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Original Article

Beta Thalassemia Mutation Analysis in Fetal Samples for Optimal Mutation Screening Strategy

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ABSTRACT

Objective: To determine the frequency of various beta-thalassemia mutations in the prenatal period and ascertain the spectrum of mutations to optimise mutation analysis with cost reduction in testing.

Study Design: Cross-sectional study

Place and Duration of Study: Department of Molecular Hematology, Armed Forces Institute of Pathology (AFIP)/National University of Medical Sciences (NUMS), Rawalpindi Pakistan from Jul 2021 to Jan 2022.

Methodology: Chorionic villus sampling (CVS) was performed at 12-16 weeks of gestation in target couples where both parents were known β-thalassemia carriers. Deoxyribonucleic acid (DNA) was extracted from fetal tissues, and polymerase chain reaction (PCR) was performed, and mutations were analysed with controls on polyacrylamide gel electrophoresis (PAGE).

Results: Out of a total of 87 CVS samples, 17(19.5%) showed no mutation, 25(28.7%) had Beta-thalassemia major, and 45 (51.7%) were beta-thalassemia trait (heterozygous). Eight mutations were detected in the study population, and the three most common mutations were Fr 8/9, IVS 1/5 and Cd 15. Comparatively less common mutations included Cd 5, Fr 41/42, Fr 16, IVS 1/1 and Cap+1. Ethnical distribution of these mutations showed high frequency in Pathans and Punjabis compared to Sindhis, Balochis, Saraikis and Kashmiris.

Conclusion: The commonly detected prevalent thalassemia mutations must be tested to provide cost-effective facilities in our resource-constrained country. This study will help in future testing strategies and optimisation of mutation analysis in our country.

Keywords: Amplification refractory mutation system (ARMS), Beta-thalassemia, Chorionic villus sampling (CVS).

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INTRODUCTION

Beta-thalassemia is a haemoglobin abnormality commonly seen in the Mediterranean region, including Greece, Italy, Cyprus, certain parts of North and West Africa, the Middle East, the Indian Subcontinent, South Far East and South East Asia and these regions are collectively called "Thalassemia belt".[1] Beta-thalassemia, which is the most common single gene disorder of haemoglobin worldwide and nearly 3% of the world population in more than 60 countries, is beta thalassemia carrier.[2] In Pakistan, the carrier rate of β-Thalassemia is about 5-8%, and approximately 10 million Pakistani population harbor beta-thalassemia.[3,4] According to the World Health Organization (WHO), if the disease rate in any area is 0.1/1000, preventive programs should be commenced.[5]

Beta-thalassemia is a heterogeneous group of disorders characterised by quantitative defects in the beta-globin chain inherited in an autosomal recessive pattern. It is characterised by reduced ($_{\beta}$+) or no (β^{0}) synthesis of the beta-globin chain of haemoglobin because of mutation on the short arm of chromosomal 11.[6,7] More than 200 distinctive thalassemia-causing mutations have been distinguished in the beta globin gene.[8] Point mutations are most frequent. However, insertion and deletion are documented.[9]

Beta-thalassemia is diagnosed at three levels. The first step is checking haemoglobin levels, red blood cell count, indices, and peripheral blood film. The second step of the investigation is Hemoglobin-studies by High-pressure liquid chromatography (HPLC) or electrophoresis. The third tier and most accurate based on which mutations are checked is molecular-based (like ARMS-PCR, DNA sequencing, etc).[5,10] In couples, where both the parents are beta thalassemia carriers, prenatal diagnosis (PND) of thalassemia is essential. This study aims to ascertain the common thalassemia mutations to help suggest and formulate an optimal mutation screening strategy in line with the available resource-constrained countries like Pakistan.

METHODOLOGY

The cross-sectional study was carried out at the Department of Molecular Hematology, Armed Forces

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Institute of Pathology, Rawalpindi Pakistan, from July 2021 to January 2022 after approval from the Institutional Review Board (IRB-certificate No: BS/HEM /READ-IRB/21/635 Dated Jun 30, 2021). The sample size was calculated by using World Health Organization (WHO) calculator taking population proportion of beta-thalassemia trait in fetuses as 6%/0.06).[11] Non-probability convenient sampling technique was used to collect the required sample size.

Inclusion Criteria: Couples who were already diagnosed as beta-thalassemia carriers, were included in this study.

Exclusion Criteria: Couples with gestational age of a fetus beyond 16 weeks were excluded from this study.

All samples were taken after informed consent from the parents. At the gestational age of 12-16 weeks, fetal samples were collected by Chorionic villous sampling in a 10ml conical tube containing normal saline. 2ml of blood from both parents was also collected in an EDTA tube. The sample was transported to a molecular laboratory. Placental tissue was isolated from fetal tissue using a dissecting microscope. The Chelex100 technique was used to extract DNA from the blood samples of both parents and CVS.

ARMS-PCR technique was used to check beta-thalassemia mutations in fetal tissues and both parents. In this technique, the first parental mutations were checked. DNAs were amplified on Proflex PCR and the ABI-2720 PCR system.

Mutations were detected on 6-8% polyacrylamide gel electrophoresis (PAGE) by staining with 0.1% silver nitrate, and findings were interpreted by comparison with known mutations (control ladder) as shown in Figure-1. Ten mutational primers were used to analyse Beta-Thalassemia mutations, including Fr 8-9, Fr 41-42, IVS 1-5, IVS 1-1, Cd 5, Cd 15, Fr 16, Cap+1, del 619 and IVS 2-1 along with 100bp (base pair) DNA ladder and 861bp internal control.

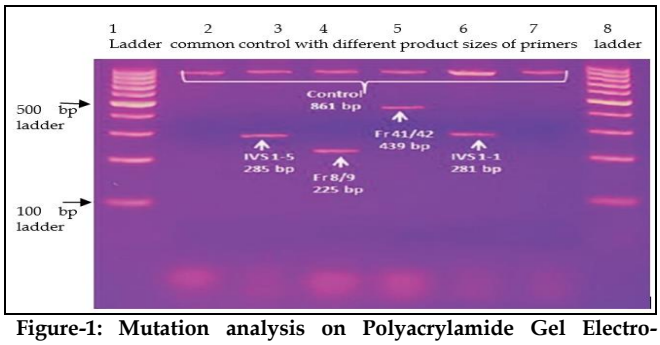

Figure-1: Mutation analysis on Polyacrylamide Gel Electrophoresis showing Control Bands and Mutations Bands

Data was analysed using the Statistical Package for Social Science (SPSS version 25). The mean standard deviation (SD) of quantitative variables like gestational age was calculated. Frequencies and percentages of qualitative variables like homozygous and heterozygous mutations were calculated. The ethnic distribution of mutations was compared using the Pearson chi-square test. The *p*-value of ≤0.05 was considered significant.

RESULTS

Out of the 87 fetal samples, 17(19.5%) were normal (having no mutation), 21(24.1%) were having homozygous beta-thalassemia mutations (phenotypically Bet-thalassemia major), 4(4.6%) showed compound heterozygous beta-thalassemia mutations (phenotypically β-thalassemia major) and 45(51.7%) were having heterozygous beta-thalassemia mutations (their phenotype was β-thalassemia trait) as shown in Figure-2.

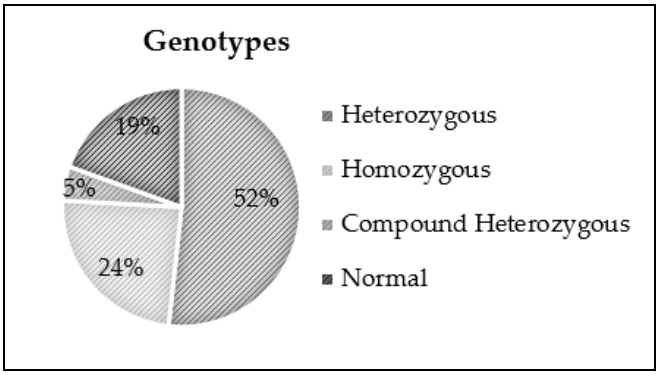

Figure-2: Frequency of Genotypes of Beta Thalassemia Mutations (n=87)

The most commonly observed mutations were Fr 8-9, IVS 1-5 and Cd 15. The distribution of frequencies of various mutations is shown in Figure-3.

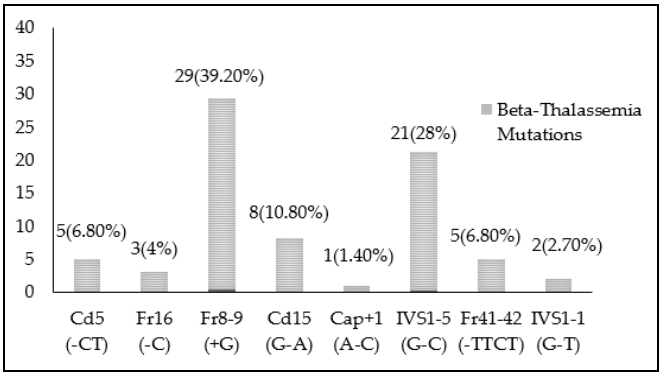

Figure-3: Frequency of observed Mutations in CVS Samples by ARMS-PCR Technique (n=87)

Beta-thalassemia mutations also showed diversity in different ethnical groups. Figure-4 shows that the mutations mostly prevail among Pathans and Punjabis. Sindhis, Balochis and Saraikis were comparatively less frequently affected. The Kashmiri population was least affected by beta-thalassemia mutations in our study.

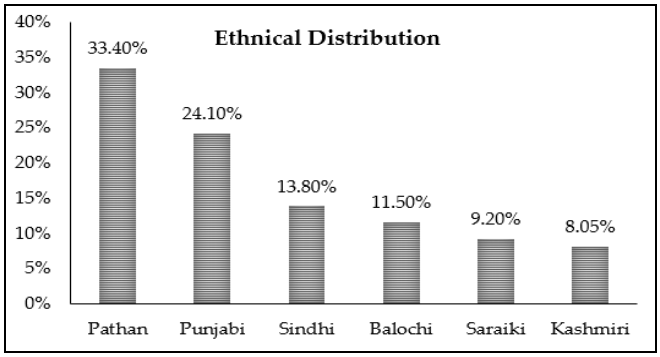

Figure-4: Ethnical distribution of Beta-Thalassemia Mutations (n=87)

Fr 8-9, IVS 1-5 and Cd 15 were the most frequent mutations in Pathans, Sindhis and Punjabis. The differences in the ethnic distribution of the Fr 8-9 mutation and IVS 1-5 mutation were found to be statistically significant, as shown in Table.

Table: Pattern of distribution of various Beta-Thalassemia Mutations observed according to Ethnicity in Pakistan (n=87)

Mutations	Pathan	Punjabi	Sindhi	Balochi	Saraiki	Kashmiri	*p*-value
Fr 8-9	15(17.2%)	8(9.2%)	1(1.1%)	0(0%)	3(3.5%)	2(2.3%)	0.02
IVS 1-5	3(3.5%)	3(3.5%)	9(10.3%)	6(6.9%)	0(0%)	0(0%)	0.00
Cd 15	1(1.1%)	3(3.5%)	0(0%)	1(1.1%)	2(2.3%)	1(1.1%)	0.34
Fr 41-42	1(1.1%)	2(2.3%)	1(1.1%)	1(1.1%)	0(0%)	0(0%)	0.81
Cd 5	3(3.5%)	1(1.1%)	0(0%)	0(0%)	0(0%)	1(1.1%)	0.55
Fr 16	2(2.3%)	0(0%)	0(0%)	0(0%)	0(0%)	1(1.1%)	0.37
IVS 1-1	0(0%)	0(0%)	0(0%)	1(1.1%)	0(0%)	1(1.1%)	0.37
Cap+1	0(0%)	1(1.1%)	0(0%)	0(0%)	0(0%)	0(0%)	0.67

DISCUSSION

Beta-thalassemia is an autosomal recessive single-gene disorder of the beta-globin chain of haemoglobin. The mutations are found in the beta-globin gene on chromosome,[11] which decreases or abolishes the production of the beta-globin chain. Although many advancements have been made in treating beta-thalassemia, including Bone marrow transplant, prenatal diagnosis and pregnancy termination of affected fetuses are important steps towards its prevention.[12]

This study evaluated the most prevailing mutations and their distribution in various ethnic groups. In our study, the genotypic distribution of beta thalassemia was found to be 51.7% for heterozygous mutation (Beta Thalassemia trait), which was most frequent, 25.3% for homozygous mutation, 4.6% for compound heterozygous mutation and 18.4% were having no mutation. These results have similarity up to some extent, with the study conducted by Yasmeen Ehan *et al.* in Nov 2020, as their results revealed 34.1% heterozygous mutation, 31.7% compound heterozygous mutation, 21.9% homozygous mutation and 12.2% normal cases.[13]

In our study, out of a total of 87 CVS samples, 26(29.9%) were beta thalassemia major, 45(51.7%) were beta thalassemia trait and 16(18.4%) were normal cases. However, the study conducted by Ansari *et al.* in 2012 showed 31.5% beta thalassemia major, 62.2% represented beta thalassemia trait, and 6.3% of cases were normal.[14]

Diversity was found in the distribution of beta thalassemia among various ethnical groups. Pathans and Punjabi were most frequently affected. Sindhis, Balochis, Saraiki and Kashmiris were comparatively less affected. According to one study, Punjabis are most affected, followed by Pathans, Sindhis and Balochis.[15]

In our present study, the most common mutations were Fr 8-9, IVS 1-5, Cd15 and Cd5. Other less common mutations were Fr 41-42, Fr 16, IVS1-1 and Cap+1. A study by Zafar *et al.* 2018 showed that the most common mutations were Fr 8-9, IVS 1-5, Cd5, Fr41-42 and Del 619. There was no significant difference between the results of our study and this study.[16] The study conducted by Kanwal *et al.* in 2017 showed that IVS1-5, Fr8-9 and Fr41-42 were the prevailing mutations and uncommon mutations were Del 619, IVS1-1, Cd15 and Cd16. There was some difference in the results of our study and this study.[17]

We found in the present study that Fr8-9 was the most common mutation, followed by IVS1-5, Cd15 and Cd5. Nevertheless, according to a study conducted by Tariq Moatter 2012, IVS1-5 was most common, followed by Cd8-9, Del 619 and IVS1-1 mutations.[18] According to a study by Muhammad *et al.* in 2017, the most common mutations detected were Fr 8-9, Cd 41-42, IVS1-5 and IVS1-1. The result of this study nearly matches the result of our study.[19]

LIMITATION OF THE STUDY

The ethnic distribution of the thalassemia mutations does not represent the true distribution as the study was conducted in northern Pakistan.

CONCLUSION

Thalassemia can be prevented by pre-marital screening, prenatal diagnosis and educating people about this disorder

by genetic counselling. The mutation analysis results can help optimise thalassemia testing panels, especially in a resource-restricted country like Pakistan. The common mutations detected should be initially screened to decrease the cost of testing, and an extended panel of mutations should be used in selected cases.

Conflict of Interest: None.

Author's Contribution:

Following authors have made substantial contributions to the manuscript as under:

MUA: Conception, study design, drafting the manuscript, approval of the final version to be published.

MB: Data acquisition, data analysis, data interpretation, critical review, approval of the final version to be published.

SSS & SHS: Critical review, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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