Research Article

Analysis of a Single Nucleotide Polymorphism of HBBP1 Gene (rs2071348) with HbF Level in β-Thalassemia Major Patients of Sialkot, Pakistan

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Abstract | HBBP1 (Hemoglobin Subunit Beta Pseudogene 1) is a pseudogene that plays a transcriptional role in erythroid lineage development by binding RNA-binding proteins, triggering β-globin gene expression, and presumably influencing the severity of β-thalassemia, thereby making the polymorphism marked by SNP rs2071348 (NC_000011.10:g.5242916T>G) a substantial indicator for assessing HbF levels in affected patients. In this study we determined to explore the single nucleotide polymorphism of HBBP1 rs2071348 in β-thalassemia major patients of Sialkot, Pakistan. Clinical and demographic data was collected by a specially designed data collection form. Blood samples from 125 β-thalassemia patients including 40 undergoing HU therapy were taken by using EDTA tubes. Patients were categorized into high HbF (n = 73; HbF>60%) and low HbF (n = 44; HbF< 60%) groups based on HbF levels. DNA extraction from peripheral blood samples was performed using the QIAamp® DNA Mini Kit, and quantitative analysis was conducted with the MultiskanSkyHigh spectrophotometer, while qualitative analysis utilized 1% agarose gel electrophoresis. Genotyping was carried out by using PCR based amplification. To determine the genetic association between the HBBP1 polymorphism (rs2071348), variations in HbF levels, and HU drug response among individuals with β-thalassemia, statistical analysis was performed using the chi square test and the web application SNPstats. The current research found insignificant connection between the rs2071348 (T/G) polymorphism of HBBP1 gene with either HbF levels (p-value=0.92) or HU treatment effectiveness (p-value=0.16). However, demographic factor age (p=0.001) and clinical factor splenomegaly (p=0.03) was significantly associated with HU response. This study paves the way for further investigation of the HBBP1 gene in the Pakistan to find an association with β-thalassemia severity because insignificant associations were found may be due to sample size limitations.

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Introduction

Hemoglobinopathies are a set of inherited ailments characterized by abnormal hemoglobin structure or synthesis. Hemoglobin (Hb) a heterotetramer protein, present in red blood cells (RBCs) binds oxygen in the alveoli and transports it to tissues; it binds to four oxygen molecules at a time (Shafique *et al.*, 2021). It comprised of two alpha (α) globin and two non-alpha globin chains (beta-like globin chains) with one heme molecule linked to each globin for oxygen binding (Rosenberg and Rosenberg, 2012). The α-globin locus on chromosome 16 comprises the α1 and α2 genes, whereas the β-globin locus on chromosome 11 includes the G gamma, A gamma, delta, and β loci (Uthman, 2009). Globin gene expression varies by developmental stage, with the predominant hemoglobin tetramer shifting from α2γ2 in foetal to α2β2 in maturity (Utsugisawa Kanno, 2022). Globin gene diseases are distinguished by structural changes, such as sickle cell anemia, or by decreased globin chain production in erythroid cells (thalassemia) during hematopoiesis. Hematopoiesis occurs in the yolk sac during embryonic development, then in the liver of fetal, and lastly in bone marrow throughout adulthood (Sankaran and Orkin, 2013).

Three distinct types of hemoglobin present in adults are: HbA (95%), HbA2 (1.5-3.5%), and HbF (≤1%) (Ali *et al.*, 2021). The most prevalent monogenic autosomal recessive hematological disorder, thalassemia, is caused by a partial or completely lack of synthesis of either of the two globin chains which comprise up the primary adult hemoglobin (HbA), a tetramer (α2β2) consisting of two α and two β-globin chains (Nasiri *et al.*, 2020). The most prevalent genetic condition is β-thalassemia, which is inherited recessively by autosomal pattern and is caused by insufficient β-globin chain production (Tari *et al.*, 2018). The Greek word thalassemia is taken from the words thalassa, which means "the sea," and haimas, which means "related to blood." It was created because individuals of Mediterranean ancestry were the first to be diagnosed with the illness known as "Mediterranean anemia (Menzel and Thein, 2019; Betts *et al.*, 2020).

β-thalassemia is caused by base exchanges on axons, introns, and promoter areas of β-globin genes (Tripathi, 2022). The quantity and kind of altered β-globin genes determine the severity of β-thalassemia. When only one β-globin gene is impacted, the condition known as β-thalassemia minor; to have moderate clinical phenotype (β^+/β, β^0/β); symptoms are usually minor or undetectable. A clinical phenotype intermediate between the two, thalassemia is caused by diverse genetic changes that permit some production of the β chain (β^+/β^0, β^+/β^+, etc.). Conversely, a severe clinical phenotype known as thalassemia major, or Cooley's Anemia, is present in patients who are homozygous/compound heterozygous for more acute β-chain mutations (severe β^+/β^+ mutations, β^+/β^0, β^0/β^0) (Jaing *et al.*, 2021). β-thalassemia major is highly prevalent in different areas of the world e.g., the Mediterranean region, South Korea, USA, Australia, Southeast Asia, the Indian subcontinent, Middle East and Africa (Musallam *et al.*, 2023; Tuo *et al.*, 2024; Rao *et al.*, 2023). Worldwide 7% population is thalassemia carrier and in Pakistan its percentage is 5-8% (Chauhan and Zennadi, 2023; Ali *et al.*, 2021). Approximately 5,000 children are diagnosed with β-thalassemia major (β-TM) in Pakistan every year (Asif and Hassan, 2016). To treat such large infected population blood transfusion, gene therapy, iron chelation, drugs (Hydroxyurea), splenectomy and bone marrow transplantation are effective treatments (Ali *et al.*, 2021; Tuo *et al.*, 2024; Ansari *et al.*, 2019; Amjad *et al.*, 2020). People with severe types of thalassemia show jaundice, paleness, black urine, poor appetite, severe anemia, facial bone deformities and yellow skin coloration (Lauer and Houtenille, 2018; Hassan *et al.*, 2018; Hassan, 2018; Iqbal *et al.*, 2018). Carrier identification, prenatal diagnosis, genetic counseling and by avoiding cousin marriages may all help to prevent β-thalassemia (Jaing *et al.*, 2021; Ghafoor *et al.*, 2023; Origa, 2021). Inheritable genetic components that resemble functional genes but do not encode proteins, pseudogenes are non-functional genes. Tissue-specific expression of pseudogene was observed, particularly in the bone marrow. The β-globin gene cluster on chromosome 11 spans ~70 kb and includes genes ε-Gγ -Aγ-ψβ- δ-β arranged 5′ to 3′ (Ma *et al.*, 2021).

There are different genetic variants associated with hemoglobin switching such as hemoglobin subunit beta pseudogene1 (HBBP1) which is a pseudogene restricted to bone marrow present in intergenic region between the Aγ- and δ-globin genes involve in erythropoiesis by binding the RNA-binding protein (RBP) (Ma *et al.*, 2021). β-globin locus, the (HBBP1) region forms fetal stage-specific

contacts with 3′HS1 (hypersensitive) and 5′HS regions of chromosome 11 (Ikawa *et al*., 2019). The heterogeneous nuclear riboproteinA1 (HNRNPA1) gene, which is accountable for up regulating the TAL1 transcription factor and a critical regulator of erythropoiesis binds to HBBP1. This is why considerable functional experiments have revealed that HBBP1 is required for erythropoiesis. Due to the interaction between HBBP1/TAL1 patients with β-thalassemia show milder signs or symptoms (Ma *et al*., 2021; Hattangadi *et al*., 2011; Porcher *et al*., 1996; Robb *et al*., 1995).

Genome-wide association studies (GWASs) identified two single nucleotide polymorphisms (SNPs) that present within the second intron of HBBP1 and are associated with increased fetal hemoglobin levels that cause severe phenotype of β-thalassemia (Ma *et al*., 2021). According to data from the ENCODE project and In silico analysis, the HBBP1 region contains a number of transcription factor binding motifs, such as GATA1, BCL11A, ZBTB7A, KLF1, and SOX6, which are important regulators of globin switching and erythropoiesis, so it is a candidate for additional functional genomics and β-thalassemia modifier research (Moleirinho *et al*., 2013; Heshusius *et al*., 2022).

Selected polymorphism of HBBP1 gene (rs2071348) was not studied in β-thalassemia patients of Pakistani population. The current study was conducted with the objective to summarize the association analysis of single nucleotide polymorphism of HBBP1 with HbF level and HU effectiveness in β-thalassemia patients of Punjab, Pakistan.

Materials and Methods

Experimental area

The Government College Women University, Sialkot (GCWUS) Ethical Institutional Review Board (EIRB) authorized this study with letter no. D/REG/EIBR/23/573.The present research work was carried out in the Research Lab of Department of Zoology and Central Research Lab of Government College Women University, Sialkot.

Patients recruitment

Overall, 125 β-thalassemia patients were recruited from the Kalsoom Society and Sundas Foundation Sialkot, Punjab, Pakistan, 40 were taking hydroxyurea therapy and 85 were not taking hydroxyurea therapy. The responders for hydroxyurea were 13 patients, while 27 were non-responders. The patients with high HbF level were n=73 (HbF≥60) and with low HbF were n=53 (HbF<60) from data which was collected. The verbal and written consent was taken from β-thalassemia patients by the specially designed consent form with mentioned data for gender, age, caste etc.

DNA extraction and qualitative analysis

Peripheral blood samples of β-thalassemia patients were collected by an expert phlebotomist in 5ml ethylenediaminetetraacetic acid (EDTA). Column based genomic DNA extraction kit of QIAamp (Qiagen) DNA Mini Kit (cat. nos. 51304 and 51306) was used to extract DNA. A qualitative analysis of the extracted DNA was conducted by using 1% agarose gel and visualized under UV light after ethidium bromide staining (Figure 1).

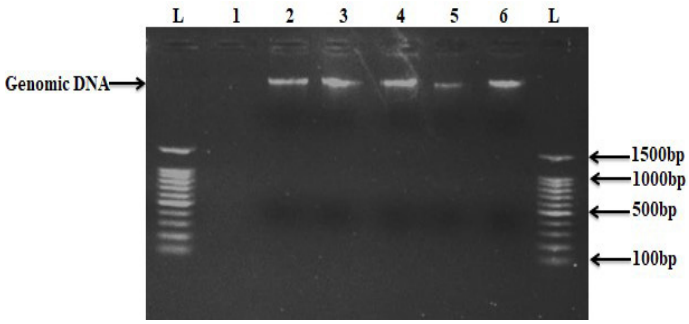

Figure 1: *Post agarose gel electrophoresis analysis of DNA extracted from blood samples of β-thalassemia patients. L= 100bbp DNA ladder, 1= Negative control, 2= ZBT-93, 3=ZBT-526, 4=ZBT-441, 5=ZBT-517, 6=ZBT-560, L= 100bp DNA ladder.*

Quantitative analysis and PCR (Polymerase chain reaction) amplification

Quantitative analysis of genomic DNA was done by Multiskan Sky High spectrophotometer (Thermo Scientific). The rs2071348 (T/G) sequences for the HBBP1 gene have been retrieved from Ensembl (http://www.ensembl.org/index.html) and genotyping of SNP rs20712348 in the HBBP1 gene was conducted by using forward primer (5′-CGCTGACCAAAGGAAAGATGT′-3′) with base pair length 21 and melting temperature 59.5°C and reverse primer (5′-TCAGCAGTGGGATTTTGAGAGT′-3′) with base pair length 22 and melting temperature 60.1°C. Amplification of HBBP1 gene rs2071348 (T/G) was set as, initial denaturation at 95°C for 3 minutes

followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 57°C for 20 seconds and extension at 72°C for 25 seconds. Final extension was done at 72°C for 10 minutes. The size of amplicon containing rs2071348 SNP of HBBP1 is 211 base pairs. For the confirmation of amplification of desired region of HBBP1 gene, 7µl of the PCR products were ran on 2% agarose gel prepared in 1x TAE (Tris-acetate EDTA) along with DNA marker of 100 bps for 40 minutes at 95 volts (Figure 2).

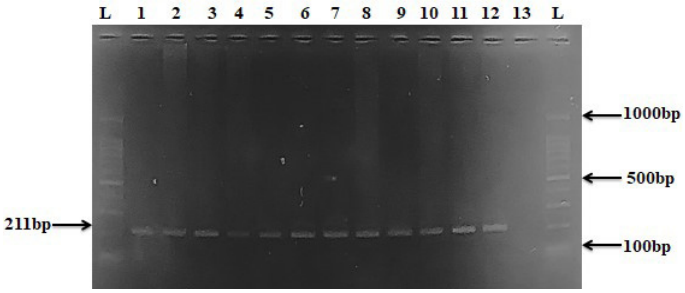

Figure 2: *The PCR results of HBBP1 gene polymorphism rs2071348 on 2% agarose gel stained with ethidium bromide. L= 100bp DNA ladder, 1= ZBT- 510, 2= ZBT-469, 3= ZBT-308, 4= ZBT-51, 5= ZBT-480, 6= ZBT-428, 7= ZBT-475, 8=ZBT-14, 9= ZBT-519, 10= ZBT-517, 11= ZBT-459, 12= ZBT-471, 13= Negative Control, L= 100bp DNA ladder.*

PCR-RFLP based analysis

In order to perform RFLP (Restriction fragment length polymorphism) based genotyping of rs2071348 (T/G) polymorphism of HBBP1 gene Mph1103I (Nsil) restriction enzyme (10U/µl) with special buffer (10x buffer R) was used. Treated samples were incubated overnight (12-16 hours) at 37°C followed by inactivation at 65°C for 20 min. After incubation, restriction digestion products were run on 2.5% agarose gel with a negative control (T/T). Samples were electrophoresed at 90 volts for 60 minutes. After completion 60 minutes, gel was visualized in Gel Doc (U: Genius[3]) gel documentation system (Figure 3).

Statistical analysis

Data was analyzed by using Statistical Package for Social Sciences (SPSS) version 20 for windows and also SNPStats: your web tool for SNP analysis program. The chi-square ($\chi 2$) test was used to represent qualitative data in percentages that can then be used for comparisons. The odds ratio (OR) and related 95% confidence intervals (CI) were used to calculate the strength of the statistical connection, and a p-value of less than 0.05 was regarded as a significant statistical value.

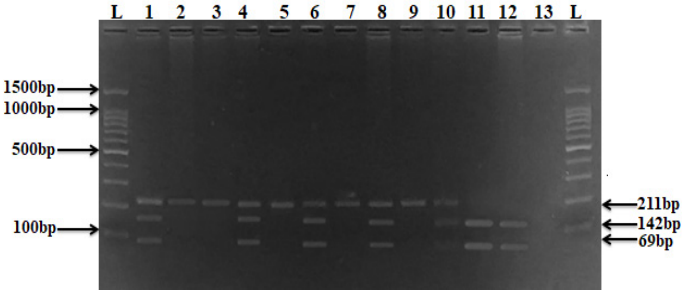

Figure 3: *PCR-RFLP based genotyping results of rs2071348 (T/G) polymorphism of HBBP1 gene on 2.5% agarose gel stained with Ethidium bromide. L= 100bp DNA ladder, 1= ZBT- 217, 2= ZBT-475, 3= ZBT-471, 4= ZBT-459, 5= ZBT-480, 6= ZBT-450, 7= ZBT-475, 8=ZBT-202, 9= ZBT-519, 10= ZBT-517, 11= ZBT-469, 12= ZBT-308, 13= Negative Control, L= 100bp DNA ladder.*

Note: *The five samples ZBT-480, ZBT-475, ZBT-519, ZBT-459 and ZBT-471 shows homozygous T/T (Normal, wild-type) genotype by producing a single band of size 211bp. Samples ZBT-469 and ZBT-308 shows two bands of size 142bp and 211bp for G/G (Homozygous variant) genotype. Samples ZBT-517, ZBT-475, ZBT-450, ZBT-202 and ZBT-517 reflect heterozygous T/G (Heterozygous variant) genotype by giving three bands of 211bp, 142bp and 69bp.*

Results

Evaluation of the association between HbF levels and HU response with the genetic polymorphism of the HBBP1 gene (rs2071348)

We firstly demonstrated the possibility of HbF levels correlation with SNP rs2071348. Data showed that genotyping distribution of rs2071348 (T/G) polymorphism of the HBBP1 gene is insignificant as OR=1.26, 95% CI=0.33-4.80, p-value=0.92 (p-value ≥0.05) and does not significantly correlate with either high or low HbF levels in patients with β-thalassemia. In β-thalassemia patients with elevated HbF levels, results showed that the homozygous TT genotype was more prevalent (68.2%) and the homozygous GG genotype was less prevalent (13.6%) same as TT genotype (68.8%) and the GG genotype (10.4%) in β-thalassemia patients with low HbF levels (Table 1).

Next, a comparative study of genotypic frequencies for the single nucleotide polymorphism of the HBBP1 gene (rs2071348, T/G) in HU responders and non-responders was carried out. In our results we failed to find significant association of HU responders and non-responders as OR=0.00, 95% CI=0.00, p-value=0.16

Table 1: *Comparative analysis of genotype frequencies across HbF levels and HU response.*

HBBP1 gene polymorphism	Genotype	HbF levels		OR (95% CI)	p value	HU therapy		OR (95% CI)	p value
		High (n%)	Low (n%)			Non-responders (n%)	Responders (n%)		
rs2071348 T/G	T/T	15 (68.2%)	33 (68.8%)	1.00	0.92	22 (71%)	8 (88.9%)	1.00	0.16
	T/G	4 (18.2%)	10 (20.8%)	1.26 (0.33-4.80)		6 (19.4%)	0 (0%)	0.00	
	G/G	3 (13.6%)	5 (10.4%)	0.89 (0.18-4.44)		3 (9.7%)	3 (9.7%)	0.73 (0.06-8.92%)	

HBBP1, Hemoglobin subunit pseudogene 1; HbF, Fetal Hemoglobin; HU, Hydroxyurea; OR, Odd Ratio; CI, Confidence Interval, n%, Number Percentage

Table 2: *Genetic models of comparison groups using genetic association analysis between high and low HbF levels and HU therapy.*

HBBP1 gene polymorphism + Models	Genotype	HbF levels		OR (95%)	p value	HU therapy		OR (95% CI)	p value
		High (n%)	Low (n%)			Non responders (n%)	Responders (n%)		
rs2071348 T/G dominant	T/T	15 (68.2%)	33 (68.8%)	1.00	0.86	22 (71.0%)	8 (88.9%)	1	0.19
	T/G- G/G	7 (31.8%)	15 (31.2%)	1.10 (0.36-3.43)		9 (29.0%)	1 (11.1%)	0.25 (0.03-2.48)	
rs2071348 T/G recessive	T/T- T/G	19 (86.4%)	43 (89.6%)	1.00	0.82	28 (90.3%)	8 (88.9%)	1	0.96
	G/G	3 (13.6%)	5 (10.4%)	0.83 (0.17-4.00)		3 (9.7%)	1 (11.1%	1.07 (0.09-12.69)	

HBBP1, Hemoglobin subunit pseudogene 1; HbF, Fetal Hemoglobin; HU, Hydroxyurea; OR, Odd Ratio; CI, Confidence Interval, n%, Number Percentage.

(p-value ≥0.05) in patients with β-thalassemia. In both HU responders and HU non-responders, TT genotype was more common and TG genotype less common (Table 1).

Genetic models with varying HbF values and Hydroxyurea (HU) response to show disease severity in β-thalassemia patients

This study examined SNP polymorphism rs2071348 (T/G) of HBBP1 gene genetically in both high and low HbF levels using dominant and recessive genetic models. The low akaike information criterion (AIC) value of these models made them the most suitable genetic models. The HBBP1 polymorphism rs2071348 (T/G) in the dominant model showed an odds ratio (OR) of 1.10 with an associated confidence interval (CI) of 0.36-3.43, meaning that individuals with the T/G and G/G genotypes have a 1.10 times greater probability of having a high HbF level. The dominant model revealed that there was no significant correlation between the HBBP1 gene's rs2071348 (T/G) polymorphism and HbF levels in patients with β-thalassemia, as demonstrated by the p-value of 0.86. Conversely, in the recessive model, the odds ratio of 0.83 with a corresponding confidence range of 0.17–4.00 showed that a person with the G/G genotype will have 0.83 times lower chance of having a high HbF level. Additionally, the recessive model's p-value=0.82 is not significant, indicating that there was no significant correlation between the HBBP1 gene's rs2071348 (T/G) polymorphism and either high or low HbF levels in individuals with β-thalassemia (Table 2).

The dominant and recessive genetic models generated through SNPStats: Your web tool for SNP analysis (online web tool) were used to investigate the genetic connection of HBBP1 gene polymorphism rs2071348 (T/G) in β-thalassemia patients across HU responders and non-responders. A person with the T/G and G/G genotypes will have a 0.25 times higher chance of responding to HU treatment, based on the dominant model for the rs2071348 (T/G) polymorphism of the HBBP1 gene, which has a value of 0.25 with an associated confidence interval 0.03-2.48. Furthermore, in β-thalassemia patients, p-value= 0.19 shows a negligible correlation between the HBBP1 gene polymorphism rs2071348 (T/G) and HU medication response. The odds ratio of 1.07 in the recessive model, together with the corresponding confidence range of 0.09–12.69, indicates that a person with the G/G genotype will have a 1.07 times less probability of responding to HU treatment. Similarly, in individuals with β-thalassemia, the rs2071348

(T/G) polymorphism of the HBBP1 gene does not significantly correlate with HU treatment response, as indicated by the recessive model's p-value= 0.96. Odds ratios have extremely wide CIs, e.g., recessive model OR=0.83 (0.17-4.00), indicating severe under powering, so these results might be inconclusive (Table 2).

Discussion

This study is the first to investigate the association of HBBP1 (rs2071348) with HBF and HU response in Pakistani β-thalassemia patients. Approximately 5,000 children are diagnosed with β-thalassemia major (β-TM) in Pakistan every year (Asif and Hassan, 2016). People with severe types of thalassemia show jaundice, paleness, black urine, poor appetite, severe anemia, facial bone deformities and yellow skin coloration (Lauer and Houtenille, 2018; Hassan *et al*., 2018; Hassan, 2018; Iqbal *et al*., 2018). The quantity and kind of altered β-globin genes determine the severity of β-thalassemia (Jaing *et al*., 2021).

Recent research indicated that via controlling the expression of the β-globin gene, HBBP1 has a significant role in increasing HbF in β-thalassemia patients. According to data from the ENCODE project and In silico analysis, the HBBP1 region contains a number of transcription factor binding motifs, such as GATA1, BCL11A, ZBTB7A, KLF1, and SOX6, which are important regulators of globin switching and erythropoiesis, so it is a candidate for additional functional genomics and β-thalassemia modifier research (Moleirinho *et al*., 2013; Heshusius *et al*., 2022). The relationship between the polymorphism of the HBBP1 gene and the levels of HbF and HU response in patients with β-thalassemia in the Pakistani community has not been examined previously. In several populations, β-thalassemia patients HbF levels have been reported to be correlated with the HBBP1 genetic polymorphism; however, there is a deficiency of research on this relationship in the Pakistani community. So, the current study was designed in order to analyze the association analysis of HBBP1 genetic polymorphism rs2071348 with HbF levels and also with HU response in β-thalassemia patients.

Present study examined the association between the HBBP1 genetic polymorphism and HbF levels as well as the HU response in 125 β-thalassemia patients. Out of the 125 individuals with β-thalassemia, 40 were undergoing HU treatment, while the remaining 85 were not. HU treatment patients were further classified as responders (n = 9) or non-responders (n = 31) based on how well they responded to HU after receiving blood transfusions. β-thalassemia patients HbF data was utilized to categorize their HbF levels as high or low. Individuals with HbF levels more than or equal to 60% (n = 44) were classified as having a low HbF level, whereas those with HbF levels high 60% (n= 73) were classified as having a low HbF level. Comparative analysis of genotype frequencies of HBBP1 gene (rs2071348, T/G) insignificantly associated (p=0.92) with HbF levels shown n=15 (68.2%) for low HbF and n=33 (68.8%) for high HbF levels, n=4 (18.2%) for low HbF and n=10 (20.8%) with high HbF levels, n=3 (13.6%) with low HbF and n=5 (10.4%) with high HbF levels for TT, TG, and GG genotypes respectively. Similarly, comparative analysis of genotype frequencies of HBBP1 polymorphism (rs2071348, T/G) insignificantly associated (p= 0.16) with HU therapy in both HU non-responders having genotypes TT (n= 22, 71.0%), TG (n= 6, 19.4%), and GG (n= 3, 9.7%) and HU responders having genotypes TT (n= 8, 88.9%), TG (n= 0, 0%) and GG (n= 1, 11.1%). Additionally, a study evaluated the correlation between the clinical and demographic characteristics of individuals with β-thalassemia, their HbF levels, and their HU response.

Results of statistical analysis showed that the HbF level and HU response were not significantly associated with basic demographic characteristics, including gender, caste, socioeconomic position, and education. However, the demographic character age (p=0.001) exhibits significant outcomes with HU treatment but insignificance results with HbF level (Table 3). Splenomegaly was significantly correlated with HU treatment in terms of clinical features as p value = 0.03 ($p < 0.05$). Due to the fact that by trapping and destroying erythroid cells, splenic sequestration disrupts erythropoiesis leading to elevates erythropoietin stimulation and ineffective erythropoiesis. Since sequestration reduced the survival of HU-induced HbF-producing cells, this may decrease the haematologic response to hydroxyurea (HU). Because there is less RBC destruction in patients with splenic dysfunction or those who have had a splenectomy, they usually show better HU response (Ware *et al*., 2017; Gulbis *et al*., 2005).

Table 3: *Association analysis of demographic Cohort characteristics with HbF levels and Hydroxyurea response.*

S. No.	Characteristics	p-value (HbF)	p-value (HU response)
1	Gender	0.298	0.970
2	Age	0.498	0.001
3	Caste	0.355	0.082
4	Socioeconomic status	0.857	0.615
5	Education	0.366	0.760

HbF, Fetal Hemoglobin; HU, Hydroxyurea.

Table 4: *Association analysis of clinical Cohort characteristics with HbF levels and Hydroxyurea response.*

S. No.	Characteristics	p-value (HbF Levels)	p-value (HU response)
1	Anemia	0.204	0.423
2	Splenomegaly	0.993	0.03
3	Splenectomy	0.307	0.900
4	Hepatomegaly	0.218	0.283
5	Growth and development	0.164	0.120
6	Facial bone deformity	0.618	0.377
7	Physical activity	0.915	0.368
8	Fatigability	0.915	0.368
9	Cardiac function abnormalities	0.430	0.399
10	Transfusion frequency	0.420	1.00
11	Family history	0.360	0.274

HbF, Fetal Hemoglobin; HU, Hydroxyurea.

In contrast, there was no significant correlation found between HbF levels and HU response in β-thalassemia patients and other clinical characteristics, including anemia, splenectomy, hepatomegaly, growth and development, facial bone deformities, physical activity, fatigue, abnormal cardiac function, blood transfusion frequency, and family history (Table 4).

A comparable study conducted in Hellenic origin (Greece) investigated the possible association of rs2071348 with β-thalassemia disease severity in a group of β-thalassemia major patients and β-thalassemia intermedia patients, results were compared with non thalassemic people of same place. Peripheral blood was collected from 95 adults β-TM and 11 adults β-TI patients, as well as 94 ethnically matched adult healthy (non thalassemic) donors of Western Greek origin. In addition, study recruited an independent sample comprising 35 HbS [β6(A3) Glu!Val, GAG>GTG]/β-thalassemia compound heterozygous patients of Western Greek origin receiving HU as an HbF-augmenting agent in their treatment regimen. These patients were divided into two groups according to the level of induction of HbF levels before and after HU treatment: for less than a 3-fold change patients were regarded as "non-responders," whereas for higher than a 3-fold change, patients were regarded as "responders."This study was approved by the local hospital ethics committee. The rs2071348 (g.5264146 A>C) polymorphism on the HBB pseudogene (HBBP1) as a variant significantly associated with a milder disease phenotype β0-thalassemia/hemoglobin (Hb) E (β0-thal/Hb E (GAG>AAG) patients patients receiving HU. Results suggested that the rs2071348 polymorphism is associated with higher HbF levels and a milder β-thal disease phenotype. However, the rs2071348 polymorphism in the HBBP1 gene does not correlate with response to HU treatment same as in our study (Giannopoulou *et al.*, 2012).

On contrary of our results, Roy *et al.* (2012) found significant association with A>C rs2071348 (HBBP1) gene present on chromosome 11, genetic alteration for high HbF phenotypes of 91 Indian patients those were screened for polymorphisms by sequencing and restriction fragment length polymorphism (RFLP) analysis (Roy *et al.*, 2012).

As the previous studies shows significant association with HbF level in β-thalassemia patients of rs2071348 (A>G) and rs2071348 (A>C) as compared to our study that results are insignificant for HbF (p=0.92) and HU level (p=0.16). Differences in sample size, local linkage disequilibrium patterns, or different genetic modifiers such as BCLA11A or HBS1L-MYB variants not assessed in this study might all be contributing factors to the data diversification. A distinct research methodology and statistical analysis might be an additional effect. Larger sample size and other laboratory and clinical parameter setups, sequencing based genotyping and multi-SNP haplotyping are required to evaluate the findings of this research.

Conclusion

The present study documented that rs2071348 (T/G) polymorphism of HBBP1 gene was not significantly associated with HbF levels in β-thalassemia patients. Furthermore, the HBBP1 gene polymorphism (rs2071348, T/G) is not associated with the

effectiveness of hydroxyurea (HU) therapy. Although, HU therapy has been found to prevent anemia in patients who exhibit a therapeutic response. On the whole, this research work suggests that rs2071348 (T/G) polymorphism of HBBP1 gene was not found to be a significant predictor of either HbF levels or HU drug effectiveness in β-thalassemia patients of Sialkot, Punjab, Pakistan; however, due to limited power, further studies are needed.

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Novelty Statement

This study is the first to investigate the association between fetal hemoglobin levels and the rs2071348 SNP in the HBBP1 gene, specifically in patients with β-thalassemia major from Sialkot, Pakistan. It offers new genetic insights into disease severity modifiers that are specific to this population.

Author's Contribution

Tahara Ashraf: Performed lab work, wrote paper.
Muhammad Hassan Siddiqi: Supervised this research.
Rabia Afzal: Help in lab work and conclude data.
Shabana Khadim: Help in paper writing.
Ali Amar: Contributed by providing valuable resources.

Conflict of interest
The authors have declared no conflict of interest.

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