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Original article

The molecular characterization of β-Thalassemia in Afridi and Wazir tribes of inbreed Pashtoon population showed different patterns, indicating genetic heterogeneity for β-Thalassemia

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Abstract: β-Thalassemia (β-thal) is a monogenic disease resulting from mutations in the HBB gene, leading to aberrant globin production and causing hypochromic and microcytic anemia. The current study aimed to assess and compare the incidence of the most frequently occurring mutations of beta-thalassemia in sub-tribes Afridi and Ahmadzai of the Wazir tribe belonging to the Pashtoon Ethnicity, additionally the study investigated the inheritance pattern of these mutations in affected patients and the prevalence of consanguinity among the parents. Furthermore, this study holds potential significance in the context of prenatal diagnosis (PND), Genetic counseling, and carrier screening to control the occurrence of affected births not only in these tribes but in the entire Pashtoon Population. During the current research, 300 peripheral blood samples of affected patients, their parents, and siblings were compiled both from families having at least one transfusion-dependent child and sporadic patients from Ahmadzai Wazir residing in different areas of Wazir subdivision Banu Khyber Pakhtunkhwa (KPK), Pakistan. The same procedure was followed for collecting 300 peripheral blood samples from Afridi Wazir residing in different areas of Wazir sub-division Banu and Nort Waziristan Tribal District (NWTD), KPK, Pakistan. These samples were analyzed for the six most common β-thalassemia mutations found in the Pashtun population via the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) technique. Results obtained were a bit unique as the most common mutation detected in Afridi Wazir were Codons 41/42 (– TTCT), interestingly followed by frameshift codons FSC 8/9 (+G) (HBB: c.27_28insG), IVS-I-5(G>C) and FSC-5 (–CT). While results obtained for Ahmadzai Wazir were different than the above, and interestingly reported IVS-I-5 (G > C) to be the most recurrent followed by FSC-5 (–CT) among the six reported mutations. The findings of the present study show differences within the geographically adjacent situated sub-branches of highly inbreed Wazir tribe of Pashtoon Ethnicity, clearly demarcating genetic heterogeneity for β-Thalassemia. These observations need serious consideration in implementing parental meetings about disease recurrence in the future, large-scale mutation screening, and PND for the population of the Wazir tribe and the whole Pashtun ethnicity as well.

Keywords: β-thalassemia (β-thal); Hemoglobin(Hb), Prenatal Diagnosis (PND).

1. Introduction

Beta-thalassemia is a blood-related autosomal recessive genetic disorder, characterized by a mutation in β-globulin gene clusters positioned at the short arm (p) of chromosome number 11 band 11p15.4 - 11p15.5 [1]. The replica of the β-globin gene has 3 coding sequences (exons) sandwiched between codons 30 to 31 with a length of 1,600 bp. Any change in the β-globulin gene leads to a decrease (β+) or absence (β0) of a β-globulin chain of hemoglobin tetramer resulting in oxygen disruption in the body [2]. The exact overall β-gene mutation frequency in the world is still indistinct but according to the Itha Genes database, about 400 different types of β-thalassemia mutations throughout the world have been described of which 35 mutations are prominent in Pakistan [3-5]. Molecular genetics studies of various thalassemia populations indicate that each group tends to have its own set of mutations. Mutations that are mostly found in Pakistani populations are IVS-I-5(G-C), FSC 8-9 (+G), CD 41/42 (–TTCT), IVS-I-1 (G-T), FSC-5 (–CT), and CD 15 (G-A). About 3% of the world's population carries the β-thalassemia gene. In Pakistan, around about 9,000 (5-8 %) thalassemia patients are born every year in different ethnic groups [6]. The exact frequency of β-thalassemia mutations in the human population helps in the initiation of health control programs for the disease. With control programs like screening population, meeting with parents to know about the possible risks of genetic disorders, and tests before birth, many countries of the world substantially reduced the birth rates of homozygous β-thalassemia [7]. The aims and objectives of the current study were to judge and compare the incidence of the most frequently occurring mutations of beta-thalassemia in, Geographical adjacent subbranches of inbreed Wazir tribe of Pashtoon Ethnicity, their inheritance pattern in patients, and consanguinity among the parents. Moreover, this study could be valuable for prenatal diagnosis (PND), genetic counseling, and carrier screening to control affected births in these tribes and the whole Pashtoon population as well.

2. Methodology

In this study, 600 peripheral blood samples were collected, comprising 300 samples each from the Ahmadzai and Afridi branches of the Wazir tribe. The samples included affected patients, their parents, and siblings from families with at least one transfusion-dependent child, as well as sporadic patients requiring regular blood transfusions. These samples were sourced from various regions of the Wazir sub-division in Bannu and NWTD, Khyber Pakhtunkhwa (KP), Pakistan. The study population included 348 (58%) males and 252 (42%) females. Each sample was collected in a 5 mL vacuum-sealed EDTA (Ethylenediamine tetra acetic acid) anticoagulant tube. The samples were obtained from the Thalassemia Center for Women and Children, Bannu, KP, Pakistan. Clinical and demographic data, including blood transfusion frequency, thalassemia signs and symptoms, age, sex, and family history, were recorded through direct interaction with patients. Written informed consent was obtained from the patients and their family members [6]. Ethical approval was granted by the Bio-Ethics Committee of the University of Science and Technology, Bannu, KP, Pakistan [8]. The molecular analysis of the blood samples was conducted at the Laboratory of Human Genetics, Department of Biotechnology, University of Science and Technology, Bannu, KP, Pakistan. Genomic DNA was extracted from all blood samples using the organic phenol/chloroform method [9]. The ARMS-PCR technique, utilizing six common allele-specific ARMS primers along with control A, B, and C primers (internal control forward and reverse primers), was employed to detect β-thalassemia mutations (Table 1). In a single polymerase chain reaction, two internal control primers (Control A and B, forward and reverse) and a common primer (Common C,

forward), along with either mutant (Mt) or normal (N) allele-specific primers acting as reverse primers, were used. The total PCR reaction volume was 20 µL, containing 0.4 µL (2.5 units) of Taq DNA polymerase, 0.5 µL of deoxynucleotide triphosphates (0.2 mM), 1 µL (100 ng) of genomic DNA, 2 µL of 1X Taq polymerase buffer (10 mM Tris-HCl, pH 9.0), 1.2 µL (1.5 mM) of MgCl2, 50 mM KCl, 0.1% Triton X-100, 0.4 µL (10 pmol) of each of the four primers (Control A, Control B, Common C, and Mt or N), with the remaining volume made up with PCR water. The mixture was gently vortexed before amplification. The PCR reaction was performed for 27 cycles with the following conditions: initial denaturation at 95°C for six minutes, denaturation at 94°C for one minute, annealing at 65°C for one minute, and extension at 72°C for 1 minute and 30 seconds. A final extension step was carried out at 72°C for 10 minutes. Five microliters of the PCR product were resolved on a 2% agarose gel electrophoresis at 110 volts for 35 minutes, and the gel was visualized under UV light at a wavelength of 254 nm.

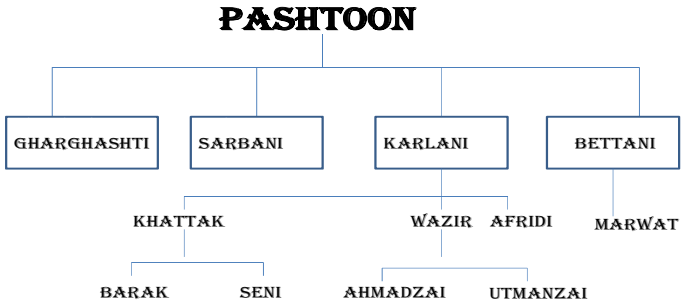

Figure 1. Family tree for Pashtoon Ethnic group

Table 1. Allele-specific primers for amplification refractory mutation system, polymerase chain reaction, and allele were used in this study.

Primers	Sequences (5' > 3')	Product Size (bp)
Control A	CAATGTATCATGCCTCTTTGCACC	861
Control B	GACTCAAGGCTGAGAGATGCAGGA	861
Common C	TCACTTAGACCTCACCCTGTGGAGCCAC	-
Codons 41/42 (Mt)	GAGTGGACAGATCCCCAAAGGCCTTGTTAG	439
Codons 41/42 (N)	GAGTGGACAGATCCCCAAAGGACTCAAAGA	-
IVS-1-5 (Mt)	CTCCTTAAACCTGTCTTGTAACCTTGTTAG	285
IVS-1-5 (N)	CTCCTTAAACCTGTCTTGTAACCTGATACGAAA	-
IVS-1-1 (Mt)	TTAAACCTGTCTTGTAACCTTGATACGAAA	280
IVS-1-1 (N)	GATGAAGTTGGT GACGCCCRG GGTAGG	-
FSC 8/9 (Mt)	CCTTGCCCCACAGGGCAGTAACGGCACACC	215
FSC 8/9 (N)	CCTTGCCCCACAGGGCAGTAACGGCACACT	-
Codon 15 (Mt)	CACCAACTTCATCCACG5TCACCTTGGCCT	500
Codon 15 (N)	CACCAACTTCATCCACGTTCACCTTGGCCC	-
FSC-5 (Mt)	ACAGGGCAGTAACGGCAGACTTCTACTCG	170
FSC-5 (N)	ACAGGGCAGTAACGGCAGACTTCTCATCAG	-

IVS: Intervening sequence; FSC: frameshift codon; Mt: mutant; N: normal

3. Results

3.1 Molecular Characterization of β-Thalassemia in Mehsud and Wazir tribes

The molecular characterization of β-thalassemia in the Mehsud and Wazir tribes has revealed distinct patterns of mutations. Several known mutations were detected in both tribes, including point mutations and small deletions in the β-globin gene. However, the prevalence of specific mutations exhibited differences between the two tribes, indicating genetic heterogeneity in β-thalassemia. A total of 920 alleles, with 460 alleles from both Mehsud and Wazir tribes each, were investigated for the presence of six β-thalassemia mutations. Among these six beta-thalassemia mutations, four mutations were found to be most prevalent in the Mehsud tribe. As indicated in Table 2, among the 230 samples, 82 individuals were identified as Homozygous for four β-thalassemia mutations while 126 were reported heterozygous for the same mutations. The data shown in Table 2 also revealed that ARMS-PCR could not characterize 13 individuals (9.59%) while 9 individuals (3.42%) did not exhibit any mutation on the β-globulin gene. The HBB: c.126_129delCTTT mutation was observed to be significantly prevalent in both heterozygous and homozygous states. The results shown in Table 3 revealed that the occurrence of HBB: c.126_129delCTTT mutation was the highest (36.9 %) followed by HBB: c.27_28insG mutation (32 %), HBB: c.92+5G>C (21.8 %), and HBB: c.17_18delCT (9.3 %)within the studied area.

Table 2. Homozygous, Heterozygous, β-thal mutations, uncharacterized mutation & normal alleles were reported in the studied individuals from the sub-tribe Afridi of the Wazir tribe.

S. No	HbVar Nomenclature	Mutations	Number of homozygous individuals	Number of heterozygous individuals	Uncharacterized Patients	Number of homozygous normal individuals
1	HBB: c.126_129delCTTT	Codons 41/42 (– TTCT)	47	69		
2	HBB: c.27_28insG	FSC 8/9 (+G)	34	52	……	
3	HBB: c.92+5G>C	IVS-I-5(G>C)	17	29	…..	
4	HBB: c.17_18delCT	FSC-5 (–CT)	9	17	…..	
5		Uncharacterized patients	…..	----	15	
6		homozygous normal individuals				11
Total No			107	167	15	11

Table 3. The frequency of mutant alleles in the studied individuals from sub-tribe Afridi of the Wazir tribe

S. No.	HbVar	Mutations	Homozygous	Heterozygous	Total alleles (n)	Frequency (%)
1	HBB: c.126_129delCTTT	Codons 41/42 (– TTCT)	47	69	163	42.8
2	HBB: c.27_28insG	FSC 8/9 (+G)	34	52	120	31.5
3	HBB: c.92+5G>C	IVS-I-5(G>C)	17	29	63	16.5
4	HBB: c.17_18delCT	FSC-5 (–CT)	9	17	35	9.2
Total			107	167	381	100.00%

Molecular analysis for the Wazir tribe was interestingly different from close close-situated Marwat tribe and reported IVS-I-5 (G > C) to be the most recurrent followed by FSC-5 (–CT) among the six reported mutations. It is evident from Table 4 that out of 300 samples, 85 individuals were found Homozygous for two β-thalassemia mutations i.e. IVS-I-5 (G > C) and FSC-5 (–CT) while for the remaining two mutations only 24 individuals were found homozygous. The data shown in Table 4 also revealed that 14 individuals (4.7%) were un-characterized by ARMS-PCR while 13 (4.3%) did not show any mutation on the β-globulin gene. The percentage of HBB: c.92+5G>C mutation was seen as high both in hetero and homozygosity. The results shown in Table 5 revealed that the frequency of HBB: c.92+5G>C mutation was seen higher (43 %) followed by HBB: c.17_18delCT mutation (33 %), HBB: c.126_129delCTTT (15 %) and HBB: c.27_28insG (9 %) in studied area.

Table 4. Homozygous, Heterozygous, β-thal mutations, uncharacterized mutation & normal alleles were reported in the studied individuals from the sub-tribe Ahmadzai of the Wazir tribe.

S. No	HbVar	Mutations	homozygous individuals	heterozygous individuals	Uncharacterized Patient	homozygous normal individuals
1	HBB: c.92+5G>C	IVS-I-5(G>C)	48	69	-	-
2	HBB: c.17_18delCT	FSC-5 (–CT)	37	52	-	-
3	HBB: c.126_129delCTTT	Codons 41/42 (– TTCT)	15	27	-	-
4	HBB: c.27_28insG	FSC 8/9 (+G)	9	16	-	-
5	-	Uncharacterized	-	-	14	-
6	-	Normal	-	-	-	13
Total	-	-	109	164	14	13

Table 5. The frequency of mutant alleles in the studied individuals from the sub-tribe Ahmadzai of the Wazir tribe.

S. No.	HbVar Nomenclature	Mutations	Homozygous	Heterozygous	mutant alleles (n)	Frequency (%)
1	HBB: c.92+5G>C	IVS-I-5(G>C)	48	69	165	43
2	HBB: c.17_18delCT	FSC-5 (–CT)	37	52	126	33
3	HBB: c.126_129delCTTT	Codons 41/42 (–TTCT)	15	27	57	15
4	HBB: c.27_28insG	FSC 8/9 (+G)	9	16	34	9
Total			109	164	382	100.00%

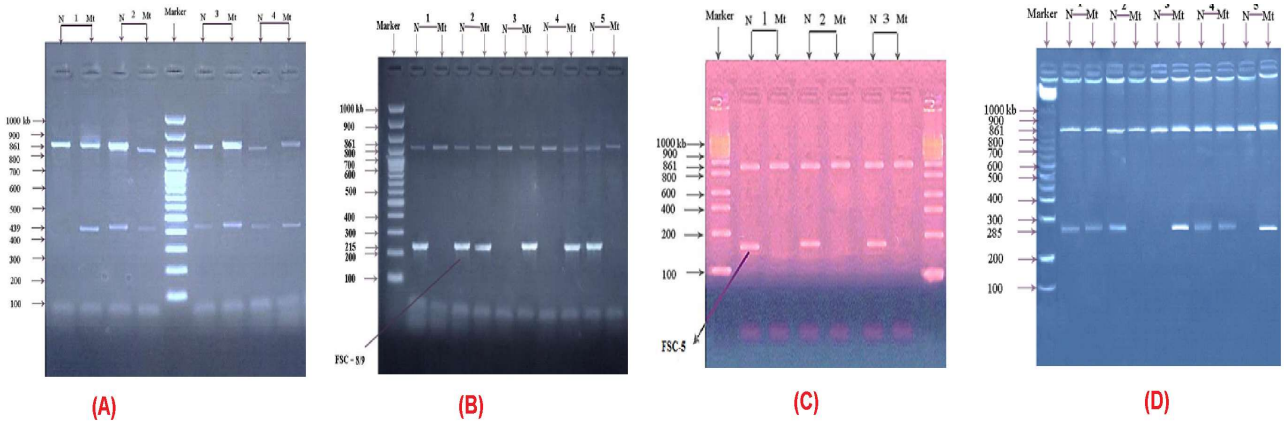

Figure 1: illustrates the ARMS PCR process and the subsequent electrophoresis analysis employed for detecting **(A)** Codons 41/42 mutation **(B)** FSC 8/9 mutation **(C)** FSC-5 mutation **(D)** IVS-I-5 mutation

4. Discussion

β-thalassemia is one of the most prominent and extensively studied genetic disorders globally, with approximately 80 million individuals affected and 23,000 new cases of major β-thalassemia reported annually [10]. Comparatively, in Pakistan, the prevalence is alarmingly high, with 5,000 thalassemia-affected births annually, accounting for 5–6% of all live births [11]. While nearly 400 β-thalassemia alleles have been identified worldwide, only about 40 mutations are responsible for 90% of global cases [12]. However, the mutation distribution varies significantly among ethnic groups, reflecting the genetic and cultural diversity across populations [13]. In the Asian subcontinent, over 40 β-thalassemia mutations have been identified, with Pakistan contributing to this diversity through 35 reported alterations. Notably, five mutations account for 90% of β-thalassemia cases in the country. These include HBB: c.27_28insG, HBB: c.92+5G>C, HBB: c.126_129delCTTT, HBB: c.92+1G>T, and HBB: c.17_18delCT [4]. Such genetic diversity underscores the importance of region-specific studies to understand mutation patterns better. Treatment strategies for β-thalassemia, such as blood transfusions, chelation therapy, bone marrow transplantation, and gene therapy, remain costly and insufficient to eradicate the disease. Pakistan introduced prenatal diagnostic (PND) facilities in 1994, allowing the detection of fetal β-

thalassemia mutations during pregnancy [15]. This has enabled parents to make informed decisions about continuing or terminating pregnancies in cases of homozygous or compound heterozygous mutations [16]. Techniques such as ARMS-PCR, utilizing allele-specific primers, have proven instrumental in facilitating prenatal diagnoses. Despite these advancements, the high prevalence of β-thalassemia in developing countries, including Pakistan, is attributed to limited public awareness, insufficient thalassemia centers, and the lack of premarital and prenatal screening programs [7,15]. This study identified four common mutations in the Afridi sub-tribe of the Wazir tribe, presenting a deviation from previous findings [17-19]. Notably, HBB: c.126_129delCTTT was the most prevalent mutation (38.35%) in the Marwat tribe, contrasting with prior reports from the Charsada and Kohat regions of Khyber Pakhtunkhwa (KPK), Pakistan [8, 20]. Among the Ahmadzai sub-tribes, IVS-I-5 (G>C) emerged as the most recurrent mutation, followed by FSC-5 (–CT). These findings reveal a distinct molecular heterogeneity within the Wazir tribe, despite the close geographical proximity, shared social structures, and cultural homogeneity of the Afridi and Ahmadzai sub-tribes. The observed differences in β-thalassemia mutation patterns between these closely related sub-tribes highlight the complex genetic architecture of the Wazir tribe, a population characterized by high consanguinity. Such molecular heterogeneity within a connected population is particularly intriguing and underscores the necessity for targeted interventions. These include large-scale mutation screenings, PND programs, and community-based parental counseling to mitigate disease recurrence. Furthermore, expanding these initiatives across the entire Pashtun ethnicity could provide valuable insights into the broader genetic landscape and improve disease management strategies for β-thalassemia.

5. Conclusions

This study explains the demarcation of molecular heterogeneity of Beta Thalassemia and elaborates that the distribution of mutations is not the same in adjacently situated tribes of the Pashtun population. While implementing programs like parental meetings about disease recurrence in the future, large-scale mutation screening, and PND in the Pashtoon population, these findings must be considered carefully.

6. Conflicts of Interest

The authors declare no conflicts of interest.

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