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Research Article

Distribution Pattern of Mutations Causing β-Thalassemia in Districts Swabi and Mansehra, Pakistan.

Naveed Khan[1,2,*], Ihteshamul Haq[3], Sajid Ul Ghafoor[2]

[1]Department of Biotechnology, Abdul Wali Khan University, Mardan, Pakistan
[2]Department of Biotechnology and Genetic Engineering, Hazara University, Mansehra, Pakistan
[3]College of Life Sciences and Technology Beijing University of Chemical Technology, Beijing, China

***Corresponding Author**: Naveed Khan, Email: naveedkhan@awkum.edu.pk

ABSTRACT

The present study analyzed 100 alleles from 50 unrelated families from Districts Swabi and Mansehra through ARMS PCR for the nine most common mutations in the gene for β-globin (*HBB*), reported in Pakistan. In general, 89% of alleles were completely characterized, and 11% remained uncharacterized. More specifically, in samples collected from Swabi, 08% of mutations remained uncharacterized, and 92% were characterized entirely, while in samples from Mansehra, 86% of the total alleles were characterized, and 14% remained uncharacterized. Overall, the IVSI-5 (G>C), with a frequency of 33%, was identified as the most prevalent, while IVSI-1 (G>A), with a frequency of 0.0%, was the least prevalent allele. The frameshift codons 8/9 (+G) [also known as FSC- 8/9 (+G)] and Cd (codons) 41/42 (-TTCT) alleles were observed to be the second and third most common alleles, respectively. The 619 bp deletion accounted for nine percent of the total alleles investigated. In Mansehra, the IVSI-5 (G>C) was found to be the most predominant allele. The same allele was observed as the second most common allele in Swabi, where the FSC- 8/9 (+G) was found to be the most prevalent type. The FSC- 8/9 (+G) was the second most common mutation in Mansehra. These variations in mutation rates could be attributed to the small sample size or possibly to the different genetic backgrounds of these two regions.

Keywords: β-Thalassemia, ARMS- PCR, β-globin, common mutations, Swabi, and Mansehra

INTRODUCTION

β-thalassemia is an autosomal recessive blood disorder caused by mutations in the *HBB* gene that reduce or completely prevent the synthesis of β-globin chains required for the formation of adult hemoglobin (HbA, α2 β2), resulting in hypochromic hemolytic anemia that requires blood transfusion for survival [1]. The severity of the condition correlates directly with the extent of imbalance in the α- and β-globin chains' production [4][5][6]. The underlying molecular basis of the disease is highly heterogeneous, with over 380 mutations in the β–globin gene leading to the disease [2]. The distribution pattern and frequency of these mutations vary among different populations and are influenced by the pattern of immigration within each country [3].

Beta thalassemia is the most common single gene disorder in Pakistan, having an overall 5-7 % carrier frequency (with over 10 million carriers), and nearly 5000 new cases are

reported each year in the country [7][8]. The high incidence of the disease in Pakistan can be attributed to various factors. In most previously screened regions, the affected population mainly comprises individuals with a strong rural background, low literacy rate, and low income. These individuals often practice the local tradition of consanguineous marriages, marrying within the same caste or with relatives [8][9]. The lack of awareness programs and the absence of screening, genetic counseling, and prenatal diagnosis facilities for local communities contribute to a growing incidence of consanguineous or carrier marriages in different parts of Pakistan, leading to a rise in affected births [9]. β-thalassemia is increasingly burdening the healthcare system in Pakistan. As in many other developing Asian countries, due to limited national resources, it is impossible to provide regular blood transfusions, iron chelation therapy, or bone marrow transplantation, which, in developed countries, have resulted in extended disease or symptoms-free survival for most of the patients [10][11]. In countries like Pakistan, preventive measures, such as genetic counseling, carrier screening, and prenatal diagnosis, are the most effective and least expensive ways of dealing with β –thalassemia [12][13]. Investigating the molecular basis and natural history of the disorder is a prerequisite for the establishment of such facilities [12]. Previous screening of different ethnicities and regions has identified 21 variants that account for almost 100% of mutations responsible for the disease in the Pakistani population [9][14][15][16][17][18][19]. Although the three most common mutations, IVS-I-5(G>C), Frameshift codons (FSC) 8-9 (+G), and Cd 41-42 (-TTCT), account for 86% of all the mutations causing the disease, significant ethno-geographic variation, in the distribution pattern of different mutations, exists in the country [17][18][19][9]. In light of this, we analyzed 100 alleles from 50 different families with at least one child with β –thalassemia from districts Swabi and Mansehra (previously unscreened areas), Khyber Pakhtunkhwa, Pakistan. The objectives of the study were to identify the prevalent types of mutations in the regions and to create awareness among people about the hereditary nature and preventive measures of the disease.

MATERIALS AND METHODS

This study was conducted in 2010 at the Department of Genetics, Hazara University Mansehra.

Subjects

A total of 126 buccal epithelial cell samples were collected from 50 unrelated families in Districts Swabi and Mansehra, each with at least one transfusion-dependent child suffering from β-thalassemia. The collected samples comprised carrier parents and their β-thalassemic child, diagnosed through red cell indices, Hb electrophoresis, and HbA2 levels. Sample collection from the patients was optional, and only children who could follow the sample collection protocol were sampled. In the case of the availability of previous molecular diagnoses for the mutations investigated in this study (Table 1), information on the type of mutation was recorded. in addition, upon our visits for sampling, we took this opportunity to provide guidance and to educate the families about the hereditary nature of the disease, the risk associated with marrying within the families, the importance of carrier screening in families already having affected children, prenatal diagnosis services as well as about the management and treatment of the condition.

Table 1: **Frequencies of β-thalassemia mutations in the study**

Mutations	No. of alleles and freq. (%)	No. of homozygotes	No. of heterozygotes
IVSI-5 (G>C)	33 (33%)	12	09
FSC-8/9 (+G)	26 (26%)	09	08
Cd 41/42 (-TTCT)	13 (13%)	05	03
619 bp deletion	09 (09%)	02	05
Codon 30 (G>A)	04 (04%)	02	00
IVS-I-1, (G>T)	02 (02%)	00	02
IVSII-l (G-A)	01 (01%)	00	01
Codon 30 (G>C)	01 (01%)	00	01
IVSI-1 (G>A)	00 (00%)	00	00
Uncharacterized	11 (11%)	00	11
Total	100 (100%)	30	40

Sampling and DNA isolation

The buccal cell samples were collected in tubes containing TNE (Tris-Cl, NaCl, and EDTA) solution and stored until DNA extraction. A modified protocol, originally developed by AIDAR and Sergio Roberto Peres LINE, was followed for sampling and DNA purification from buccal epithelial cells [20].

DNA analysis

We modified and standardized an allele-specific Amplification Refractory Mutation System (ARMS) PCR-based method to characterize 8 out of 9 common mutations (Table 1) of the β-globin's gene (*HBB*) reported in Pakistan [14][21][22]. Additionally, we used a PCR-based method to directly detect a 619 bp deletion at the 3′ end of the β-globin's gene [23]. For details about the primers used in this study, please refer to Baig *et al*., 2006a [19]; Varawalla *et al*., 1991[22]; Old *et al*., 1990 [21]; Varawalla *et al*., 1991a [14]; Baysal *et al*., 1994[23]. For the detection of IVSI-5 (G>C) and Cd (codons) 41/42 (-TTCT) mutations, multiplex ARMS PCR was used. The PCR was performed in 15 µL reaction volume having in final concentrations 1X PCR buffer, 0.92 µM of each of the three (two control and one mutation specific) or four primers (two control and two mutations specific, for multiplex PCR), 2.5 mM $MgCl_2$, 200 µM of each of the four dNTPs, 0.5 to 1 µg of template DNA, and 1U of Taq polymerase. For ARMS-PCR reactions, 33 cycles were carried out, each cycle involved denaturation for 40 seconds at 94 °C, primer annealing for 40 seconds at 65 °C, and extension for 80 seconds at 68 °C. Additionally, for detecting the 619 bp deletion, the PCR reaction also consisted of 33 cycles, and each cycle consisted of denaturation for 50 seconds at 94 °C, primers annealing for 45 seconds at 52 °C, and extension for 1 minute at 72 °C. In both cases, the initial denaturation and final extension were prolonged for 5 and 6 minutes, respectively. After amplification, the entire amplified product was mixed with 1X loading dye (3 µL) and electrophoresed on Ethidium Bromide (EtBr) containing 2% agarose gel for 45–60 min at 70-100 V, followed by visualization and photographing under UV light (Figure 1).

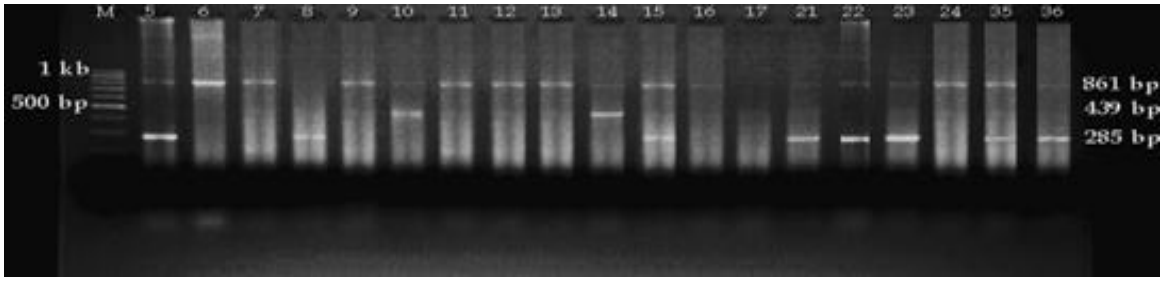

Figure 1: Ethidium bromide-stained gel pictures of the amplified products/multiplex PCR of 19 DNA samples with the primers for IVSI-5(G>C) and Cd41/42 (-TTCT) plus internal control primers. The 861 bp product is of control primers. The 439 bp and 285 bp fragments in some of the products indicate the presence of IVSI-5 (G> C) and Cd41/42 (-TTCT) mutations, respectively. The size of the amplified fragments is on the right, while those of the 100 bp ladder are on the left of the pictures.

RESULTS

In the present work, 100 alleles from 50 unrelated families from District Swabi and Mansehra were characterized. About 80% of these families were known to be consanguineous, and the parents of the affected child were related. Out of these, 45% were first cousins, and the others were second cousins or far related. In 20% of these families, the parents of the affected children have no blood relation (figure 2). At the molecular level, 89% of the total 100 alleles were completely characterized, while 11% remained uncharacterized. A total of eight different mutations were found in the subjects. The percentage prevalence of these eight mutations shows that throughout the study, the most predominant allele was IVSI-5 (G>C), which accounted for 33% of the total alleles. The FSC- 8/9 (+G) with 26% and Codons 41/42 (-TTCT) with 13% frequencies were identified as the second and third most common mutations, respectively. The 619 bp deletion at the 3′ end of the β-globin gene was found in 9% of the total alleles and was the fourth prevalent mutation in the study. In addition, 60% of the patients were found to be true homozygotes, having the same mutation in both copies of the gene, and the remaining 40% were compound heterozygotes, having two different mutations, one per each copy of the gene. The numbers of each allele encountered, together with frequency and the number of patients homozygote and compound heterozygote for it, are presented in Table 1.

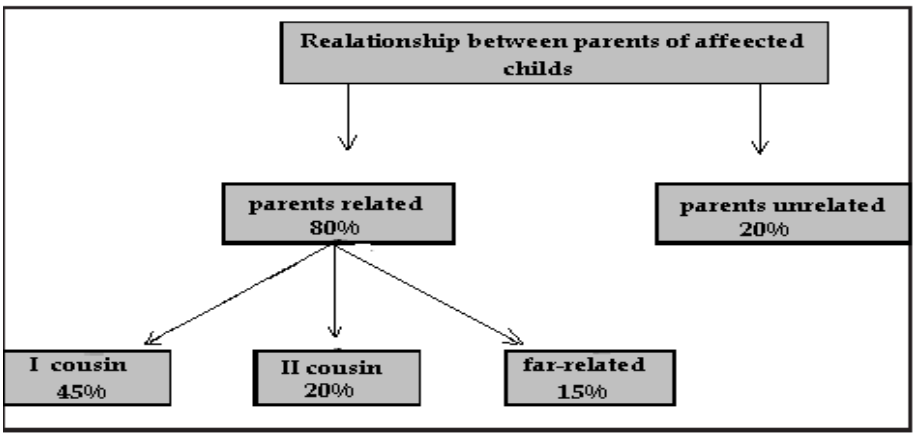

Figure 2: Relationship between parents of affected children

Discussion

The frequencies and distribution of different β-thalassemia mutations vary across populations. There are previous studies on the distribution pattern of different β-thalassemia mutations in various regions of Pakistan. In our study, like many other regions of Pakistan, the most prevalent mutation was IVSI-5 (G>C). Prior studies have also identified this mutation as the most common one, with a combined frequency of 42.1% in Faisalabad, Bahawalpur, and Multan and 45.09% in D.G Khan [9][18]. This mutation was the second most common mutation in Rawalpindi, Islamabad, Lahore, and Sargodha, with an overall frequency of 33.8% [19]. The second most common mutation in the present study was FSC-8/9 (+G), having a frequency rate of 26%. The already reported frequencies of this mutation are 34.8% in Rawalpindi, Islamabad, Lahore, and Sargodha where it occurred as a most common mutation; 40.35% in Faisalabad, Bahawalpur and Multan; 36.6% in D.G. Khan; 44% in KP region; 41% in Northern areas; and 4.5% in Karachi [24][9][17][18][19]. The third most common allele (with 13% frequency) in the present study was Cd 41/42 (-TTCT), with previously reported frequencies of 15.0% in Rawalpindi, Islamabad, Lahore, and Sargodha; 7.4% in Faisalabad, Bahawalpur, and Multan; 5.9% in D.G. Khan [9][17][18][19]. The district-wise comparison of our results shows that in Mansehra, the most common mutation was IVSI-5 (G>C), while in Swabi, the same mutation was found as a second common mutation. In Swabi, the most common mutation was FSC-8/9 (+G), which was the second most common mutation in Mansehra. The third prevalent mutation in Mansehra was Cd 41/42 (-TTCT), which was also the third prevalent mutation in Swabi, observed at an equal frequency with 619 bp deletion. Together, these eight mutations comprised 92% of the total mutations in Swabi and 86% of the total mutations in Mansehra. The comparative frequencies of the mutations analyzed in the study are presented in Table 2. In conclusion, we observed considerable differences in mutation rates in these two regions. However, a more precise understanding of mutation patterns in these areas requires a thorough and comprehensive study with a larger population size and a higher number of mutations to help reduce potential biases due to a small population size and a limited number of mutations.

Table 2: Comparative analysis of β-thalassemia mutations in various regions of Pakistan

Mutations	*Mansehra*	*Swabi*	*Swabi Mansehra*	*Rawalpindi Islamabad Lahore Sargodha*	*Faisalabad Bahawalpur Multan*	*D.G. Khan*	*Karachi*	*North West and Northern area of Pakistan*	*total*
IVSI-5 (G>C)	40%	26%	33%	33.8%	42.1%	45.0%			38.47%
FSC-8/9 (+G)	20%	32%	26%	34.8%	40.35%	36.6%	4.5%	42.5%	30.79%
Cd41/42 (-TTCT)	14%	12%	13%	15.0%	7.4%	5.9%			10.32%
619 bp deletion	06%	12%	09%	1.8%	2.5%	0.6%			3.47%
Codon 30 (G>A)	00%	08%	04%	0.2%	------	-----			2.1%
IVS-I-1, (G>T)	02%	02%	02%	1.8%	0.6%	5.2%			2.4%
IVSII-l (G-A)	02%	00%	01%	1.0%	0.9%	-----			0.96%
Codon 30 (G>C)	02%	00%	01%	1.3%	------	0.7%			1.0%
Total	86%	92%	89%	89.7%	93.85%	94%		---------	89.51

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