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ORIGINAL ARTICLE

BETA-THALASSEMIA MUTATIONS IN PUNJAB, PAKISTAN (2016-2022), HIGHLIGHTING RARE VARIANTS DURING COVID-19 (2019-2020)

Shahida Parveen[1], Amna Aziz[2], Yasmeen Ehsan[3],Daniyal Warraich[4],Rubaida Mehmood[5]

[1]Associate Professor, Department Gynecology& Obs , Ward no 17, Nishtar Medical University & Hospital, Multan, Punjab
[2]Assistant Professor, Department Gynecology& Obs,. Ward no 17, Nishtar Medical University & Hospital, Multan, Punjab
[3]Additional Director, Punjab Thalassemia Prevention Programme Fatima Jinnah Medical University, Lahore,Punjab
[4]MBBS, USMLE Student. Sahiwal Medical College Pakistan. Sahiwal, Punjab
[5]Principal Scientist, Head, Department of Diagnostics Labs, MINAR Cancer Hospital, Multan, Punjab

Correspondence:
Rubaida Mehmood
Email: rubaidasana@gmail.com

Abstract

Background: Beta-thalassemia is one of the most common inherited hemoglobin disorders in Pakistan, with a heterogeneous mutation spectrum that varies across different geographic regions, making region-specific mutation data essential for accurate prenatal diagnosis and genetic counseling.

Objective: This study evaluated the frequency, regional diversity, and rare beta-thalassemia mutations in Punjab, Pakistan, during 2016–2022, emphasizing implications for prenatal diagnosis, genetic counseling, and possible COVID-19-related temporal variations.

Material and Methods: A retrospective study was performed from 2016–2022 at Nishtar Hospital Multan. Chorionic villus samples from 1000 pregnant women with gestational ages of 12–14 weeks undergoing prenatal diagnosis for beta-thalassemia were collected along with parental blood samples. Samples were analyzed at the Punjab Thalassemia and Other Genetic Disorders Prevention and Research Institute, Lahore, for 17 beta-thalassemia mutations using PCR-based molecular techniques. Genomic DNA extraction and beta-globin gene amplification were performed. Descriptive statistics and chi-square analysis were applied to compare mutation frequencies across years and demographic groups.

Results: IVS 1-5 (G-C) was the most prevalent mutation (39.06%), followed by Fr 8-9 (+G) (30.94%) and Cd-15 (G-A) (6.6%). Rare mutations included IVS1-1 (G-T) (2.8%), Exon mutation (1.1%), and Cap+1 (A-C) (0.8%). Rare variants including HbS, HbD, Cd-16, and Exon mutations appeared intermittently during 2016–2020. Exon mutation frequency increased during late 2019–2020, coinciding temporally with the COVID-19 pandemic period; however, no causal association could be established. Mutations were most common in women aged 25–29 years and gravida three or more.

Conclusion: The study revealed significant regional variation in common and rare beta-thalassemia mutations in South Punjab, supporting improved prenatal diagnosis, targeted mutation screening, and genetic counseling, while further multicenter studies are needed to explore possible COVID-19-related associations.

Keywords: Chorionic villus sampling; beta-thalassemia; spectrum of mutations; prenatal diagnosis; COVID-19.
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Introduction:

Thalassemia inherited autosomal recessive disorders in which reduced synthesis of one or more goblin chains. It results in partial or complete suppression of the production of normal hemoglobin[1].There are four clinical types of Beta Thalassemia, two of them named as Carriers and Beta Thalassemia minor, which do not show any symptoms and are called silent types. The remaining two types are Beta Thalassemia major and intermedia, which require medical attention that needs blood transfusion for survival[2]. It shows a diversity of medical appearance, starting from asymptomatic to severe blood transfusion dependence. It is commonly found among populations in all Mediterranean countries, as well as in Southeast Asia, India, Africa, Central America and the Middle East.

Beta Thalassemia is a well-known monogenic disorder prevalent in more than 60 countries and affecting about 5% of the world population. Thalassemia Carrier rate is 5 to 8 % in Pakistan, posing an increase burden for health-services. However, it is estimated that 5,000-

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9,000 children with thalassemia are born per year. Almost 9.8 million carriers are estimated in wide-ranging[3]. Consanguineous marriages, illiteracy and lack of awareness are major factors leading to high carrier ratio. Consanguinity significantly increases the probability of homozygosity for β-thalassemia mutations, thereby amplifying the expression of severe phenotypes such as β-thalassemia major within families and restricting the genetic diversity of mutation patterns observed in affected populations. Socioeconomic constraints, including limited access to premarital screening and genetic counseling, further contribute to the persistence and intergenerational transmission of pathogenic variants. World Health Organization says that effective screening program should be initiated, if the birth rate of affected infants exceed0.1/1000 for any disease[4].

It is a big misfortune that Pakistan is lacking a uniform national policy to prevent thalassemia. However, Punjab Thalassemia Prevention Program (PTPP) is only project in Punjab funded by the Government since 2009. At PTPP, many services are available including prenatal diagnosis, extended family screening, mutation analysis and premarital screening for beta thalassemia[5]. People can barely afford the expensive treatment of this disease.

So, for a third world country prevention program is more economical to combat the disease and control the affected births. Pakistan started its clinical services for prenatal diagnosis in 1994 in Lahore. Now this facility is being offered in Rawalpindi, Lahore and Karachi, Bahawalpur and Multan[6].Prenatal diagnosis become more rapid and reliable after the introduction of chorionic villus sampling (CVS)along with PCR-based technique, i.e. amplification refractory mutation system-polymerase chain reaction(ARMS-PCR) at early stage of pregnancy[7]. Single-strand conformation polymorphism and gene sequencing are more reliable tool to detect rare mutations.

Almost more than 200 mutations are reported in the HBB gene (hemoglobin subunit beta) that are associated with β-thalassemia according to the Human Gene Mutation Database (HGMD®). Mutations that cause absence of β-globin chains production are nonsense, missense, initiation codon, frameshift and splice-site junction mutations; resulting in β0thalassemia.Mutations that results in reduction in β-globin chains leading to β+-thalassemia, are in 5'/3'-UTRs and in the promoter region[8].Twenty one beta-globin gene mutations are spotted in Pakistan. Among them three-point mutations frameshift codon (FSC) 8/9, intervening sequence (IVS) 1-5 and Cd-41/42 appear in almost 86% of cases.

In Punjab, particularly in the central and southern regions, the most frequently reported mutations include IVS-I-5 (G>C), CD-41/42 (-TTCT), and FSC 8/9, while IVS-I-1 and CD-15 mutations are also observed at lower frequencies[9]. This regional distribution highlights a strong founder effect and supports the need for population-specific mutation panels for efficient prenatal diagnosis and cost-effective screening strategies.

The beta-globin gene has three exons and two intervening sequences (IVS). There is geographic diversity in the prevalence of these mutations. Globally IVS-I is most susceptible to mutations followed by exon 1 and exon 2, and IVS-II less frequent and exon 3 being the least common[10].

The prevalence of beta thalassemia mutations ranges from <1.0 to 16.0%, being 3.8% in South China, 1.5–3.0% in North Africa, 3.0–4.0% in India and 4–11.0% in Middle East countries. The Mediterranean countries also have high frequency of beta thalassemia, Cyprus has frequency of 17.2%, However, drop down to 12.1% in last two decade with the implementation of prenatal screening. Multan is Pakistan's 7th largestcity. As of 2017 census, Multan's population jumped to 1.871 million[11]. People from nearby divisions are referred in Nishtar hospital for prenatal diagnosis of Thalassemia. Targeted screening of beta -thalassemia gene mutation is required to reduce the cost burden.

Focusing on rare mutations in this region is particularly important because it enables identification of atypical or previously underreported alleles that may not be detected by standard mutation panels. This is crucial in Punjab, where population heterogeneity, endogamy, and inter-family marriages can introduce unique or low-frequency variants that influence disease severity and diagnostic accuracy. Inclusion of rare mutation profiling enhances the effectiveness of prenatal diagnosis programs and prevents misclassification of at-risk pregnancies.

This study aimed to describe the molecular characteristics of thalassemia in prenatal diagnosis (PND). It will help to determine the regional spectrum of beta Thalassemia mutations and identification of some rare mutations. So, we will have a targeted approach for the screening. No data on CVS is published yet from this Centre by Punjab thalassemia Prevention Program (PTPP).

Many countries around the world have been severely impacted by the coronavirus disease outbreak (COVID-19).SARSCoV2 infection poses unique challenges and risks to patients with hemoglobinopathies[12]. Globally, the prevalence of COVID-19 appears to be lower in thalassemia patients than in the general population. There was also a low prevalence and fatality rate of COVID-19 in countries with a high rate of hemoglobinopathy[13].A regression analysis revealed that the heterozygote population of beta thalassemia is related to immunity against COVID-19[14].

Edouard Lansiaux recently demonstrated that subjects from the Italian regions of Puglia, Sardinia, and Sicily have a high prevalence of Beta-thalassemia and, as a result, are more resistant to the coronavirus pneumonia known as SARSCoV2, (formerly known as 2019-nCoV), which causes COVID19 disease[14].

Material and Method:

Study Design and Setting: This retrospective cross-sectional study was conducted using prenatal diagnostic records from Nishtar Hospital, Multan, with molecular analysis performed at the Punjab Thalassemia and Other Genetic Disorders Prevention and Research Institute (PTPP), Lahore, Pakistan. The study evaluated the spectrum of β-thalassemia mutations among high-risk pregnancies referred for prenatal diagnosis from different divisions of Punjab.

Study Duration: The study was conducted over a six-year period from January 2016 to December 2022.

Ethical Considerations: The study was approved by the Institutional Ethics Committee of MINAR Cancer Hospital, Multan (Reference No. M-3(13)/2018). All study procedures were conducted in accordance with the ethical principles of the Declaration of Helsinki for research involving human participants. Written informed consent was obtained from all participating couples prior to sample collection, and the confidentiality of participant information was strictly maintained throughout the study.

Inclusion Criteria:

Pregnant women with a gestational age of 12–14 weeks undergoing prenatal diagnosis for β-thalassemia.

Couples in whom both partners were confirmed or suspected β-thalassemia carriers.

Cases with complete demographic, clinical, and molecular diagnostic records.

Chorionic villus samples and parental blood samples available for molecular analysis.

Exclusion Criteria:

Pregnant women beyond the recommended
Gestational age for CVS.

Cases with incomplete clinical or laboratory records.

Samples with inadequate DNA quality or unsuccessful molecular analysis.

Pregnancies in which informed consent was not obtained.

Sample Size and Sampling Technique:

A total of 1,000 chorionic villus samples (CVS) were collected from pregnant women with a gestational age of 12–14 weeks who met the study eligibility criteria and were referred for prenatal diagnosis of β-thalassemia between 2016 and 2022. The sample size comprised all eligible prenatal diagnostic cases received during the study period. A non-probability consecutive (convenience) sampling technique was employed, whereby all eligible high-risk pregnancies presenting for prenatal diagnosis were included after obtaining written informed consent. Peripheral blood samples from both parents were also collected in EDTA tubes[15] for molecular analysis and transported to the Punjab Thalassemia Prevention Programme Overall analysis of prenatal diagnostic data from 2016–2022 demonstrated that IVS 1-5 (G-C) remained the predominant β-thalassemia mutation in Southern Punjab, accounting for 39.06% of all detected (PTPP), Lahore, where DNA extraction and mutation analysis were performed. [19]

Selection of Mutations: A total of 17 β-globin gene mutations were selected for analysis. These mutations were chosen based on their previously reported high prevalence in Pakistan and South Punjab, as documented in regional molecular epidemiological studies and the PTPP mutation database. The selected panel primarily included common mutations (such as IVS-I-5 G>C, CD-41/42 -TTCT, FSC 8/9, CD-15 G>A, and others) responsible for the majority of β-thalassemia cases in the local population, ensuring cost-effective and targeted prenatal screening.

DNA Extraction and PCR Analysis: Genomic DNA was extracted from leukocytes of EDTA-anticoagulated maternal and paternal blood samples using either a standard phenol-chloroform method or a commercial silica-based extraction kit, following the manufacturer's instructions. Mutation analysis was performed using the Amplification Refractory Mutation System–Polymerase Chain Reaction (ARMS-PCR) technique. Each PCR reaction mixture consisted of template DNA, mutation-specific primers, deoxynucleotide triphosphates (dNTPs), Taq DNA polymerase, $MgCl_2$ and reaction buffer. The PCR amplification protocol included an initial denaturation step at 95°C for 5 minutes, followed by 30–35 cycles of denaturation at 95°C for 30 seconds, annealing at 58–62°C (optimized according to each mutation-specific primer set) for 30–45 seconds, and extension at 72°C for 45 seconds, with a final extension step at 72°C for 5–7 minutes. The amplified PCR products were then separated by agarose gel electrophoresis (2–3%) stained with ethidium bromide or an alternative safer dye and visualized under UV transillumination to determine the presence or absence of specific β-thalassemia mutations.

Data Collection: A structured questionnaire was used to collect detailed information from the participating couples, including demographic characteristics, socioeconomic status, ethnicity, educational level, antenatal history, blood group, degree of consanguinity, and obstetric history.

Statistical Analysis: Data were analyzed using SPSS software (version 2022). Mutation frequencies were calculated as percentages. Associations between categorical variables such as consanguinity, ethnicity, and mutation type were assessed using the Chi-square test. Where appropriate, Fisher's exact test was applied. A p-value < 0.05 was considered statistically significant. Descriptive statistics were used to summarize demographic and clinical characteristics of the study population.

Results:

mutations, followed by Fr 8-9 (+G) (30.94%) and Cd-15 (G-A) (6.6%). Among the less frequent variants, IVS1-1 (G-T) constituted 2.8%, Del (619) 2.83%, Fr16 (-C) 2.26%, Exon mutation 1.13%, and Cap+1 (A-C) 0.75%. Rare mutations such as Hb D, Hb S, Cd-16, and neighboring regional populations; however, their occurrence in prenatal samples from Southern Punjab highlights their clinical relevance for genetic counseling and targeted mutation screening programs.

Year-wise analysis showed variation in mutation patterns across the study period. In 2016, IVS 1-5 (G-C) was the most common mutation (37.4%), while rare mutations included Cd-5 (-CT), Hb D, and IVS-1-1 (G-A), each occurring at low frequency (0.7%). In 2017, IVS 1-5 (G-C) further increased to 45.5%, with Hb D and Fr 41/42 (-TCTT) identified as rare variants. In 2018, Fr 8-9 (+G) became the dominant mutation (36.7%), and Cd-16 appeared as a rare mutation. No Exon mutation or Hb S cases were detected during 2016–2018(Table 1).

Table No 1:Year wise data of beta thalassemia mutations in different divisions of southern Punjab(Pakistan)

							DG Khan Division										
Year	Fr89 (+ G)	IVS 1-5 (GC)	Del (619)	Cd 30 (GC)	Cd 30(GA)	Fr 4142(-CTT	Fr 16 (-C)	Cd-15 (GA)	Cd- 5 (CT)	IVS1-1 (G-T)	IVS-1 - 1(G-A)	Hb D	Cd15(-CT)	HBS	Exon mutation	Cap+1 (A-C)	Cd16
2016	16	29	1	2	2	3	2	2	2	1	1	1	1	0	0	0	0
2017	9	29	1	2	0	1	1	0	3	1	0	1	0	0	0	0	0
2018	16	19	3	2	0	0	1	4	3	2	0	0	0	0	0	2	1
2019	27	68	3	6	1	5	3	9	2	3	0	0	0	1	2	1	0
2020	45	5	16	10	3	0	8	8	0	0	0	0	0	0	0	0	0
2021	35	2	16	2	1	5	6	4	0	0	0	0	0	0	0	0	0
2022	10	2	3	1	2	0	2	4	0	0	0	0	0	0	0	0	0
Multan division																	
2016	18	19	3	3	2	8	1	7	2	4	0	0	0	0	0	0	0
2017	14	7	1	1	0	0	1	1	2	1	0	0	0	0	0	0	0
2018	7	7	2	0	0	0	1	3	0	0	0	0	0	0	0	0	0
2019	26	11	1	0	0	8	1	5	2	0	0	0	0	0	1	1	0
2020	20	56	19	3	2	0	5	50	0		0	0	0	0	0	0	0
2021	16	3	23	4	1	0	3	4	0	0	0	0	0	0	0	0	0
2022	28	5	17	7	3	4	6	10	0	0	0	0	0	0	0	0	0
Bahawalpur division																	
2016	6	4	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
2017	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2018	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2019-20	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Faisalabad Division																	
2016	2	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2017	2	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
2018	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2019-20	4	3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Sargodha Division																	
2016	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2017	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
2018	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
2019-20	5	1	0	0	0	1	1	2	0	1	0	0	0	0	2	0	0
Others																	
2016	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2017	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2018	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2019-20	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

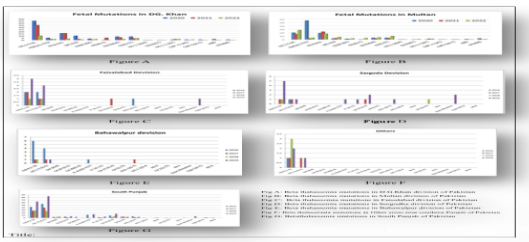
A shift in the mutation spectrum was observed during 2019–2020. In 2019, IVS 1-5 (G-C) remained the predominant mutation (39.4%), while Cd 30 (G-A) was detected as a rare variant. Hb S first appeared in early 2019 (0.7%), whereas Exon mutation and Cap+1 (A-C) IVS-1-1 (G-A), and Cd15 (-CT) were observed in very low frequencies (<1%), indicating substantial genetic heterogeneity in the studied population. These rare mutations have previously been reported in Pakistani mutations were identified during late 2019–2020, particularly in DG Khan and Multan divisions. However, these rare variants were not detected in the 2021–2022 data. Statistical analysis showed that the year-wise variation in mutation frequency was not

significant overall (p = 0.117), suggesting that the major mutation spectrum remained relatively stable over time despite fluctuations in rare variants (Table 1 &Figure 2).

Although some rare mutations appeared during the COVID-19 period (2019–2020), the present data do not establish a direct causal association between COVID-19 and mutation emergence. The temporal overlap may reflect regional or sampling variations rather than a biological effect of SARS-CoV-2 infection. Therefore, the relationship between COVID-19 and mutation frequency should be interpreted cautiously, and larger molecular epidemiological studies are needed to confirm any possible association.

Regional analysis demonstrated that DG Khan and Multan divisions showed the highest mutation burden throughout the study period. DG Khan particularly exhibited greater diversity of uncommon mutations, including Exon mutation, Hb S, and Cap+1 (A-C) (Figure 1A and 1B), whereas Faisalabad, and Sargodha, andBahawalpur divisions demonstrated comparatively lower frequencies and less diversity in mutation types (Figure1C,1D and 1E). These findings indicate possible regional clustering of specific β-thalassemia mutations in Southern Punjab(Figure 1F and 1G).

Demographic analysis revealed that β-thalassemia mutations were most frequently detected in women aged 25–29 years. In addition, mutations were more common among women with gravida 3 or higher (Figure 2A and 2B).These findings may reflect the increased probability of carrier-carrier marriages and repeated reproductive exposure in multiparous women within populations with high consanguinity rates. The observed association suggests that maternal demographic factors, particularly reproductive history, may contribute to the transmission pattern and prenatal detection frequency of β-thalassemia mutations. Therefore, targeted premarital counseling and prenatal screening programs should particularly focus on high-risk reproductive age groups and families with multiple pregnancies.



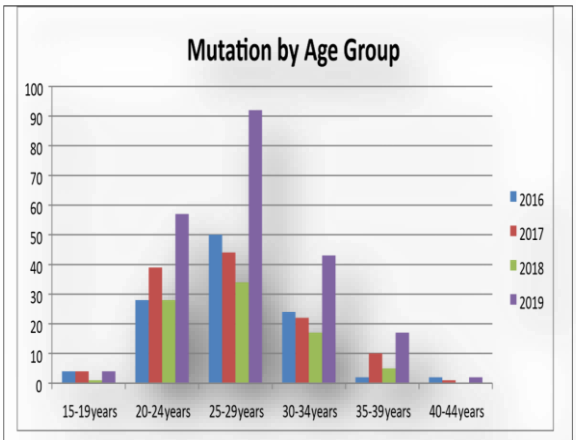


Figure A: Beta thalassemia mutations according to gestational age in South Punjab of Pakistan

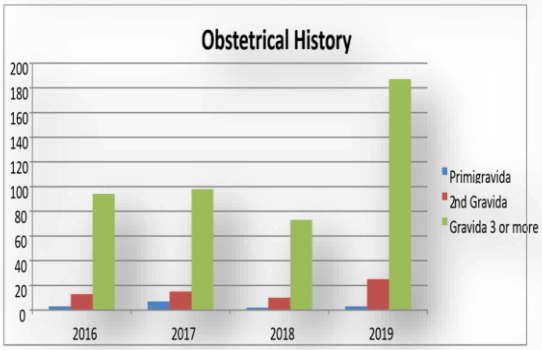


Figure B: Beta thalassemia mutations according to obstetrical history(Year wise data)

Table 2: incidence of beta thalassemia mutation in south Punjab

Common Mutations		Uncommon Mutations	
IVS 1-5 (G-C)	39.06%	Del (619)	2.83%
Fr8-9 (+ G)	30.94%	IVS1-1 (G-T)	2.83%
Cd-15 (G-A)	6.6%	Fr 16 (-C)	2.26%
Fr 4142 -TCTT	5.09%	Exon Mutation	1.13%
Cd-5(-CT)	3.4%	Cd 30 (G-A)	0.94%
Cd-30(G-C)	3.02%	Cap+1 (A-C)	0.75%
		Hb D	0.37%
		Cd 15 (-CT)	0.19%
		IVS-1 -1 (G-A)	0.19%
		hb S	0.19%
		Cd-16	0.19%

Discussion:

Thalassemia is a crucial public health issue in Pakistan, with over 5000 children with β-thalassemia major being born each year. A number of studies have been carried out for the detection of β-thalassemia mutations in various ethnic groups of Pakistan on the basis of which prenatal diagnosis and genetic counseling are currently being offered. Our main goal of the study was to assess the year wise prevalence of different β-thalassemia mutations along with their geographic dissemination and appearance of rare mutations on basis of prenatal diagnosis. This study will also help us in genetic counseling and in turn will minimize frequency of this tormenting disorder.

The spectrum of β-thalassemia mutations in the Pakistani population is highly diverse. In addition to the commonly reported mutations, several rare mutations were identified in our cohort, including Hb S, Hb D, Cap+1 (A-C), IVS-1-1 (G-A), Cd-16, and Exon mutations[16].These rare mutations are clinically important because they may complicate prenatal diagnosis, influence disease severity, and affect transfusion dependency and genetic counseling strategies. Although most of these mutations have previously been reported in Pakistani and neighboring populations, their detection in prenatal samples from Southern Punjab emphasizes the need to expand local mutation panels for more accurate screening and prevention programs.

Previous studies in Pakistan have shown that the prevalence of β-thalassemia is 5–7% . High prevalence (18.5%) has been noted in Dera Ismail Khan[17], and even higher prevalence has been reported in Abbottabad (58.2%)[18]. Our results showed the occurrence of Fr 8-9 (+G) mutation (30.94%) in Southern Punjab regions of Pakistan, whereas a study conducted in 2018 reported this mutation at 29.8%[19]. Yasmeen *et al*. also reported the occurrence of this mutation as 35.5% in 2016[20], indicating persistent predominance of this mutation in the Pakistani population.

Overall data represented the occurrence of IVS 1-5 (G-C) mutation (39.06%), whereas a study conducted at Bahawalpur center of Punjab Thalassemia Prevention Program (PTPP) showed that the most common mutation was Fr 8-9 (+G), accounting for 29.8%, followed by IVS 1-5 (G-C), which was 28.9% [19]. This comparison indicates that IVS 1-5 (G-C) mutation is becoming increasingly prevalent in Southern Punjab.

A study in India found that 90.5% of patients carried one of five common mutations, namely IVS 1-5 (G-C), codon 26 (G-A), codon 30 (G-C), codon 15 (G-A), and FS 41-42 (-CTTT) [21]. Similar mutation patterns were observed in our study, further supporting the regional similarity in β-thalassemia mutation distribution across South Asia.

There was no indication of Exon mutation or Hb S mutation during 2016–2018 in our study population. However, during 2019–2020, rare mutations such as Hb S, Exon mutation, and Cap+1 (A-C) were identified, particularly in DG Khan and Multan divisions. Although these mutations appeared during the COVID-19 period, the present study does not provide sufficient evidence to establish a direct causal relationship between COVID-19 and mutation emergence. The observed temporal association may reflect random variation, regional clustering, or increased molecular surveillance during the pandemic period. Therefore, the possible relationship between COVID-19 and mutation frequency should be interpreted cautiously, and further multicenter molecular studies are required to validate this observation.

A study conducted in Bangladesh in 2018 also identified rare mutations in a small proportion of cases, including Cd16 (-C), IVS1-130 (G-C), and Cd15 (G-A) [22], which is comparable to the rare mutation spectrum observed in our population. Similarly, studies from China[23] , Arab countries [24], Iran [25,] and Thailand have demonstrated considerable geographic diversity in β-thalassemia mutations, supporting the importance of region-specific mutation databases.

Year-wise analysis in our study demonstrated fluctuations in mutation frequencies from 2016 to 2022, although the overall variation was not statistically significant ($p = 0.117$). IVS 1-5 (G-C) remained the dominant mutation throughout most years, while Fr 8-9 (+G) showed increased frequency during 2018. Rare mutations appeared sporadically during specific years and regions, particularly in DG Khan division. These findings indicate relative stability of common mutations over time, with periodic emergence of less frequent variants. More advanced statistical analyses involving larger datasets and regional comparisons may help identify significant temporal trends and mutation clustering patterns in future studies.

Overall data indicated that β-thalassemia mutations were most frequently observed in women aged 25–29 years. In addition, the prevalence was higher among women with gravida 3 or more. This observation may be associated with the higher reproductive exposure and increased probability of carrier-carrier marriages in populations with frequent consanguinity. Multiparous women may also have greater chances of repeated prenatal screening due to previous affected pregnancies. These demographic findings highlight the importance of targeted premarital counseling, antenatal screening, and awareness programs in reproductive-age women, especially those with multiple pregnancies and positive family history of thalassemia.

Incidence of β-thalassemia mutations was particularly higher in DG Khan region according to our study, and this region also demonstrated greater diversity of rare mutations compared to other divisions. The regional clustering of uncommon mutations suggests the possible influence of local ethnic background, consanguinity patterns, and genetic drift on mutation distribution. Therefore, region-specific prenatal diagnostic strategies may improve the effectiveness of β-thalassemia prevention programs in Pakistan.

LIMITATIONS: This study has several limitations that should be considered while interpreting the findings. The retrospective study design and use of non-probability convenience sampling may have introduced selection bias and limited the generalizability of the results. In addition, the data

were collected mainly from Southern Punjab divisions referred to a single prenatal diagnostic center, which may not fully represent the mutation spectrum of the entire Pakistani population. The absence of a control group and incomplete demographic data also restricted detailed statistical comparisons and trend analysis across regions and years.

Another important limitation is the proposed association between the emergence of rare mutations and the COVID-19 period. Although some uncommon mutations appeared during 2019–2020, the study does not provide sufficient evidence to establish a direct causal relationship between COVID-19 and mutation frequency. Other contributing factors such as regional clustering, ethnic variation, consanguinity patterns, and increased diagnostic surveillance during the pandemic may have influenced the findings. Therefore, further multicenter and longitudinal studies are required to validate these observations and provide stronger epidemiological evidence

Conclusion:

This study demonstrated that IVS 1-5 (G-C) and Fr 8-9 (+G) are the most prevalent β-thalassemia mutations in Southern Punjab, while several rare mutations were also identified in specific regions and years. The findings emphasize the significant geographic and temporal diversity of β-thalassemia mutations in Pakistan and highlight the importance of region-specific mutation databases. These results may improve genetic counseling, premarital screening, and prenatal diagnostic strategies by enabling the development of targeted screening panels based on the regional mutation spectrum.

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