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Original Article

Associations between *BCL11A* and *HBS1L-MYB* polymorphisms and thalassemia risk

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الملخص

أهداف البحث: تناولت هذه الدراسة التحقيق في العلاقة بين ثلاثة متغيرات جينية: آر اس 4671393 و آر اس 1427407 و آر اس 11886868 تقع داخل جين "بي سي ال 11 أ" ومتغير واحد آر اس 9399137 في جين "اتش بي اس 1 ال-ام واي بي"، وارتباطهم المحتمل بمرض الثلاسيميا لدى المرضى من سكان البنجاب، باكستان.

طريقة البحث: تم استقطاب مجموعة مكونة من 600 مشارك من مستشفيات مختلفة في البنجاب، باكستان. وقد ضمت المجموعة 300 مريض بالثلاسيميا و300 من الضوابط الأصحاء المطابقين في العمر والجنس. وتم استخراج الحمض النووي من عينات الدم الكاملة لجميع المشاركين. وتم تضخيم مناطق الحمض النووي المحددة التي تحتوي على أربعة متغيرات وراثية ذات أهمية باستخدام تفاعل البوليميراز المتسلسل.

النتائج: تشير الترددات الجينية تعدد أشكال النوكليوتيد المفردة لـ آر اس 4671393 من جين "بي سي ال 11 أ" إلى أن النمط الجيني المتغاير (أ ج) لـ تعدد أشكال النوكليوتيد المفردة كان مرتبطا بشكل كبير بزيادة خطر الإصابة بالثلاسيميا بمقدار الضعف تقريبا مقارنة بالمجموعة المرجعية. كما أكد الجمع بين جميع الأنماط الجينية في نموذج مشترك وجود ارتباط كبير بزيادة خطر الإصابة بالثلاسيميا. وعلى غرار آر اس 4671393، أظهر النمط الجيني المتغاير(س ت) لـ آر اس 11886868 أيضا ارتباطا كبيرا بزيادة خطر الإصابة بالثلاسيميا بمقدار الضعف تقريبا. كشف تحليل كلا النمطين الجينيين معا عن ارتباط طفيف مع زيادة بمقدار ضعف واحد في خطر الإصابة بالثلاسيميا للأفراد الذين يحملون أي أليل متغير من آر اس 11886868. أظهرت حالات التوزيع الأليلي لـ آر اس 1427407 للجين "بي سي ال 11 أ" أن النمط الجيني المتغاير (ت ج) لـ آر اس 1427407 أظهر ارتباطا مهما بزيادة في خطر الإصابة بالثلاسيميا بمقدار الضعف تقريبا مقارنة بالمجموعة المرجعية. أكد الجمع بين جميع الأنماط الجينية في نموذج مشترك وجود ارتباط مهم بين وجود أي أليل متغير لـ آر اس 1427407 وزيادة في خطر الإصابة بالثلاسيميا بمقدار الضعف. أظهرت تردادات التوزيع الجيني المتغاير (ت س) لـ آر اس 9399137 لجين "اتش بي اس 1 ال-ام واي بي" مشابهة لـ آر اس 1427407، أظهر النمط الجيني المتغاير (س ت) لـ آر اس 9399137 ارتباطا مهما للغاية بزيادة في خطر الإصابة بالثلاسيميا بمقدار الضعف تقريبا. أظهر تحليل كلا النمطين الجينيين معا وجود ارتباط كبير مع زيادة خطر الإصابة بالثلاسيميا بمقدار ضعف واحد للأفراد الذين يحملون أي متغير من أليل آر اس 9399137.

الكلمات المفتاحية: اعتلالات الهيموجلوبين؛ الهيموجلوبين الجنيني؛ الثلاسيميا؛ جين بي سي ال 11 أ؛ التبديل الجنيني

Abstract

Objectives: This study investigated the associations of the rs4671393, rs1427407, and rs11886868 genetic variants of the *BCL11A* gene and the rs9399137 variant of the *HBS1L-MYB* gene with thalassemia in patients from the population of Punjab, Pakistan.

Methods: A cohort of 600 participants, comprising 300 patients with thalassemia and 300 age- and sex-matched healthy controls, was recruited from various hospitals in Punjab, Pakistan. DNA was extracted from whole blood samples from all participants. Specific DNA regions containing four genetic variants of interest were amplified with polymerase chain reaction.

Results: The genotypic frequencies of the rs4671393 SNP of *BCL11A* indicated that the heterozygous (AG) genotype of this SNP was significantly associated with a nearly two-fold increased thalassemia risk, with respect

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to the control group (OR = 1.77; 95% CI = 1.77−2.80; p = 0.01). Combining all genotypes into a joint model further confirmed their significant association with thalassemia risk. Similarly to the findings for rs4671393, the heterozygous (CT) genotype of rs11886868 exhibited a significant association with thalassemia risk (approximately two-fold increased risk. Analysis of both genotypes together revealed a marginally significant association (one-fold increased risk) with thalassemia in individuals carrying any variant allele of rs11886868. The allelic distribution of the rs1427407 SNP of *BCL11A* indicated that the heterozygous (GT) genotype of this SNP was significantly associated with thalassemia (approximately two-fold increased risk, with respect to the control group). Combining all genotypes into a joint model confirmed a significant association between the presence of any variant allele of rs1427407 and thalassemia (two-fold increased risk). The genotypic distribution frequencies of the heterozygous (CT) genotype for the rs9399137 SNP of *HBS1L-MYB* was similar to that of rs1427407, and exhibited a highly significant association with thalassemia risk (nearly two-fold increased risk). Analysis of both genotypes together revealed a significant association with thalassemia risk (one-fold increase) for individuals carrying any variant allele of rs9399137.

Conclusion: *BCL11A* and *HBS1L-MYB* polymorphisms were significantly associated with increased thalassemia risk.

Keywords: *BCL11A* gene; Fetal hemoglobin; Fetal switch; HbF; Hemoglobinopathies; rs4671393

Introduction

Thalassemia is an inherited blood disorder involving abnormal hemoglobin.[1] According to prior studies, 1.5% of the world's population are thalassemia carriers, and this number is continually increasing as 60,000 new carriers are born every year.[2] Thalassemia unfortunately is the most common diseases in Pakistan. Approximately 100,000 individuals across the country have been estimated to be living with this condition, and the number is increasing yearly. The thalassemia carriage rates are also concerning, with 5−7% of the population carrying the genetic trait.[3]

The amount of hemoglobin present in adults is 0.6%. Two alpha and gamma globin molecules combine to form HbF. After birth, gamma globin disappears. As gestation begins, the production of gamma globin genes increases and outpaces beta globin gene copying. After birth, a fetal switch occurs, in which gamma globin is replaced with adult beta globin. Bauer & Orkin, 2015 discovered an authentic repressive expression for HbF is BCL11A gene (B cell leukemia/lymphoma 11A). Genome-wide association studies have revealed a strong link between variations in the BCL11A gene and elevated HbF levels in adults.[4] HbF level is a quantitative trait in adult humans and consequently is controlled by and depends upon different gene loci.[5] HbF quantitative trait locus (QTL) studies have identified three major loci contributing to approximately 20−25% of HbF variation in numerous populations, including XmnI-HBG2, HBS1L-MYB, and BCL11A on chromosome 6q23, and *BCL11A* on chromosome 2p16.[6] Our goal was to explore three variations in BCL11A (rs11886868, rs4671393, and rs1427407) and one in HBS1L-MYB (rs9399137) among patients with thalassemia of all ages in Punjab, Pakistan. **Three distributed disequilibrium blocks of these polymorphisms exist, denoted HBS1L-MYB intergenic polymorphism (HMIP) blocks 1, 2, and 3. The alleles in these blocks are associated with HbF expression.[7] The association of rs28384513 and rs9399137 with HbF levels was first reported in people of northern European descent, whereas the association of rs4895441 was identified in a large non-anemic Sardinian population. These single nucleotide polymorphisms (SNPs) are associated with HbF levels in sickle cell disease as well as thalassemia in African American and Brazilian populations. The minor allele frequency of rs9399137 (C) is considered most significantly associated with HbF expression in an African population, with frequencies of 1−2%. Similarly, a 3-bp (TAC) deletion, which is in complete LD with the minor allele of rs9399137 and considered the functional motif for this QTL, is also more common in non-African populations.**[8] Herein, we investigated HbF levels in patients, considering factors such as age; sex; and family history regarding BCL11A, specifically the rs4671393 variant.

Materials and Methods

A total of 600 participants (300 patients with thalassemia and 300 healthy controls) were matched by age and sex. Patients with thalassemia were recruited from three locations in Punjab: Halal e Ahmar center in Sargodha, DHQ Sargodha, and THQ Bhalwal. Controls were recruited from hospital checkups. Yamane's formula was used for calculating population size. $n = [N/(1+N\ (e)^2]$ ensured a statistically relevant sample size. The exclusion criteria included the presence of other genetic diseases. Blood samples were randomly collected in patients with various ages, family histories, and smoking statuses. Informed consent was obtained from participants. Blood samples of 2−3 ml were collected in EDTA vacutainers and stored at 4 °C at TUOL laboratory (University of Lahore Zoology Department).

This study used a previously described phenol-chloroform method with modifications[9] to isolate DNA from 3 to 4 ml blood samples from all participants. Extracted DNA was quantified with 2% ethidium bromide gel electrophoresis and then stored at −20 °C. Specific primers for BCL11A and HBS1L-MYB polymorphisms were designed with bioinformatics tools, and PCR genotyping was performed for all selected polymorphisms in both patients and controls.

PCR conditions

To achieve optimal PCR conditions for the primers for detecting each polymorphism, we adjusted the primer concentration, $MgCl_2$ concentration, and annealing temperatures. The success of these adjustments was confirmed through 2%

agarose gel electrophoresis. The optimized PCR profile for this experiment involved an initial 5-min melting step at 94 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at the optimized annealing temperature, and 1 min at 72 °C. A final extension step of 10 min at 72 °C was then performed, and reactions were subsequently held at 4 °C.

Amplification of sample and control DNA

DNA samples from 300 patients and 300 healthy controls were amplified in 10 µl reaction volumes to detect gene polymorphisms. A Gene Amp PCR System 9700 (Applied Biosystem, USA) and Verity 96-well thermal cycler (Applied Biosystem, USA) were used for both the optimization and amplification processes. Details of reaction mixtures are given in Table 1.

Statistical analysis

Age is presented as mean ± standard deviation. Sex, smoking status, and family history are categorized as male/female, smoker/non-smoker, and yes/no, respectively. Pearson's χ^2 chi-square test was used to assess the association between patients and controls for genotypic and allelic frequencies in SPSS 17.1. Statistical tests appropriate for the data structure were used for analysis. According to Hardy-Weinberg law, allelic frequencies, mutations, and genotypes for all genes were calculated in both controls and patients. MEDCLAC software was used to determine p-values, odds ratios (ORs), and 95% confidence intervals (CIs), and to perform multivariate analysis.

Results

The SNP analyses for the *BCL11A* and HBS1L-MYB genes included 300 patients with thalassemia and 300 healthy controls. Detailed demographic characteristics for the controls and patients are given in Table 2. The major features of the study group were as follows:

Both healthy controls and patients with thalassemia included participants 1−26 years of age. The average age of patients with thalassemia was 14 years, whereas healthy controls averaged more than 14 years. We analyzed both groups, stratified into categories of >14 and < 14 years. Notably, 83% of patients with thalassemia were >14 years of age, compared with 65% of healthy controls in the same age group i-e >14 years. In contrast, 18% of patients with thalassemia were <14 years of age, whereas 35% of healthy controls were within this age range. Statistically significant differences in average age were observed between the patients with thalassemia and healthy controls. For age <14 years, a two-fold increased thalassemia risk was observed (OR = 2.53; 95% CI = 1.47−4.34; p = 0.0008), whereas for age >14 years, a protective role was observed (OR = 0.39; 95% CI = 0.23−0.67; p = 0.0008). The sex composition in both healthy control and thalassemia patient groups was analyzed. Among healthy controls, 46% were male, and 54% were female. In contrast, the thalassemia patient group comprised 58% males and 42% females. A statistically significant association was observed between sex and the presence of thalassemia. Males showed a nearly two-fold increased thalassemia risk (OR = 1.62; 95% CI = 1.02−2.55; p = 0.0380), whereas females showed a significantly decreased thalassemia risk (OR = 0.61; 95% CI = 0.39−0.97; p = 0.0380). Family history analysis revealed clear differences between healthy controls and patients with thalassemia. Among healthy controls, only 15% reported a family history of thalassemia, whereas 85% did not. In contrast, 36% of patients with thalassemia had a family history of the condition, and 64% did not. A statistically significant association was observed between family history and thalassemia diagnosis.

Individuals with a family history of thalassemia had a significantly greater risk of developing the condition than those without a family history (OR = 3.27; 95% Cl: 1.86−5.74; p = 0.0001). A family history of thalassemia was associated with a more than three-fold greater likelihood of developing thalassemia than observed in those without a family history. In contrast, individuals without a family history of thalassemia had a significantly lower risk than those with a family history, as reflected by the OR of 0.30 (95% Cl: 0.17−0.53; p = 0.0001). Thus, having no family history associated with an approximately 70% lower likelihood of developing thalassemia than observed in individuals with a family history of this disease.

Analysis of genotypic frequencies for the SNP rs4671393 of BCL11A (Table 3) revealed a significant association between heterozygosity (AG genotype) and elevated thalassemia risk. Individuals with this genotype were nearly twice as likely to develop thalassemia than those in the control group (OR = 1.77; 95% CI = 1.77−2.80; p = 0.01). Interestingly, the homozygous mutant genotype (GG) of the same SNP did not show a statistically significant association with thalassemia risk (OR = 0.85; 95% CI = 0.51−1.40; p = 0.52). This finding suggested that the presence of both mutated alleles might not directly influence thalassemia susceptibility in the same manner as the heterozygous genotype. Further analysis using a joint genotype model for rs4671393 confirmed the overall risk increase associated with this SNP. Individuals carrying any variant allele (AG or GG genotypes) had a nearly two-fold higher thalassemia risk than individuals in the control group (OR = 1.73; CI = 1.03−2.91; p = 0.03).

Allelic frequencies of the SNP rs11886868 of *BCL11A* showed that the heterozygous (CT) genotype was significantly associated with thalassemia risk (two-fold increase; OR = 1.79; 95% CI 1.07−3.00; p = 0.0280). However the homozygous mutant (TT) genotype of the same SNP did not show any significant association with thalassemia risk (OR = 0.97; 0.61−1.54; p = 0.9063). A combined genotype model of rs11886868 further indicated a significant association with thalassemia risk (one-fold increase; OR = 1.62; 1.00−2.65; p = 0.049). The allelic distribution of the rs1427407 SNP of *BCL11A* showed that the heterozygous

Table 1 PCR reaction mixture composition (per reaction).

SNPs for All Genes	DNA Template	Reverse	Forward	PCR Master Mix	Water
	1 µl (80−200 ng)	1 µl	1 µl	3 µl	4 µl

Table 2: Demographic characteristics of patients and controls.

Variables	Patients (n = 300)	Controls (n = 300)	Adjusted Odds Ratio (95 % CI)	p-value
Age (Y) mean ± standard deviation				
<14, n (%)	248 (82.6 %)	196 (65 %)	2.53 (1.47−4.34)	0.0008
>14, n (%)	52 (17.3 %)	104 (34.6 %)	0.39 (0.23−0.67)	
Sex				
Male, n (%)	174 (58 %)	138 (46 %)	1.62 (1.02−2.55)	0.0380
Female, n (%)	126 (42 %)	162 (54 %)	0.61 (0.39−0.97)	
Family history				
Yes	108 (36 %)	44 (14.6 %)	3.27 (1.86−5.74)	0.0001
No	192 (64 %)	256 (85.3 %)	0.30 (0.17−0.53)	

OR odds ratio; CI, confidence interval. OR, CI, and p-value were calculated through regression analysis.

(GT) genotype was significantly associated with thalassemia risk (up to two-fold increase; OR = 2.08; 95% CI 1.16−3.76; p = 0.0147), whereas the homozygous mutant (TT) genotype of rs1427407 did not show any significant association with thalassemia (OR = 1.2; 95% CI: 0.79−2.04; p = 0.3321). A joint genotype model of rs1427407 again showed a significant association with thalassemia risk (up to two-fold increase; OR = 1.97; 1.24−3.12; p = 0.0039). The genotypic distribution frequencies of the heterozygous (CT) genotype of the SNP rs9399137 of *HBS1L-MYB* showed a significant association with thalassemia risk (nearly two-fold increase; OR = 1.78; 95% CI = 1.30−2.42; p = 0.0002), whereas the homozygous mutant (TT) genotype of rs9399137 showed a non-significant association with thalassemia risk (OR = 0.85; 95%CI = 0.58−1.25; p = 0.4306). A joint genotype model of rs9399137 also showed a highly significant association with thalassemia risk (one-fold increase; OR = 1.31; CI = 1.10−1.56; p = 0.0022).

In males carrying the rs4671393 SNP of the BCL11A gene, the heterozygous (AG) genotype showed a non-significant association with elevated thalassemia risk (OR = 1.42; CI = 0.98−2.07; p = 0.06). Similarly, the homozygous mutant (GG) genotype displayed no significant association with thalassemia risk (OR = 0.81; 95% CI = 0.40−1.62; p = 0.55). In females, the homozygous mutant (GG) and heterozygous (AG) genotypes of rs4671393 showed non-significant associations with the disease (OR = 0.82; 95% CI = 0.39−1.71; p = 0.61, and OR = 1.75; CI = 0.89−3.42; p = 0.09, respectively). The rs11886868 male heterozygous (CT) and homozygous (TT) mutant genotypes for *BCL11A* showed non-significant associations with the disease (OR = 1.80; 0.87−3.71; p = 0.1122, and OR = 0.99; 0.52−1.88; p = 0.0.9647, respectively). Similarly, in females, the heterozygous (CT) genotype showed a non-significant association (OR = 1.88; 0.89−3.98; p = 0.0977, and OR = 1.00; 0.51−1.98; p = 0.9829, respectively).

The rs9399137 SNP of the HBS1L-MYB gene exhibited a sex-specific association with thalassemia risk. In males, the heterozygous CT genotype was associated with a significantly greater thalassemia risk (OR = 2.57, CI = 1.30−5.05, p = 0.006) than the homozygous TT genotype (OR = 0.83, CI = 0.40−1.72, p = 0.62). Similarly, in females, the CT genotype was associated with significantly greater thalassemia risk (OR = 2.59, CI = 1.29−5.21, p = 0.007) than the TT genotype (OR = 0.78, CI = 0.371−1.68, p = 0.54). These findings suggested that the CT genotype is a risk factor for thalassemia in both males and females, whereas the TT

Table 3: Allele and genotype frequencies of selected SNPs of BCL11A and *HBS1L-MYB* in patients and controls.

SNPs	Genotypes	Patients (n = 300)	Controls (n = 300)	OR (95 % CI)	p-value
rs4671393	AA	64 (21.3 %)	96 (32 %)	Ref (1)	
	AG	144 (51.3 %)	112 (37 %)	1.77 (1.11−2.80)	0.01
	GG	82 (27.3 %)	92 (30.6 %)	0.85 (0.51−1.40)	0.52
	AG + GG	236	204	1.73 (1.03−2.91)	0.03
rs11886868	CC	82 (27 %)	114 (38 %)		
	CT	98 (32 %)	64 (21 %)	1.79 (1.07−3.00)	0.0280
	TT	120 (40 %)	122 (40 %)	0.97 (0.61−1.54)	0.9063
	CT + TT	218 (72 %)	186 (62 %)	1.62 (1.00−2.65)	0.0496
rs9399137	CC	82 (27 %)	134 (45 %)	Ref (1)	
	CT	146 (49 %)	82 (27 %)	1.78 (1.30−2.42)	0.0002
	TT	72 (24 %)	84 (28 %)	0.85 (0.58−1.25)	0.4306
	CT + TT	218	166	1.31 (1.10−1.56)	0.0022
rs1427407	GG	112 (37 %)	162 (54 %)	Ref (1)	
	GT	176 (25 %)	42 (14 %)	2.08 (1.16−3.76)	0.0147
	TT	112 (37 %)	96 (32 %)	1.27 (0.79−2.04)	0.3321
	GT + TT	188 (62 %)	138 (46 %)	1.97 (1.24−3.12)	0.0039

OR, odds ratio; CI, confidence interval. OR, CI, and p-value were calculated through regression analysis.

genotype may be protective in females but not males. In rs1427407 of *BCL11A*, the male heterozygous (GT) and homozygous (TT) mutant genotypes showed non-significant associations with the disease (OR = 1.99; 0.87−4.56; p = 0.1010, and OR = 1.31; 0.67−2.54; p = 0.4328, respectively). Similarly in females, heterozygous (GT) and homozygous (TT) mutant genotypes showed non-significant associations with this disease (OR = 2.17; 0.92−5.08; p = 0.0754, and OR = 1.30; 0.65−2.59; p = 0.4538, respectively). SNP associations with sex are shown in Table 4.

The rs4671393 SNP in the BCL11A gene exhibited age-dependent effects on thalassemia risk. In individuals younger than 14 years, carriage of one copy of the variant allele (AG) increased the thalassemia risk by 1.83-fold (OR = 1.83; 95% CI = 0.58−3.15; p = 0.02). In contrast, having two copies of the variant allele (GG) significantly decreased thalassemia risk (OR = 0.39, 95% CI = 0.23−0.69 p = 0.0008). However, neither the AG nor the GG genotype showed any association with thalassemia risk in individuals older than 14 years (OR = 1.73; 95% CI = 0.66−4.50; p = 0.25, and OR = 0.82; 95% CI = 0.29−2.36; p = 0.27, respectively). The BCL11A gene variant rs11886868 did not significantly influence thalassemia risk in either age group studied. For individuals ≤14 years of age, neither the heterozygous (CT) nor the homozygous mutant (TT) genotype of the rs11886868 SNP of BCL11A showed a statistically significant association with thalassemia risk (OR = 1.64; p = 0.1073, and OR = 1.02; p = 0.9366, respectively). Similarly, no significant association was observed for either genotype in individuals ≥14 years of age (OR = 1.66; p = 0.35, and OR = 0.92; p = 0.87, respectively). The heterozygous genotype of rs9399137 of *HBS1L-MYB* showed a highly significant association with thalassemia risk. In the younger age group (≤14 years), the heterozygous (CT) genotype of the rs9399137 SNP of HBS1L-MYB exhibited a highly significant association with thalassemia risk, with nearly twice the risk observed in the control group (OR = 2.68; p = 0.0007). However, the homozygous mutant (TT) genotype showed no significant association (OR = 0.79; p = 0.46). For individuals ≥14 years of age, the heterozygous (CT) genotype of rs9399137 again demonstrated a significant association with thalassemia risk (two-fold increased risk; OR = 2.71; p = 0.04). Similarly to the observations in the younger group, the homozygous mutant genotype did not show a significant association with decreased risk (OR = 0.74; p = 0.58).

For rs1427407 of *BCL11A*, in the ≤14 year age group, the heterozygous (GT) genotype was significantly associated with thalassemia risk (up to 2-fold increase; OR = 2.09; 1.04−4.18; p = 0.0378), whereas the homozygous mutant (TT) and heterozygous (GT) did not show significant associations with this disease (OR = 1.27; 0.73−2.23; p = 0.39).

Table 4: Association of genotype frequencies of *BCL11A* and *HBS1L-MYB* polymorphisms with sex.

SNPs	Sex	Genotype	Patients (n = 300)	Controls (n = 300)	OR (95 % CI)	p-value
rs4671393	Male	Overall	174	138	1.26 (1.01−1.57)	0.03
		AA	36	44	Ref (1)	
		AG	90	50	1.42 (0.98−2.07)	0.06
		GG	48	44	0.81 (0.40−1.62)	0.55
	Female	Overall	126	162	0.77 (0.61−0.98)	0.03
		AA	28	52	Ref (1)	
		AG	64	60	1.75 (0.89−3.42)	0.09
		GG	34	50	0.82 (0.39−1.71)	0.61
rs11886868	Male	Overall	174	138	1.62 (1.02−2.55)	0.0380
		CC	46	52	Ref (1)	
		CT	58	30	1.80 (0.87−3.71)	0.1122
		TT	70	56	0.99 (0.52−1.88)	0.9647
	Female	Overall	126	162	0.61 (0.39−0.97)	0.0380
		CC	34	64	Ref (1)	
		CT	42	34	1.88 (0.89−3.98)	0.0977
		TT	50	64	1.00 (0.51−1.98)	0.9829
rs9399137	Male	Overall	174	138	1.61 (1.02−2.55)	0.03
		CC	46	62	Ref (1)	
		CT	86	38	2.57 (1.30−5.05)	0.006
		TT	42	38	0.83 (0.40−1.72)	0.62
	Female	Overall	126	162	0.16 (0.39−0.97)	0.03
		CC	34	72	Ref (1)	
		CT	62	44	2.59 (1.29−5.21)	0.007
		TT	30	46	0.78 (0.37−1.68)	0.54
rs1427407	Male	Overall	174	138	1.62 (1.022.558)	0.0380
		GG	64	74	Ref (1)	
		GT	44	20	1.99 (0.87−4.56)	0.1010
		TT	66	44	1.31 (0.67−2.54)	0.4328
	Female	Overall	126	162	0.61 (0.39−0.97)	0.0380
		GG	46	88	Ref (1)	
		GT	32	22	2.17 (0.92−5.08)	0.0754
		TT	48	52	1.30 (0.65−2.59)	0.4538

OR odds ratio; CI, confidence interval. OR, CI, and p-value were calculated through regression analysis.

In the ≥14 year age group, the homozygous mutant (TT) and homozygous mutant genotypes did not show significant associations with this disease (OR = 0.92; 0.33−2.52; p = 0.86, and OR = 2.86; 0.090−9.04; p = 0.07, respectively). Age associations with polymorphisms are shown in Table 5. For individuals with family history, heterozygous genotype (AG) and homozygous mutant of rs4671393 SNP of the *BCL11A* gene did not show significant association with thalassemia (OR=1.88; 95% CI=0.67−5.22; p=0.22; OR=0.82; 95% CI=0.28−2.41; p=0.72).In contrast, for individuals without a family history, the heterozygous (AG) genotype exhibited a significant association with thalassemia risk, with nearly twice the risk of the control group (OR = 1.79; 95% CI = 1.04−3.07; p = 0.03). However, the homozygous mutant (GG) genotype again showed no significant association with thalassemia risk (OR = 0.81; 95% CI = 0.45−1.46; p = 0.49). Analyses for the rs11886868 SNP revealed no statistically significant associations of heterozygous (CT) or homozygous mutant (TT) genotype with thalassemia risk, regardless of family history (p = 0.46, p = 0.45; OR = 1.78; 0.98−3.26; p = 0.0598; OR = 1.00; 0.58−1.71; p = 0.7 respectively). For individuals with a family history, rs4671393 genotypes did not significantly influence thalassemia risk. In the absence of family history, the rs4671393 heterozygous genotype was associated with a nearly two-fold increase in thalassemia risk, whereas the association for the homozygous mutant genotype was non-significant. The rs11886868 SNP showed no significant associations with thalassemia in either the presence or absence of a family history. In individuals with a family history of thalassemia, the rs9399137 SNP of HBS1L-MYB showed significant associations with thalassemia risk. The heterozygous (CT) genotype carried a two-fold increase in risk (OR = 2.50; 95% CI = 1.24−5.02; p = 0.01), whereas the homozygous mutant (TT) genotype did not significantly influence risk (OR = 0.81; 95% CI = 0.377−1.74; p = 0.59). For individuals without a family history, similar results were observed. The rs9399137 CT genotype showed a two-fold increased thalassemia risk (OR = 2.66; 95% CI = 1.06−6.68; p = 0.038), whereas the TT genotype did not significantly affect risk (OR = 0.72; 95% CI = 0.24−2.10; p = 0.55). Analysis of the rs1427407 SNP revealed no significant associations with thalassemia in either the presence or absence of family history. The homozygous mutant (GG) and heterozygous (GT) genotypes did not show significant effects on thalassemia risk in individuals with a family history (OR = −, p = 0.16, and OR = 2.22; 0.057−8.65; p = 0.25, respectively). Similarly, for individuals without a family history, the homozygous mutant genotype showed no significant effects on thalassemia risk (OR = 1.26; 0.44−3.61; p = 0.67), whereas the heterozygous (GT) genotype showed a two-fold increased risk (OR = 2.04; 1.03−4.12; p = 0.04).

Table 5: Association of genotype frequencies of BCL11A and *HBS1L-MYB* polymorphisms with age.

SNPs	Age (years)	Genotype/Alleles	Patients (n = 300)	Controls (n = 300)	OR (95 % CI)	p-value
rs4671393	≤14	Overall	248	196	2.53 (1.47−4.34)	0.0008
		AA	52	64	Ref (1)	
		AG	128	72	1.83 (0.58−3.15)	0.02
		GG	78	60	1.04 (0.58−1.84)	0.89
	≥14	Overall	52	104	0.39 (0.23−0.69)	0.0008
		AA	12	34	Ref (1)	
		AG	26	38	1.73 (0.66−4.50)	0.25
		GG	14	32	0.82 (0.29−2.36)	0.27
rs11886868	≤14	Overall	248	196	2.53 (1.47−4.34)	0.0008
		CC	68	74	Ref (1)	
		CT	80	44	1.64 (0.89−3.01)	0.1073
		TT	100	78	1.02 (0.59−1.76)	0.9366
	≥14	Overall	52	104	0.39 (0.23−0.67)	0.0008
		CC	16	40	Ref (1)	
		CT	16	22	1.66 (0.57−4.81)	0.3534
		TT	20	42	0.92 (0.35−2.42)	0.8701
rs9399137	≤14	Overall	248	196	2.53 (1.48−4.34)	0.0008
		CC	66	88	Ref (1)	
		CT	122	52	2.68 (1.52−4.74)	0.0007
		TT	60	56	0.79 (0.43−1.45)	0.46
	≥14	Overall	52	104	0.39 (0.23−0.68)	0.0008
		CC	14	46	Ref (1)	
		CT	26	28	2.71 (1.02−7.25)	0.04
		TT	12	30	0.74 (0.25−2.21)	0.58
rs1427407	≤14	Overall	248	196	2.53 (1.47−4.34)	0.0008
		GG	92	106	Ref (1)	
		GT	64	28	2.09 (1.04−4.18)	0.0378
		TT	92	62	1.27 (0.73−2.230	0.39
	≥14	Overall	52	104	0.39 (0.23−0.67)	0.0008
		GG	20	56	Ref (1)	
		GT	16	14	2.86 (0.90−9.04)	0.07
		TT	16	34	0.92 (0.33−2.52)	0.86

OR odds ratio; CI, confidence interval. OR, CI, and p-value were calculated through regression analysis.

Table 6: Association of genotype frequencies of BCL11A and *HBS1L-MYB* polymorphisms with family history.

SNPs	Family History	Genotype/Alleles	Patients (n = 300)	Controls (n = 300)	OR (95 % CI)	p-value
rs4671393	Yes	Overall	108	44	3.27 (1.86−5.74)	<0.0001
		AA	22	14	Ref (1)	
		AG	56	16	1.88 (0.67−5.22)	0.22
		GG	30	14	0.82 (0.28−2.41)	0.72
	No	Overall	192	256	0.30 (0.17−0.53)	0.0001
		AA	42	82	Ref (1)	
		AG	98	94	1.79 (1.04−3.07)	0.03
		GG	52	80	0.81 (0.45−1.46)	0.49
rs11886868	Yes	Overall	108	44	3.27 (1.86−5.74)	0.0001
		CC	30	16	Ref (1)	
		CT	34	10	1.56 (0.49−4.93)	0.45
		TT	44	18	0.99 (0.36−2.72)	0.9892
	No	Overall	192	256	0.30 (0.17−0.53)	0.0001
		CC	52	98	Ref (1)	
		CT	62	54	1.78 (0.98−3.26)	0.0598
		TT	78	104	1.00 (0.58−1.71)	1.0000
rs9399137	Yes	Overall	108	192	0.32 (0.19−0.51)	0.0001
		CC	30	46	Ref (1)	
		CT	52	52	2.50 (1.24−5.02)	0.01
		TT	26	54	0.81 (0.37−1.74)	0.59
	No	Overall	44	256	0.02 (0.01−0.05)	0.0001
		CC	12	116	Ref (1)	
		CT	22	70	2.66 (1.06−6.68)	0.038
		TT	10	74	0.72 (0.24−2.10)	0.55
rs1427407	(Yes)	Overall	108	44	3.27 (1.86−5.74)	0.0001
		GG	40	24	Ref (1)	
		GT	28	6	2.22 (0.57−8.65)	0.25
		TT	40	14	1.26 (0.44−3.61)	0.67
	(No)	Overall	192	256	0.30 (0.17−0.53)	0.0001
		GG	72	138	Ref (1)	
		GT	48	36	2.04 (1.03−4.12)	0.04
		TT	72	82	1.27 (0.73−2.22)	0.39

OR odds ratio; CI, confidence interval. OR, CI, and p-value were calculated through regression analysis.

The homozygous mutant (TT) genotype in this group also did not significantly affect risk (OR = 1.27; 0.73−2.22; p = 0.39). Family history associations are shown in Table 6.

Discussion

Hemoglobinopathies are considered major health issues worldwide and major causes of death, thus burdening affected people and the health sector. This disease is common in the tropics and subtropics but, because of migration, is observed worldwide.[10] An estimated 60,000 births are affected by thalassemia worldwide, and 5% of the global population shows changes in the alpha or beta globin chains of hemoglobin, but some asymptomatic silent carriers have also been found. These numbers are not exact, because many countries are unable to diagnose this disease properly, and many affected individuals die before proper diagnosis. Some countries do not register patients with thalassemia.[11] According to a prior estimate, 1.7% of the world's population acquires thalassemia from genetic mutations, but the distribution of genetically mutated genes changed with their emergence in Africa, the Middle East, and Mediterranean regions, and extension to Asian countries.[12] This disease has markedly increased in Europe, because of global migration and intermarriage.[13] The frequency of thalassemia is 6% is higher in Pakistan than other countries of the world, because of consanguinity. In Pakistan, thalassemia has high genetic diversity, because of intermarriages and migration.[14] In Pakistan, approximately 5000 children are born with thalassemia major every year, and the carriage frequency is 5−7%.[15]

B-cell leukemia/lymphoma 11A is a protein in humans that is encoded by the BCL11A gene on chromosome 2.[16,17] This zinc finger C_2H_2 type regulatory protein binds DNA. This protein is connected to Switch/Sucrose non fermentable complex (SWI/SNF) and controls gene expression through chromatin remodeling.[18] The BCL11A gene plays a major role in switching off the expression of fetal gamma globin and beta globin chains during the shift from fetal to adult stages. Lower expression of *BCL11A* in adult erythrocytes is associated with higher expression of HbF[19] and has major effects on the brain.[20] BCL11A protein binds the regulatory region of T-Box Brain Transcriptional factor 1 (TBR1) in the neurocortex. It plays a role in hiding the expression of TBR1.[21]

In thalassemia, the conversion of HbF into adult hemoglobin is hindered, thus resulting in elevated HbF levels through effects on globin chains.[22] A major modifier of HBF levels is the minor C allele of the rs11886868 SNP of *BCL11A*. Genotypic analysis indicated three *BCL11A* genotypes: CC,

CT, and TT in rs11886868 at intron 2. This SNP might not disturb the coding series, but it includes development of new signals that interrupt the linking of some transcripts with roles in regulating gene expression.[23]

To investigate *BCL11A* and *HBS1L-MYB* polymorphisms, we collected blood samples from 300 patients at various medical centers in Sargodha, Pakistan, as well as 300 healthy individuals. We observed a significant association between the heterozygous (CT) genotype of the rs11886868 SNP of *BCL11A* and thalassemia risk (OR = 1.79; 95% CI 1.07−3.00; p = 0.0280). The outcomes of our study are analogous to those reported by Genc et al., in 2020, indicating elevated persistence of HbF and β-thalassemia in the population of Adiyaman.[24] Studies on residents of Europe and Central Asia have revealed that rs11886868 is strongly associated with elevated HbF in both normal individuals and those with thalassemia.[25] Studies have revealed that rs11886868 and rs4671393 have the most important effects on amplified HbF levels in fully grown patients. The SNP is very important in increasing HbF and the probability of blood disorders such as thalassemia.

Given that thalassemia is an autosomal recessive disorder, and consanguinity plays an important role in the initiation of this disease, our results indicated involvement of family history in the disease. Ishfaq et al., in 2015, elucidated the influence associated with households with thalassemia in the inhabitants of Southern Punjab, Pakistan.[26] In that study, nearly 500 samples were randomly collected from the population; a total of 306 people were married to their first cousins, and a small percentage of participants were not married to blood relatives. Our results indicated that thalassemia is an inherited disorder associated with the blood and runs in families. Letter et al., in 2008, also indicated that *BCL11A* gene polymorphisms are a genetic marker associated with elevated thalassemia risk and HbF. Beyond thalassemia, *BCL11A* plays roles in many other maladies.[27] For example, it increases tumor formation and cell attack, and acts as an oncogene.[2] Moreover, it promotes programmed cell death during the ovarian cycle. In addition, in type 2 diabetes, elevated *BCL11A* expression is negatively associated with insulin production and secretion.[28]

Studies have shown that variations in the non-coding region between genes GTS1L and MYB (HBS1L-MYB) on chromosome 6q influence HbF levels and other red blood cell traits. We further investigated the roles of these variations and observed that specific variants affect binding sites for key red blood cell development regulators, thereby influencing their interaction with MYB, a crucial HbF controller. Our results explain how these variations influence red blood cell features and highlight MYB as a potential target for therapies aimed at increasing HbF in conditions such as sickle cell disease and thalassemia.[29]

Our study showed the involvement of HBS1L-MYB in thalassemia with two folds increased risk of disease. Our results are consistent with the results of Genc et al., in 2020, studied the relationship of excessive amount of HbF and beta thalassemia in the population of Dairyman, Turkey.[24] In this study, rs4671393 showed a strong association with thalassemia, and was found to increase thalassemia risk. Similar observations have been reported in the Chinese Zhuang population. Lai et al., in 2017, described the associations for the SNPs rs7482144, rs28384513, and rs4895441, and confirmed that those SNPs of the HBG2, and HMIP genes have synergistic consequences on growing the extent of HbF in thalassemia patients of China.[30]

High HbF levels are effective treatments for beta thalassemia and sickle cell anemia. Studies have indicated an association of HbF with QTLs, *HBS1L-MYB*, the *HBG2* promoter, and *BCL11A*. *BCL11A* rs1427407 G>T is associated with HbF levels and contributes 23% of trait variance. *BCL11A*, but not *HMIP*, is associated with elevated HbF levels. Several genetic factors, such as mutations and SNPs, affect HbF at the *BCL11A* gene and the *HBS1L-MYB* intergenic regions regulated by microRNAs, transcription factors, and genetic variations.[31] In primary adult human erythroid cells, *BCL11A* is expressed as two major isoforms at the protein and RNA levels.[32] Our results showed an association between *HBS1L-MYB* and thalassemia risk, in agreement with Gene et al., who have described an association between high HbF levels and beta thalassemia in a Turkish population.[24] Aydınok et al., in 2018, described that SNPs in the intron 2 region of *BCL11A* are strongly associated with HbF.[33] The SNP rs1427407 is important in increasing HbF levels and the risk of blood disorders such as thalassemia. The motifs responsible for fluctuations in HbF levels or F cell numbers have been suggested to reside within or immediately adjacent to a 3 kb region bounded by rs1427407 (position 60,629,694) and rs4671393 (position 60,632,602) in intron 2 of *BCL11A*.[34]

Limitations

Although we strived to adhere to high research standards and to represent the region's population, our sample size was constrained by the four-semester timeframe of a Master's degree study. Future research will include other ethnic groups. We acknowledge potential human error in both sampling and analysis, and the possibility of environmental factors influencing results if they differ from previous studies.

Conclusion

A total of 300 patients with thalassemia and 300 healthy individuals were studied for SNP analysis. Our results indicated a significant association between *BCL11A* and *HBS1L-MYB* gene polymorphisms and thalassemia risk.

Recommendations

In light of our findings and existing research, we believe this study makes a valuable early contribution to the analysis of polymorphisms in thalassemia in Pakistan. However, further research is needed, particularly given the significant associations identified between *BCL11A* and *HBS1L-MYB* SNPs and the disease. These findings hold promise for future biomarker development. Given the rising thalassemia risk in Pakistan, exploring the genetic links between various polymorphisms and the disease through large-scale, regionally diverse studies is essential. Accelerating research efforts in this area would substantially aid in thalassemia prevention and management.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

Ethical approval was provided by the departmental ethical committee and collaborating hospitals.

Consent

Signed consent was provided by each patient before samples were collected.

Authors contributions

KB: Conceptualization, Supervision, Review & Editing; UKN: Writing—Original Draft; RS: Investigation; KA: Methodology; AI: Sample Collection; SA: Article Review; QTA: Application of Statistical Tools; AAA: Review and Finalization. All authors have critically reviewed and approved the final draft, and are responsible for the content and similarity index of the manuscript.

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