme are Evaluation of the disorder burden, create Knowledge in the people, finding of carriers and couples who are at risk and genetic counseling. Major necessity For such preventive programmes are ,early people testing, genetic counseling , prenatal diagnosis through different methods and miscarriage of affected pregnancy , and finding the basic molecular defects in β-thalassaemia[13]. Many molecular approaches for identifying point mutations have been developed based on preventive PCR amplification of the DNA target, such as restriction fragment length polymorphisms (RFLP-PCR), amplification refractory mutation system-PCR, and so on[17]. The wide ranges of mutations in the β-globins gene result in variety of phenotypically distinctive features in patients. Different mutations in beta-globin gene disseminate among each ethnic group. Recognition of such mutations help out authorities for exact evaluations and additional practical prevention programs[18]. Prenatal diagnostics relies on informative polymorphisms to distinguish between normal and mutant gene inheritance from parents to offspring. Above 20 restrictions fragment length polymorphisms be identified in the Hbb gene group and some of those are normally use in the prenatal diagnosis (PD)[19]. Pregnancy and perinatal decision-making and management depend upon information given by Prenatal genetic diagnosis .Presently, prenatal testing plan consist of quantitative fluorescence polymerase chain reaction (PCR)[20]. About twenty one β-globin mutations were identified in Pakistan. (19) ,five mainly frequent mutations in Pakistan are IVS1-5 , IVS1-1 , Fr 41-42, Fr 8-9 and deletion 619[21]. The PCR brought important change in recognition and screening of mutation , in addition to the development of PGD and non-invasive prenatal diagnosis. Currently thalassaemia are diagnosed immediately by the testing of amplified DNA from fetal trophoblast and also infrequently from amniotic fluid . Prenatal diagnosis turns into quick and accurate due to of CVS with PCR, i.e. ARMS-PCR at initial period of pregnancy. But uncommon variants can be characterized by technique such as gene sequencing, that is believed to be more accurate techniques[13]. Majority of these RFLPs are found outside of the HBB gene, either up or down stream in the beta-globin. In local population, few of these sites might have a low allele frequency, making them useless for prenatal diagnosis. Furthermore, up stream of the hbb gene is known as a hotspot used for meiotic recombination. As a result, the goal of this research is to unidentified fresh polymorphisms with high allele frequencies in the local population, ideally within the beta-globin gene, for use in prenatal diagnosis linkage analysis[19]. In this study, number of samples were screened and examined.

MATERIALS AND METHODS

This study was conducted at Department of Pathology at LUMHS Jamshoro, Diagnostic and Research Laboratory at Liaquat University Hospital Hyderabad during March 2021 to August 2021. A non-probability and Purposive Sampling Method was used during the study. Patients who are beta thalassemia minor diagnosis on routine investigation during first trimester of pregnancy. Patients who have MCV < 76 fl and MCH < 27 pg are included in this study. People with no mutations (controls) are also included in the study.

Thalassemia major patients are excluded from this study. Patients having genetic disorder suspected to be other than thalassemia are excluded. For 5% beta-thalassemia sample size calculation, The sample size is n=73, and it was calculated using Open EPI software with a proportion of 95 percent confidential interval and a margin of error of 5%.

After taking aseptic measures 6 ml blood sample is collected from patients, and Healthy control was drawn by using 10cc disposable syringe and then divided in to sterilized purple top (EDTA) vacutainer. After collection of sample patient's identification was labeled on the sample. One tube containing 3 ml of sample is used for Complete blood count (CBC) it was performed on XN 1000 by Sysmex Japan and hb- electrophoresis was performed in. Slide was prepared for Microscopic examination. Other tube containing of 3 ml of sample is used for extraction of DNA . EDTA anticoagulated samples were analyzed using a CBC done on a Sysmex XN1000i Japan, 6-part differential automated hematology analyzer. This analyzer is based on methods involving Hydro Dynamic Focusing (DC Detection), Flow-Cytometry (Semi-Conductor Laser), and SLS-Hemoglobin.

RESULTS

The study subject consisted of 146 (100%) female participants, out of which 73 (50%) Females were cases with majority having family history of Beta Thalassemia and 73 (50%) healthy controls were also taken.

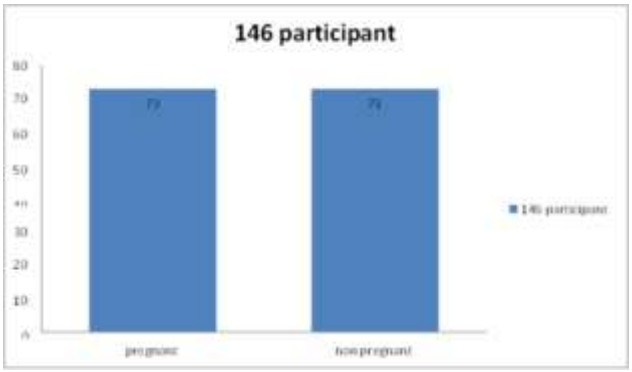

Figure 1: Association of gestation with Beta Thalassemia trait

The mean age calculated for cases was 26.83 ± 5.28. while the mean age of controls were 27.05 ± 7.15, shown in table 1.

Table-1: Mean Age of the Patients and

Subject	Mean age	Minimum	Maximum
Case	26.83 ± 5.28	18 years	38 years
Control	27.05 ± 7.15	16 years	40 years

The patients' geographical dispersion reflects the heterogeneity of beta –Thalassemia minor within division Hyderabad Sindh Pakistan's several districts. This shows that beta- thalassemia minor can affect people from many areas of life, races, and ethnic groupings. In our study total participant were 146 out of them 73 were beta thalassemia minor cases and 73 were controls. out of them 57% (42/73) cases were from Hyderabad district, 7% (5/73) cases were from Jamshoro district, 19% (14/73) cases were from DADU district, 11% (08/73) were from hala district, 3% (02/73) were from Matiyari district, 3S% (02/73) were from Tando Allah Yar district.73 were controls. out of them, 68.4% (50/73) controls were from Hyderabad district, 7% (5/73) controls were from Jamshoro district, 20.5% (15/73) were from Dadu district, 4.1% (03/73) were from Matiyari district. (figure 2)

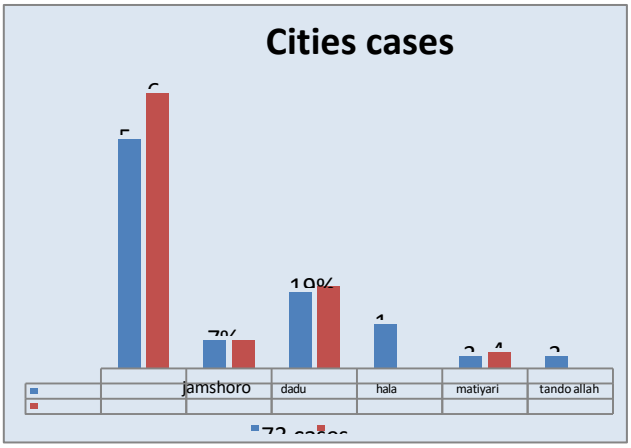

Figure-2: Association of Hyderabad district with Beta Thalassemia minor

The vast majority of the participants had a history of thalassemia in their families (74%; 54/73), however 27.4% (20/73) did not have a thalassemia family history and 26% (19/73) did not have a consanguineous marriage. (table 2)

Table-2: Association of consanguineous marriage and family history with Beta Thalassemia trait

Parameters	Study Group (73)	
	Present	Absent
Consanguineous Marriage	53(72.6%)	20(27.4%)
Family History of Thalassemia	54(74%)	19(26%)

Minimum haemoglobin (HB) level among cases was 5.20 gm/dl and maximum Hemoglobin were 13.90 g/dl and mean HB level was 10.10. While in controls mean Hemoglobin level were 12.32 gm/dl. Mean corpuscular volume and MCH in cases were also found to be on lower side as compared to controls. Mean MCV was 64.0 fl in cases and 83.74 fl in controls, while mean MCH in cases and control was 19.52 pg and 27.58 pg respectively. (Table 3)

The following mutations were found in a total of 31 cases: IVS-1-5 (42.5%), Fr 8/9 (27%), IVS-1-1 (11%), Del -619 (5.5%), CD-30 (5.5%), and CD-5 (5.5%), among others. The fifth most common mutation was Fr 41/42 (3%), which was found in only two cases of selected Beta patients.(figure 3)

Table-3: Association of CBC parameters in beta thalassemia trait cases and controls

Para Meters	Mean ± SD		Minimum		Maximum	
	Case	Control	Case	Control	Case	Contro L
HB (g/dl)	10.10 ± 2.01	12.32 ± 1.32	5.20	9.20	13.90	15.40
MCV (fl)	64.00 ± 7.04	83.74 ± 5.70	47.70	71.20	86.50	98.10
MCH (pg)	19.52 ± 2.41	27.58 ± 2.43	13.70	21.20	26.90	34.00

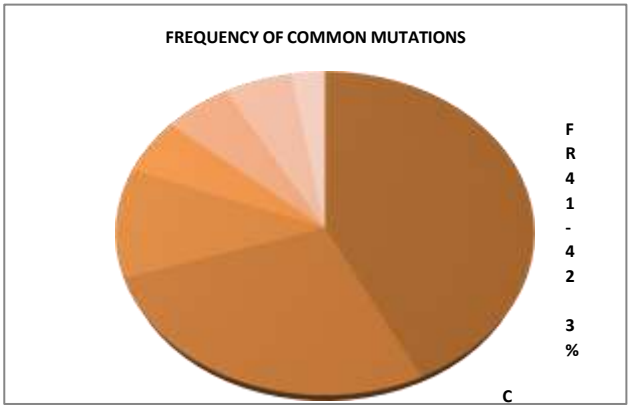

Figure-3: Percentage of mutations frequency (IVS1-5, IVS1-1, Fr 8-9, Fr41-42,CD-30, CD-5 and Del-619)

DISCUSSION

The high prevalence of beta-Thalassemia in Pakistani residents is attributed to a number of causes, including close relative marriages, human exposure to mutagens, and an elevated birth rate. In our study consanguineous marriage was found in the majority of the patients (72.6%) as the primary reason for Thalassemia occurrence in Pakistan. This is in line with a study by Khalid A. who also reported consanguineous marriages as the main cause of Thalassemia prevalence with a high rate in Pakistan, causing suffering not only for the patients but also for their families[20]. Naseem A et al also found similar finding during their research conducted on Thalassemia that the majority (64.2%) patients came from rural areas, while only 35.8% were from cities; as expected, 77.6 percent said they were in a consanguineous marriage, and which is a potential risk factor for the Thalassemia occurrence[21].

In our research the mean level of MCV and MCH in beta Thalassemia patients was 64.0 fl and 19.52 pg. Another study by Gangwani DA et al reported the similar results with mean MCV 63.5 fl and mean MCH 19.2 pg among Thalassemia patients (70). Sadiq MA et al also observed mean level of Hb 12.1 g/dl, MCV 62.7 fl and MCH 19.8 pg in Thalassemia patients[22].

In this study the genetic risk factor i.e. Beta globin gene mutations were analyzed by means of PCR and we found IVS1-5 with (43%), Fr 8-9 (27%), IVS1-1 (11%), Del -619 (5%), CD-

30 (6%), CD-5 (5%) and Fr 41/42 (3%) the most common mutations found in Pakistan. A research conducted on beta Thalassemia by Ansari SH et al in Karachi also reported eight frequent mutations: IVS1-5, Fr 8/9, Del 619bp, IVS1-1, Fr 41/42, Cd-30, Cd-5 and Cd-15, responsible for about 93.9 percent of beta Thalassemia cases. Among them the most prevalent genetic alterations found in Pakistan were IVS-I-5 (40.89 percent), Fr8/9 (15.7 percent), and IVS-I-I (8.17%)[23]. It is in resemblance with another study from Khyber

Pakhtunkhwa by Jalil T et al reported similar beta Thalassemia variation in Pakistan, this includes IVS1-5 (G–C), Fr 8-9 (+G), Fr 41-42 (–TTCT), IVS1-1 (G–T), IVSII-1 (G–A),CAP+1,

Cd-5 , Cd 16 & Cd-3 Cd-15, IVS1-1 (G–T), IVSII-1 (G–A), IVS1-1 (G–T). (6-9).

Among these the mutational research found Fr 8-9 (+G) to be the most common or sole detected mutation, whereas CD 5 (-CT) was the second most common. When looking at regional distribution, the Fr 8-9 (+G) mutation was found throughout the majority of KP's central areas, including Kurram, Khyber agencies in FATA, Peshawar Charsada, Mardan, Hangu, and Swabi. While mutations in remote locations, such as Cd 15 (G-A) in North Waziristan, ISV 1-5/Cap+1 (A-C) in Karak, and Fr 41-42 (- TTCT) in Swat, were found (24). Another study by Zafar U et al reported IVS 1-5(G-C) as the most prevalent mutation in the Bahawalpur division with 34.2 percent, following Fr 8-9(+G) 30.2 percent. Bahawalpur district had IVS 1-5 as the most frequent mutation while rest of the districts in the Bahawalpur division revealed Fr 8-9(+G) as the most prevalent mutation (G-C). Similarly, the most frequent mutation in Multan division was Fr 8-9(+G) (29.4 percent or 30/102). Except Lodharan district, where cd5 (-CT) was the most common mutation, all of the Multan division's areas included in this analysis had Fr 8-9(+G) as the most prevalent mutation. In Ghotki (Sindh) IVS 1-5(G-C) was discovered as the most frequent mutation (25). In Mardan, Pakistan, Shakeel Muhammad et al did a study on the HBB gene mutation. The investigation discovered five frequent mutations: IVS-1-5, Fr 8/9, Cd 41/42, IVS1-1, and Cd 15. In the Charsadah population, the most prevalent mutation detected was IVS-1-5 (26). According to the researcsh conducted by Waqas M et al Fr 8-9 (+G) was detected as the most prevalent mutation among beta-Thalassemia carriers in the Pakistani population, accounting for 38.2 percent, followed by IVS1-5 (G-C) at 28 percent, Fr41-42 at 8.2 percent, Cd 5 at 6.8 percent, and other mutations (18.8 percent). (27)

CONCLUSION

The study indicated that beta thalassemia was most commonly caused by mutations in IVS1-5 and Fr 8/9, although it also discovered mutations in IVS1-1, Del-619, CD-30, CD-15, and fr 41/42 in Hyderabad and the surrounding areas. One sensitive molecular diagnostic tool for thalassemia screening in our community is the ARMS PCR assay.

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