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DEMOGRAPHICS AND SPECTRUM OF BETA GLOBIN GENE MUTATIONS IN TRANSFUSION DEPENDENT β-THALASSEMIA PATIENTS TREATED WITH LOW-DOSE THALIDOMIDE

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Abstract

Background: β-thalassemia is a significant public health problem in Pakistan with higher carrier rate and disease frequencies. Its mutation prevalence is highly geographical and ethnicity dependent.
Objective: To investigate the prevalence of genetic mutations and demographics in transfusion-dependent β-thalassemia (TDT) patients in Pakistani communities.
Methodology: Samples were gathered from TDT patients who had been diagnosed and met the inclusion criteria. The SPPS 27 was applied to analyze comprehensive patient demographics, clinical history, and prognostic data. For detection of genetic mutations in TDT patients' DNA extraction and polymerase chain reaction (PCR) were applied using the relevant screening protocols.
Results: A total of 384 patients of TDT were screened from almost 79 various ethnic castes of Pakistan. The Cd 8-9 (+G) mutation was discovered to have the highest number of mutations followed by IVS 1-5. In demographics, Dera Ghazi Khan led in the disease prevalence, followed by Peshawar, Malakand, and Kohat divisions. The areas that have a remarkably high Excellent and Good (Ex+G) responders were Rawalpindi, Multan, Faisalabad, Gujrat, Sukkur, Shaheed Benazirabad, and Merged Districts (Fata). The areas that have comparatively poor Ex+G responders (≤50%) were Sargodha, Sahiwal, Loralai, Pasheen, and Gilgit-Baltistan.
Conclusion: Our findings determined the prevalence of genetic mutations and demographics of TDT patients in the diverse community of Pakistan with significant response to low-dose thalidomide in most of the Pakistani communities. For precision medicine and further ensuring the safety and efficacy of low-dose thalidomide in TDT patients, multicenter and large scale clinical trials with Bayesian approach are recommended.

Key words: Mutations, β-thalassemia, thalidomide, demographics, responders, precision medicine

1. Introduction

A β-globin gene has more than 200 different mutations that produce β-thalassemia, a category of hereditary illnesses characterized by variable clinical presentation due to impaired or missing synthesis of the β-globin chain [1]. The prominent feature of a disease in β-thalassemia is an inefficient erythropoiesis that leads to erythroid precursors late-stage apoptosis, compensates haemopoietic expansion, chronic hemolytic anemia, increased iron absorption, and hypercoagulability [2]. Current guidelines have standardized a clinical categorization of thalassemia syndromes according to the severity of the disease as measured by the patient's transfusion needs. Patients with TDT require regular blood transfusions for the rest of their lives because they develop severe anemia as early as 6 months of age. Although occasional transfusions may be necessary, people with non-transfusion-dependent thalassemia (NTDT) often keep their hemoglobin (Hb) levels between 7 and 10 g/dL. As a result of hepcidin suppression leading to erythron enlargement and increased duodenal iron absorption, these people acquire clinically substantial iron overload [3]. So, the TDT and NTDT are therefore determined by the degree to which α and β globin ratio is out of balance, which can be brought on by a range of homozygous and heterozygous state mutations.

Mutations in β-globin gene are amongst the common causes of genetic disorders in humans. So far, hundreds of mutations have been reported in humans that cause β-thalassemia [4]. β-globin gene mutations like IVS 1-1 (G–T), IVS 1-5 (G–C), Fr 41-42 (–TTCT), Fr 8-9 (+G), del 619, IVSII-1 (G–A), Cd 30, Cd 15 (G–A), Cd 5 (–CT), Fr 16 (-C) and CAP+1 have been identified as most common genetic mutations [5-7] and single nucleotide polymorphisms (SNPs) of Xmn1, HBG2, BCL11A & HBSIL-MYB as most common genetic modifiers in Pakistani population [8].

The frequency of associated mutations in β-thalassemia exhibits tremendous geographical and ethnic variations. The Middle East and Mediterranean area, as well as several Asian countries, have the greatest incidence, with certain communities claiming rates of up to 10% [9]. Carriers of the mutation are usually asymptomatic or may have moderate anaemia in these locations, but individuals with β-thalassemia major require various medical treatments and regular blood transfusions to control their condition. In Asia, the key nations with the highest frequency of mutations among TDT patients are India, Pakistan and Thailand. In India, the carrier rate for β-thalassemia ranges between 3 and 17 percent, with a significant incidence of roughly 1 in 10,000 live births [10]. In Pakistan, the incidence of TDT is estimated to be 1 in every 5,000 live births. This is greater than the global rate of 1 in 100,000 live births [11].

In the current study, we have determined the prevalence of genetic mutations and demographics of TDT patients in the diverse community of Pakistan. We were also interested to investigate the efficacy of low-dose Thalidomide therapy in various common ethnicity of Pakistan community and further categorized based on the provincial, divisional as well as district level to evaluate the clinical evidence for logical practice of thalidomide in TDT patients.

2. Materials and Methods

The current retrospective study was performed in the Blood Diseases Clinic, Peshawar and Institute of Pathology and Diagnostic Medicine (IPDM), Khyber Medical University, Peshawar. Total 384 patients were examined for eligibility from September 2021 to August 2022.

Sample selection

Blood samples were taken from all eligible participants who met the study's inclusion and exclusion criteria. Male and female TDT patients aged 3 years or older and who have been on thalidomide for at least 6 months participated in the study. Active metabolic or systemic co-morbidities, as well as those missing integral clinical characteristics, were not included in the analysis. Information from patients who did not comply was also left out of the total count.

Data Collection Procedure

Initial requirements for sample collection

Informed consent was taken from the parents/guardians. Samples were collected from the Blood Diseases Clinic (BDC), Peshawar and transported to the laboratory with standard protocols being strictly observed. For samples collection, the diagnosed cases of TDT patients with ≥3 years of age (both genders), who received thalidomide for a period of ≥6 months and on low-dose thalidomide therapy were conveniently selected. Non-compliant patients were excluded from this study (n=384).

Clinical Assessment

Participants' demographic and clinico-hematological information was compiled in a descriptive way. After doing a thorough clinical examination, a doctor filled out the proforma for each participant. Blood transfusion and low-dose thalidomide therapy were documented in full details. Patients who took thalidomide for TDT and were able to remain transfusion-free for at least two months, maintaining Hb levels of ≥9.0 g/dl were classified as Excellent Responders (ExR), while those who did so while maintaining Hb levels between 7.0 and 8.9 g/dl were classified as Good Responders (GR), those who did so while maintaining Hb levels between 6.0 and 6.9 g/dl were classified as Partial Responders (PR), and those who showed no significant improvement were classified as Non-Responders (NR). SPPS 27 was used to analyze the recorded data.

Sample Collection

Five milliliters of venous blood was collected from each patient. Two milliliters of blood was placed in a purple-top EDTA tube, and three milliliters was placed in a Gel tube for further biochemical analysis. Biochemical assays were conducted on the Cobas 6000 analyzer series, and the CBC was done on an automated hematological analyzer (Sysmex XP-100, Japan).

Genetic Assessment

A 2 ml venous blood was collected in an EDTA containing glass tube for genetic assessment. By using the GeneJET Genomic DNA purification kit (Thermo Scientific, USA), DNA was extracted as per the manufacturer's SOPs. The integrity of DNA was determined by gel electrophoresis. The DNA fragment was stored at -20^{o}C. The mutations associated with β-thalassemia were investigated using multiplex amplification refractory mutation system (MARMS) [12]. In the first step, we set to assess the identification and screening of the commonest mutations that are prevalent in our population (IVS1-1 (G–T), IVS1-5 (G–C), Cd 8-9 (+G), Fr 41-42 (–TTCT) and del 619). Then we proceeded to identify and screen rare mutations that were IVSII-1 (G–A), Cd 30, Cd 15 (G–A), Cd 5 (–CT), Fr 16 (-C), and CAP+1.

Three multiplex primer combinations were set to use with a 5 pM/μL of final concentration for each primer. Finally, to the primer mix, we added the 02 control primers and their respective standard primers. PCR products, control strain, and marker (ladder) were subjected to electrophoresis. The Gel system was used to visualize the bands.

PCR mix was made by mixing 1X PCR-buffer (10 μL), primer mixes 01, 02 and 03 (1 μL each), Taq, polymerase (0.2 μL), and genomic DNA (2.5μL). This mixture was then pipetted into PCR tubes, which was processed in a Thermocycler. The amplified product was stored at 4˚C after 25 cycles. XmnI, genotyping (-158 γG Polymorphism) was carried out using restriction fragment length polymorphism (RFLP-PCR) [13]. ARMS PCR was used for the detection of "Single nucleotide polymorphism, (SNP)" of BCL11A (rs11886868, rs4671393, rs1427407, and rs766432), and HBG2 (rs7482144) [14-16].

Results

Patient Characteristics

The reported cases were ranged in the age from 3 to 24 years, with the median being 5 years old. There were around 62 percent male and 38 percent female cases. The range of weight was 8-65 kg, with the median being 17.5 kg. A median duration of treatment with thalidomide happened to be 19.2 months (range: 6-58), and the mean thalidomide dose was 2.1±0.6 mg/kg/day. The ages ranging from the initial transfusion were 6 months to 108 months. At the time of evaluation, the interval for the last transfusion varied between 2 and 63 months, with 14 months being the median.

Demographic and Ethnic Diversity in Pakistani Population

In our study, 42 (11%) patients were from Dera Ghazi Khan Division, 39 (10%) were from Peshawar and Malakand division, and 36 (9%) were from Kohat division. Dera Ismail Khan, Rawalpindi, Mardan, Azad Kashmir, and Multan were 25 (7%), 22 (6%), 22 (6%), 20 (5%), and 18 (4%), respectively. Quetta, Merged Districts (Fata), Hazara and Bannu divisions were 13 (3%), each. Current study observed that Balochi and Yousafzai communities were 9% and 6%, Afridi, Wazir, Awan, Utmanzai and Khattak were 4% each. Mohmand, Bangash, Rajpot, and Bhitani were 3% each, while Khalil, Swati, Arab, Bannusi, Syed, Qureshi and Sheikh community were 2% each. Rest of the ethnic castes were 1% or under 1% of the total demographic distribution in the current study.

Mutation Prevalence

A total of 384 TDT screened patients from almost 79 various ethnic castes of Pakistan were evaluated in this study. The Cd 8-9 (+G) mutation was observed to have the highest number of mutations which were 44.4%, followed by IVS 1-5 with 23.6%, Cd-5 with 11.2%, Fr 41-42, 7.3% and Cd 15 with 5.2%. Fr 16-C, Cap+ 1, del 619 and Alpha 3.7 kb del were also identified but less frequently. We also identified Xmn1 (13.3%), BCL11A (6%), and HBG2 (5.5%) as potential genetic modifiers in the study population of Pakistani community. The prevalent mutations and genetic modifiers in the study population are presented in **Table 1**.

Table 1: Frequency of the prevalent mutations and genetic modifiers in the study population

		n (%)
Prevalent Mutations	Cd 8-9 (+G)	207 (44.4%)
	Fr 41-42	34 (7.3%)
	IVS1-5	110 (23.6%)
	Fr 16-C	12 (2.6%)
	Cd 30	9 (1.9%)
	Cd 15 G-A	20 (4.3%)
	Cd-5 (-CT)	52 (11.2%)
	Cap+ 1	6 (1.3%)
	del 619	12 (2.6%)
	Alpha 3.7 kb del	4 (0.8%)
Genetic Modifiers	Xmn1	51 (13.3%)
	BCL11A	23 (6%)
	HBG2	21 (5.5%)

Based on the demographics, the prevalent mutations were identified in provincial and divisional data. In provincial data, Cd 8-9 (+G) was more prevalent in Khyber Pakhtunkhwa province (n=80), followed by Punjab (39) and Fata (10) while at a divisional level, Peshawar (19), Kohat (18) and Malakand (17) were the highest amongst cases. In the case of 2nd most prevalent mutation IVS1-5, Punjab province marked (43) cases followed by Khyber Pakhtunkhwa (21), while Dera Ghazi Khan

(21), Azad Kashmir (9) and Multan marked (8) cases at the divisional level. The prevalence of provincial and division-wise identified mutations is shown in **Figure 1a & 1b** respectively.

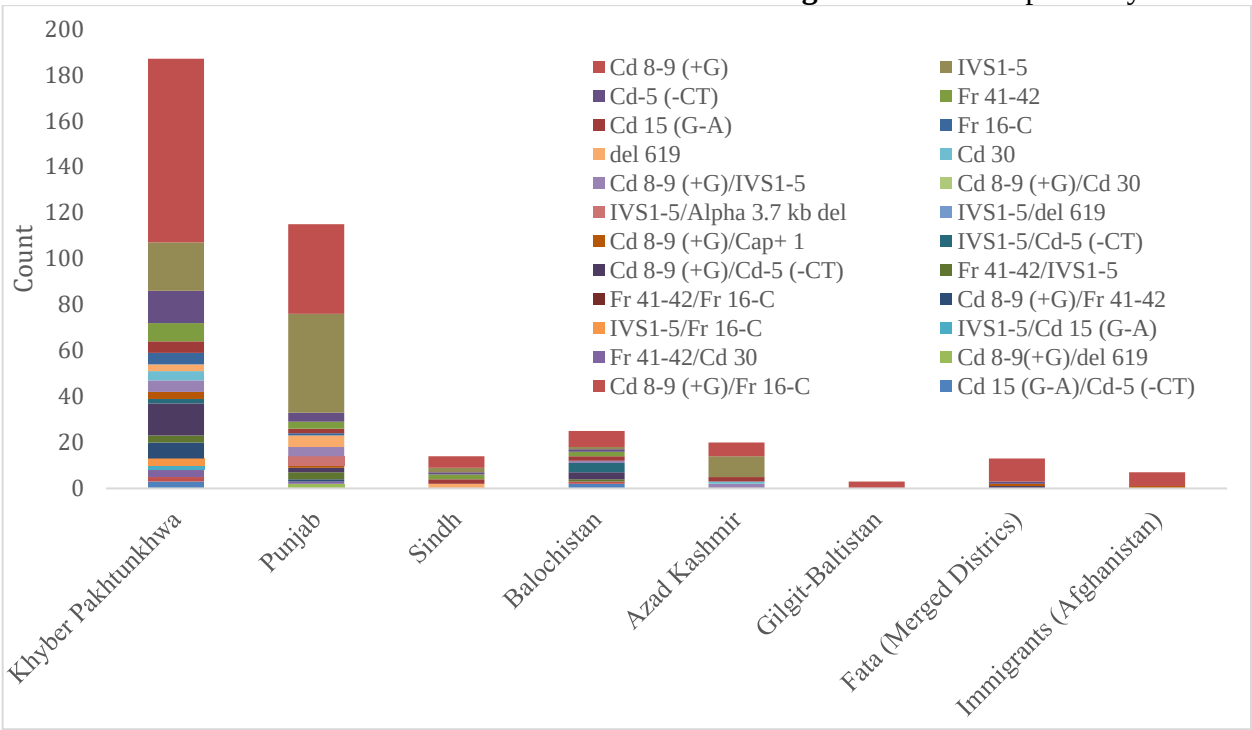

Figure 1a: Provincial-based Prevalence of Genetic Mutations in Pakistani Population

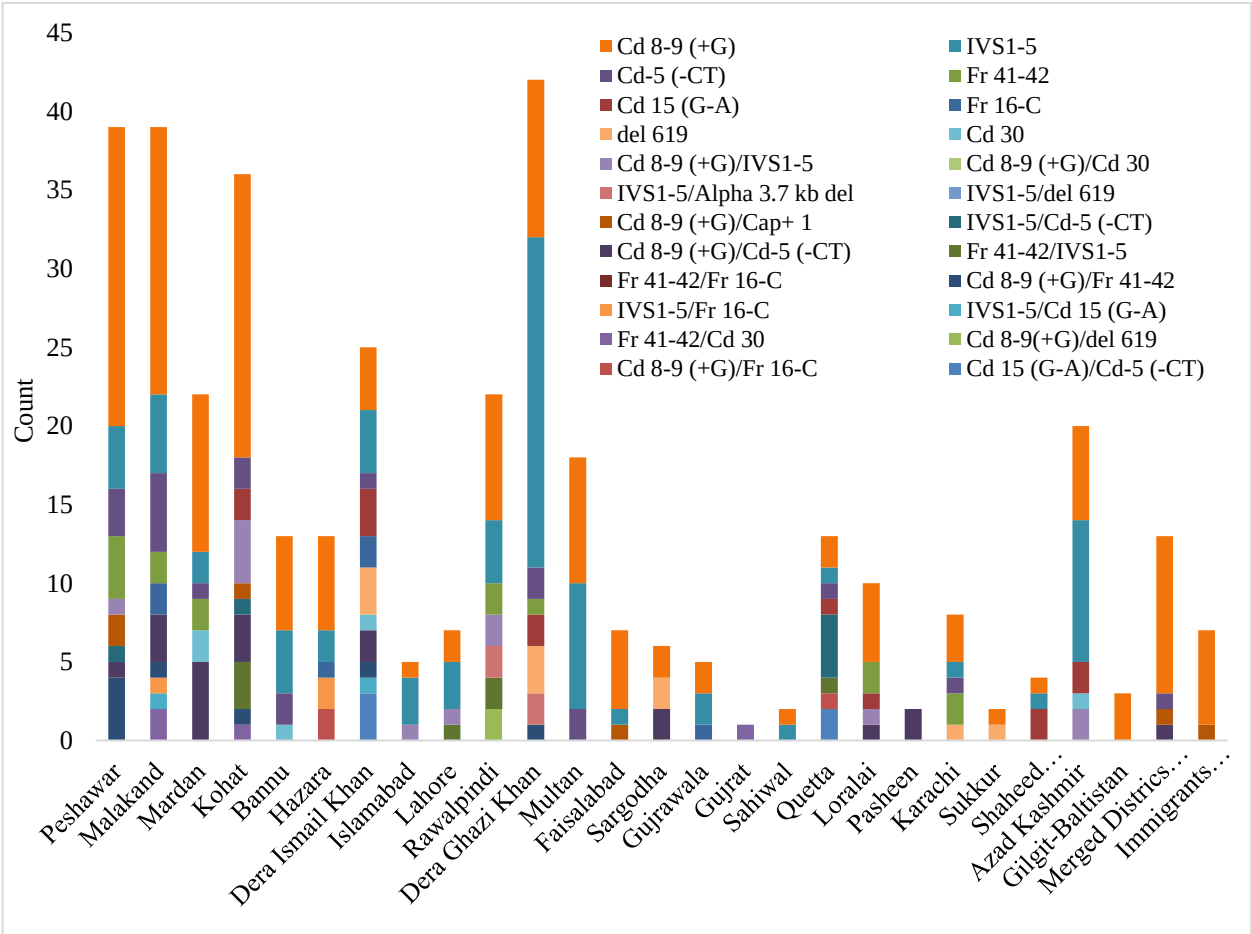

Figure 1b: Divisional-based Prevalence of Genetic Mutations in Pakistani Population

Phenotypic Response- Thalidomide Efficacy

Using the Hb level as our thalidomide response criteria, we observed that 184 patients (47.9%) were Excellent Responders, 96 patients (25%) and 60 patients (15.6%) were categorized as Good and Partial Responder respectively, whereas 44 patients (11.5%) were Non-Responders. **Figure 2** reflects a divisional-based thalidomide response for phenotypic description of the study population.

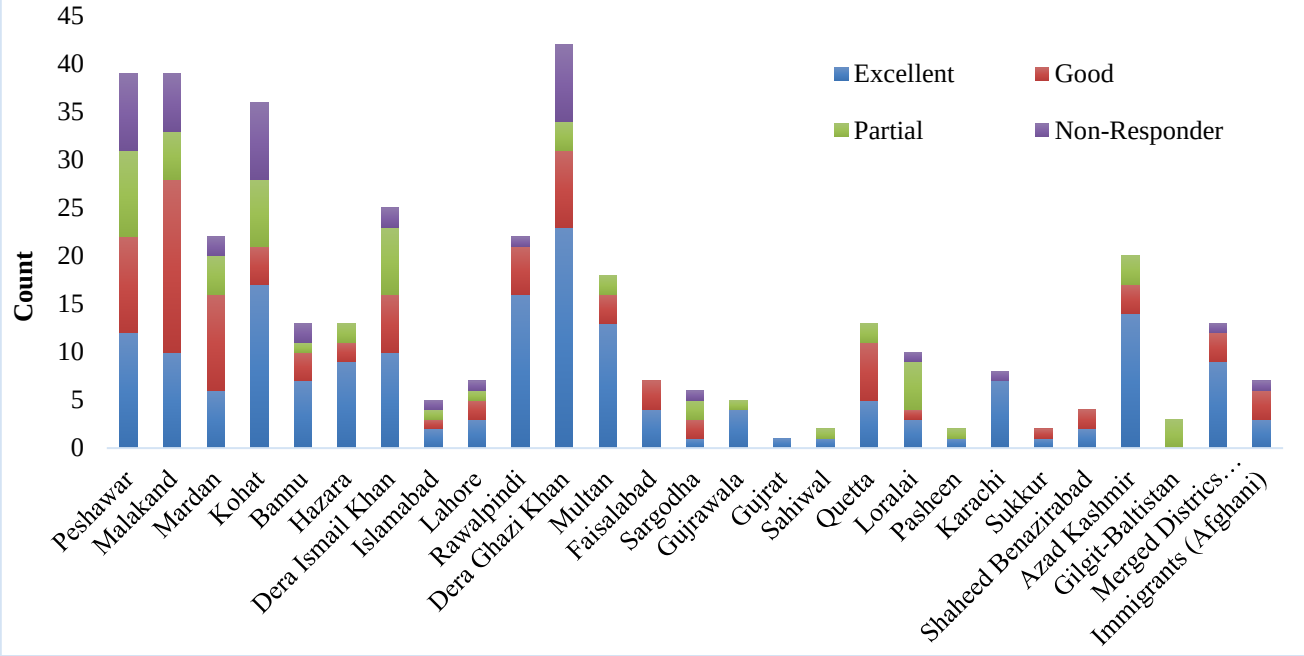

Figure 2: Phenotypic Description for Thalidomide Response in Pakistan (Divisional Data).

Division based demographic communities with merged districts and immigrants that have more than 80% Excellent and Good (ExR+GR) responders included Hazara, Rawalpindi, Multan, Faisalabad, Gujrawala, Gujrat, Quetta, Karachi, Sukkur, Shaheed Benazirabad, Azad Kashmir, Merged Districts (Fata) and Immigrants (Afghani). Although some of these communities had a lower number of cases but still, we need to mark these locations to further investigate them for a larger population size. **Figure 3** showing the division based communities with merged districts and immigrants having clinically significant high Excellent and Good responders to thalidomide therapy.

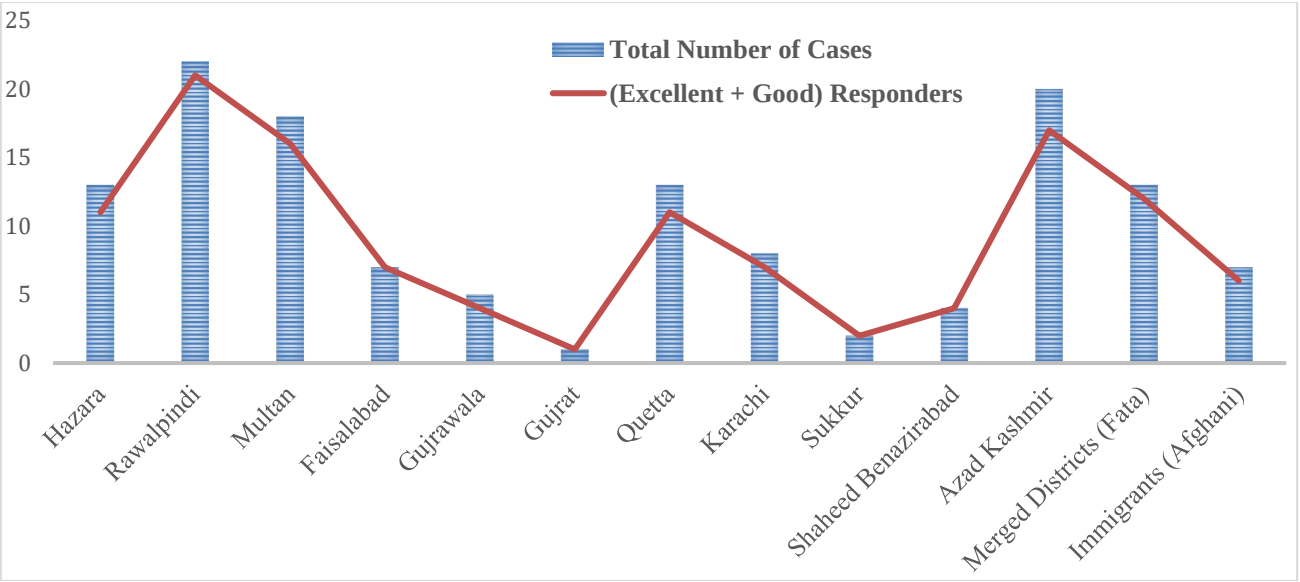

Figure 3: Division based communities with merged districts and immigrants having clinically significant high Excellent and Good Responders (≥85%)

Divisional based communities having less than 80% Excellent and Good responders included Peshawar, Malakand, Mardan, Kohat, Lahore, Dera Ismail Khan, Islamabad, Dera Ghazi Khan, Sargodha, Bannu, Sahiwal, Loralai, Pasheen, and Gilgit-Baltistan. The areas that have comparatively poor E+G responders (≤50%) were Sargodha, Sahiwal, Loralai, Pasheen, and Gilgit-Baltistan. Although some of these had a lower number of cases but still, we need to mark these locations to further investigate them for a larger population size. The comparatively low Excellent and Good responders to thalidomide therapy are presented in **Figure 4**.

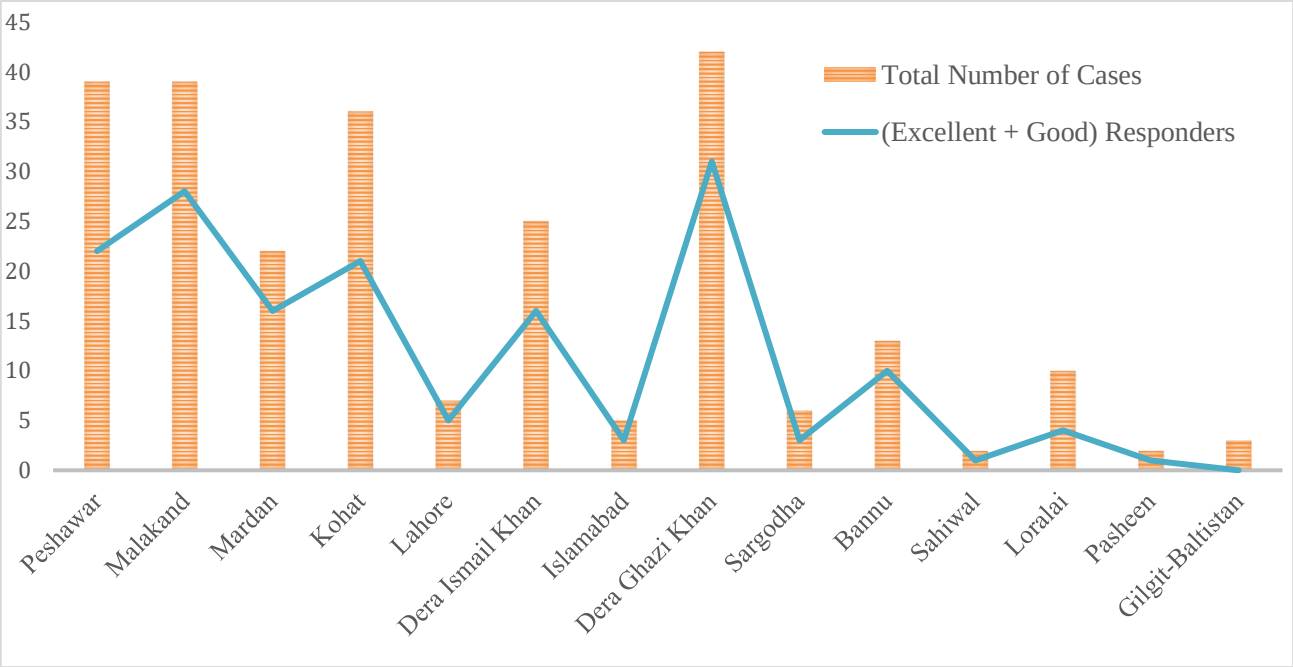

Figure 4: Divisional based communities having comparatively low Excellent and Good Responders to thalidomide therapy.

The Pakistani castes that's have 100% Excellent and Good responders to low-dose thalidomide therapy were Qureshi, Sheikh, Khattak, Peeran, Gujjar, Arab, Malak, Nasari, Qadar Khel, Kundi, Wazir, Mufti, Afghani, Painda Kheli, Rajpoot, Shinwari, Sindhi, Naurang Khel, Salarzai, Sikhani, Jadoon, Zakha Khel, Orakzai, Kakar, Jatt, Dogar, Wardaq, Arain, Borana, Bhatta, Mohana, Mahsood, Gigiani, Channa, Kubahir, Esa Kheli, Toori, Barki, Momzai, Bhutta, Tarkan, Kumhar, Sabari, Dhobian, Dawi, and Kashmiri.
Jatoi Baloch, Ahmed Zai, Wihar, Lodhi, Dawar, Giyal, Kaisrani, Katany, Chandia, and Mulian were castes that recorded Zero percent cases falling in ExR+GR, while Mohmand, Baasait, Tanoli, Degaan, Malana, and Kumhar were amongst those who had ≤50% of Exr+GR to low-dose thalidomide therapy.

2. Discussion

Pakistan is the country where there are several tribes and people from different creeds reside. Major ethno-linguistic groups of Pakistan are Punjabis, Pashtuns, Sindhis, Balochs, Muhajirs, Saraikis, Paharis and Brahuis. Besides, Pakistan is a country consisting of geographical provinces having complex ethnic diversity. A variety of mutations contribute to β-Thallasaemia, which causes a quantitative decrease in β-globin chains with healthy structural characteristics [17]. Although phenotypic presentation does not always represent genotype, yet the information from the extended genetic study may be utilized to plan proper care, offer sufficient genetic counseling, and perhaps even explore new potential targets for therapeutic intervention [16]. In the current study, Cd 8-9 (+G) mutation was discovered to have the highest number of mutations (207, 44.4%) in TDT patients followed by IVS 1-5 (23.6%), Cd-5 (11.2%), Fr 41-42 (7.3%) and Cd 15 (5.2%). Fr 16-C,

Cap+ 1, del 619 and Alpha 3.7 kb del were also identified but less frequently. Xmn1 (51, 13.3%) was the leading genetic modifier followed by BCL11A (23, 6%), and HBG2 (21, 5.5%) prevalent in the study population of Pakistani community. The prevalence of identified mutations is almost similar to the previous published data reporting the β-globin mutations with varying number of cases and geographical location of the Pakistani community [6, 18-23].

According to recent clinical research, an upsurge in keen interest for HbF inducers among doctors has been observed, and thalidomide has been investigated as a potentially useful medicine in the treatment of β-thalassemia. Patients with β-thalassemia in many different parts of the world have been the focus of several clinical studies establishing the usage, efficacy, and safety of thalidomide [24-32, 40]. Quality of life and clinical symptoms for people with β-thalassemia may improve with the use of thalidomide, a powerful HbF inducer, as reported in several retrospective research studies [28, 32-34]. According to Chen et al., [32] patients with TDT who enrolled in a multicenter, randomized, phase-II clinical study of thalidomide's safety and effectiveness had significant increases in Hb concentrations and significantly decline in RBC transfusions. The Hb of the patients was maintained at >105 g/L after 48 weeks of thalidomide medication without the need for red blood cell transfusion, and no major side effects (grades III-IV ADRs) were seen, proving the drug's safety.

The Bayesian technique is a specific method for posing and solving such issues. When applied to health care choices, it shows significant potential in making assumptions more transparent and conclusions simpler to explain and defend [35]. Many clinical decisions can benefit from this method, including those involving diagnosis, prognosis, and risk assessment [36, 37]. In order to assist clinical decision-makers in making the optimal medicine choice out of the available possibilities, we have proposed the Bayesian logarithm in β-thalassemia geographic data. The two main factors identified for the restricted application of Bayesian statistics are a lack of understanding of Bayesian techniques and a lack of clarification or advice from the authorities [38].

A Bayesian approach has been applied to the statistical data for the diagnosis of β-Thalassemia in several research studies [38, 39], but lack of evidence that apply such approaches in the treatment of TDT patients. Since, the analysis of genetic mutations in TDT patients is not a common practice in low to middle income countries due to lack of awareness and resources therefore, this current study was initiated to attract the attention of hematologists towards the significance of genetic testing in TDT and the variable effects of HbF inducers like thalidomide on the outcome of TDT patients in the context of prevalent mutations in the diversely distributed Pakistani population on the basis of ethnicity and demographics. In our study Cd 8-9 (+G) mutation happened to be the most prevalent in ethnic castes like Afridi (4.5%), Bangash (3.5%), Wazir (3.5%), and Awan (3%). The IVS 1-5 was most prevalent in ethnic casts like Balochi (12%) then Arab and Khattak (3.7%, both). The gene modifier Xmn1 was most prevalent in Arab, Wazir and Salarzai communities (11.1%, each).

This is the first type of study that categorized the β-thalassemia patients into districts and divisional level of Pakistani population with larger sample size. Data showed that our study represented the highest number of patients for Dera Ghazi Khan Division, followed by Peshawar, Malakand and Kohat divisions. As far as phenotypic response in districts level is concerned, we observed that Rawalpindi, Multan, Hazara, Karachi, Quetta, Azad Kashmir, Faisalabad, Gujrawala, Gujrat, Shaheed Benazirabad, Merged Districts (FATA), and Afghani (Immigrants) marked ≥85% of Excellent and Good responders which clearly indicates the best possible efficacy of Thalidomide in the selected Pakistani communities.

3. Conclusion

Geographic and ethnic distribution has a significant role in determining the prevalence of β-thalassemia and its variations in Pakistan. The Cd 8-9 (+G) mutation was discovered to have the highest number of mutations followed by IVS 1-5. Among ethnic diversity, Balochi and Yousafzai were the communities that contributed higher prevalence rate of TDT. Based on the demographic based prevalence, the highest TDT patients were from Dera Ghazi Khan district, which was

followed by Peshawar and Malakand division. Our results indicated that low-dose thalidomide significantly improved TDT patient outcomes, with a large percentage of respondents falling into the excellent or good responder group. Based on demographic criteria, this study will draw physicians' attention to the effectiveness of thalidomide in TDT patients. For precision medicine and to increase the effectiveness and safety of low-dose thalidomide and to give therapeutic guidance for its responsible use in TDT patients, multicenter, larger-scale clinical studies employing a Bayesian methodology are advised.

Acknowledgement
We are thankful to the consultants at the Blood Diseases Clinic and Khyber Medical University, Peshawar for allowing us free access to clinical data and patient blood sampling after acquiring informed consent and for helping us carry out our research study.

Conflict of Interest
The authors claim to be aware that the research presented in this work is not impacted by any financial or personal conflicts.

Ethical statement
The Institutional Research and Ethical Review Board (IREB) of Khyber Medical College, Peshawar, Pakistan, reviewed the study protocol before approving it under reference number 926/DME/KMC. Thorough history taking and blood drawing were performed after taking informed consent from the patient's father/guardian.

Authors' contribution
Muhammad Idrees conceptualized the study, conducted research work and prepared original draft under the supervision of Yasar M Yousafzai. Muhammad T M Khan, Asif Ali and Inayat U Rahman assisted in data collection, Sstatistical analysis and methodology, respectively.

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