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ORIGINAL ARTICLE

Association between BCL11A, HSB1L-MYB, and XmnI γG-158 (C/T) gene polymorphism and hemoglobin F level in Egyptian sickle cell disease patients

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Abstract

Sickle cell disease (SCD) is a monogenic disease characterized by multisystem morbidity and highly variable clinical course. Inter-individual variability in hemoglobin F (HbF) levels is one of the main modifiers that account for the clinical heterogeneity in SCD. HbF levels are affected by, among other factors, single nucleotide polymorphisms (SNPs) at the BCL11A gene and the HBS1L-MYB intergenic region and Xmn1 gene. Our aim was to investigate HbF-enhancer haplotypes at these loci to obtain a first overview of the genetic situation of SCD patients in Egypt and its impact on the severity of the disease. The study included 100 SCD patients and 100 matched controls. Genotyping of BCL11A (rs1886868 C/T), HBS1L-MYB (rs9389268 A/G) and Xmn1 γ^{G}158 (rs7842144 C/T) SNPs showed no statistically significant difference between SCD patients and controls except for the hetero-mutant genotypes of BCL11A which was significantly higher in SCD patients compared with controls. Baseline HbF levels were significantly higher in those with co-inheritance of polymorphic genotypes of BCL11A + HSB1L-MYB and BCL11A + Xmn1. Steady-state HbF levels, used as an indicator of disease severity, were significantly higher in SCD-Sβ patients having the polymorphic genotypes of HSB1L-MYB. Fold change of HbF in both patient groups did not differ between those harboring the wild and the polymorphic genotypes of the studied SNPs. In conclusion, BCL11A, HSB1L, and Xmn1 genetic polymorphisms had no positive impact on baseline HbF levels solely but had if coexisted. Discovery of the molecular mechanisms controlling HbF production could provide a more effective strategy for HbF induction.

Keywords SCD · BCL11A · rs11886868 · HSB1L- MYB · rs9389268 · Xmn1 γ^{G}158 · rs7842144 · Egypt

Introduction

Sickle cell disease (SCD) is an inherited blood disorder characterized by abnormal hemoglobin production, hemolytic anemia, and intermittent small blood vessel occlusion, leading to tissue ischemia and chronic organ damage [1]. It is caused by a single point mutation in the β-globin gene that alters the hemoglobin structure [2]. SCD patients experience acute vaso-occlusive events including pain crisis, splenic sequestration, acute chest syndrome, and stroke, contributing to increased morbidity and early mortality. Complications are related to polymerization of the abnormal hemoglobin S (HbS), leading to erythrocyte sickling and vaso-occlusive crises (VOCs) [3]. The clinical presentation of sickle cell anemia (SCA) shows marked phenotypic variability, involving several genetic and environmental factors [4].

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The two major modifiers of the clinical severity in SCD are concomitant α-thalassemia and persistent HbF (α2γ2) production. α-Thalassemia modulates SCD by lowering the intracellular HbS concentration, which reduces HbS polymer-induced cellular damage and in turn ameliorates hemolysis. High γ-globin expression inhibits HbS polymerization and reduces HbS concentration in SCD patients [4]. During fetal life, HbF is the predominant hemoglobin produced that switches to adult hemoglobin (HbA, α2β2) around birth [5]. By the end of the first year of life, HbF level drops to less than 1% of total hemoglobin [6]. HbF is usually elevated in the β-globin gene disorders due to stress erythropoiesis but can be further boosted by common genetic factors. In addition, factors directly affecting the expression of HBG1 and HBG2, the genes encoding the γ chain of HbF, account for HbF variability [7]. This inter-individual HbF variability accounts for the clinical heterogeneity in SCD patients [8].

In SCD and β-thalassaemia, high γ-globin expression inhibits HbS polymerization and α-globin precipitation, respectively. One of the major ameliorating factors of clinical severity, in both conditions, is the persistence of HbF ($\alpha_2\gamma_2$) production. HbF regulation is a complex genetic process controlled by genetic factors linked to the β-globin gene-like cluster and quantitative trait loci (QTL) located on chromosomes 6, 8, and X-chromosome. Other regulatory loci are also likely to exist as well as epigenetic and cellular factors [9, 10]. Genetic association studies have identified SNPs in major loci that are associated with the variation of HbF levels in patients with SCD or β-thalassaemia [11]. QTLs are associated with elevated HbF levels and consequently influence the clinical course of SCA and account for the inter-individual HbF variability [12]. The first identified QTL was the − 158 C > T, XmnI site (rs7482144), located at the 5′ to HBG2 [13]. SNP (rs2071348) in the -globin locus is in a tight linkage disequilibrium with rs7482144 (HBG2) and is also associated with increased HbF levels. Another SNP (rs5006884) in olfactory receptor (OR) genes (OR51B5 and OR51B6), located upstream of the -globin gene cluster, had been reported in several populations. [12]. Two other QTLs, located in the BCL11A gene and the HBS1L-MYB intergenic region are either implicated in HbF gene silencing in adult life or in cell proliferation and differentiation [14, 15]. HbF-enhancer alleles at all three loci have subsequently been demonstrated to alleviate the severity of SCD and β-thalassemia [16].

Thus, analysis of SNPs that have influence HbF levels can provide crucial genetic information enabling patient stratification and can help predict the severity of disease in β-thalassaemia and SCD patients [9]. Understanding the molecular mechanisms that control HbF production and developing such therapeutic targets for SCD has been a major ongoing research effort for decades [17]. This evidence has led us to hypothesize that genetic variability at loci influencing HbF production has been demonstrated to ameliorate the clinical course of SCD. In the current study, we aimed to investigate the prevalence of three major HbF enhancer haplotypes at 3 loci; BCL11A, HSB1L-MYB, and X*mn*1 in a cohort of pediatric Egyptian SCD patients and their possible influence on HbF levels and disease associated morbidity.

Patients and methods

The study population

The present work included 100 patients diagnosed as SCD and 100 age and sex-matched healthy children with normal hemoglobin electrophoresis as a control group. All patients attended regular follow up visits at the Pediatric Hematology Clinic, Children Hospital, Cairo University (CHCU) between April 2016 and April 2017. The patients group included both sickle cell phenotypes, sickle homozygous (SS) (66%) (Group I) and sickle β-thalassemia (Sβ) (34%) (Group II). The mean age of the patients was 13.68 ± 8.91 years. An age cut-off of > 2 years was applied so that "adult-type" HbF production would not be confounded with residual "fetal-type" HbF synthesis present during the perinatal period. Among the 100 patients, 54 were males (54%). All cases were subjected to complete history-taking, comprehensive clinical examination, and laboratory evaluation at presentation and regular follow up visits. For diagnosis of SCD, hemoglobin profiling was performed by hemoglobin electrophoresis and/or high performance liquid chromatography (HPLC) method. The working definition of vaso-occlusive crises (VOC) was used to assess the severity. VOC is defined as pain in the extremities, back, abdomen, chest, or head that led to an unscheduled clinic or emergency room visit, required hospitalization, and could not be explained except by SCD. Hand-foot syndrome, pain, swelling, and tenderness of digits due to vaso-occlusive episodes leading to ischemia and finally infarction of the distal portions of the extremities, acute chest syndrome, osteomyelitis, and any episode of pain that was treated entirely at home were excluded. Baseline laboratory data at presentation and before the initiation of hydroxyurea (HU) therapy were recorded as well as in the follow up visits (steady-state records). Informed consent was obtained from all subjects' legal guardians in accordance with the 1964 Helsinki Declaration. The research protocol was approved by the Research Ethics Committee of Pediatrics and Clinical Pathology Departments, Faculty of Medicine, Cairo University.

BCL11A, HBS1L-MYB, and Xmn1 γ^G-158 genotyping

BCL11A (rs1886868 C/T), HBS1L-MYB (rs9389268 A/G), and Xmn1 γ^G 158 (rs7842144 C/T) single nucleotide polymorphism (SNP) were tested using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Briefly, DNA extraction from peripheral blood

leucocytes was carried out using the AxyPrep Blood Genomic DNA Miniprep Kit (Cat. No AP-MN-BL-GDNA-50, Axygen Biosciences, USA). DNA was stored at − 20 °C until use. PCR-RFLP was performed on Perkin Elmer thermo-cycler (No 9600, USA) using the PCR Master Mix (Cat. No 101005-Bioron GmbH, Germany). The primers sequences used for amplification were listed in Table 1. Identification of the alleles at each polymorphic site was performed by incubating the PCR product with the following restriction enzymes: MboII, Ssil (Acil), and Xmn1 restriction endoneuclease enzyme (Thermo Scientific Cat. No ER 0821, ER 1791 and ER 0821) for the candidate SNP respectively, followed by electrophoresis through a 2% agarose gel. These PCR products and digested fragments were separately visualized in agarose gel with 2% ethidium bromide staining under UV transillumination. For BCL11A SNP, the wild allele (C) was restricted into 3 fragments, 119 bp, 62 bp, and 42 bp, while the variant allele (T) was restricted into 2 fragments 181, bp and 42 bp. For HBS1L-MYB SNP, the wild allele (A) was identified by one fragment, 277 bp, while the variant allele (G) was presented by 2 fragments, 277 bp and 53 bp. For Xmn1 γ^{G}-158 SNP, the wild allele (C) was identified by one fragment, 650 bp, while the variant allele (T) was restricted into 2 fragments, 450 bp and 200 bp.

Statistical analysis

Data were analyzed using the SPSS with statistical package version 24. Numerical data were expressed as the mean and standard deviation or median and range as appropriate while qualitative data were expressed as frequency and percentage. For quantitative data, non-parametric Kruskal-Wallis and Mann-Whitney tests were used to compare between two groups. For comparison of serial measurements within each patient, the non-parametric Wilcoxon signed rank test was used. The genotype and allele frequencies were analyzed and compared between the patients and controls using the Chi square (χ^2) test. Exact test was used instead when the expected frequency is < 5. Correlations between quantitative variables were done using Spearman correlation coefficient. A p value < 0.05 was considered statistically significant.

Results

Clinical and hematological characteristics of SCD patients

The clinical and hematological baseline characteristics of SCD patients are shown in Table 2. Baseline laboratory data at presentation and before the initiation of HU were recorded as well as in follow up visits (steady-state records). All our

Table 1 Used primers (Fermentas, Lithuania), thermo-cycler program and the size of the PCR products

Genes	Primers	Thermo-cycler Conditions					Final product		
		Initial Denaturation	35 cycles of PCR reaction			Final extension	Wild	Hetero-mutant	Homo-mutant
			Denaturation	Annealing	Extension				
BCL11A	5'-ACCCTCTGACCCCTTCCTTCTAAC-3'	95 °C	94 °C	55 °C	72 °C	72 °C	C/C	C/T	T/T
rs11886868 C/T	5'- TGAATGGATAAACGCCCTATGG-3'	5 min	30s	30 s	45 s	6 min	119 bp + 62 bp + 42 bp	181 bp + 119 bp + 62 bp + 42 bp	181 bp + 42 bp
HBS1L-MYB	5'- ATTCTCGTGCCTCAGCCTCC -3'						A/A	A/G	G/G
rs9389268 A/G	5'-GGTGCCAAAGGTAGCAGATTAGTTAC -3						277 bp	277 bp +224 bp + 53 bp	277 bp + 53 bp
Xmn1 γ^{G}-158	5'-AAC TGT TGC TTT ATA GGA TTT T-3'						C/C	C/T	T/T
rs7482144 C/T	5'-AGG AGC TTA TTG ATA ACC TCAGAC-3'						650 bp	650b +450 bp +200 bp	450 bp +200 bp

Table 2 Clinical and laboratory data of all SCD patients studied, sickle homozygous (SS) and sickle β thalassemia (Sβ) patient groups

Variables	All SCD patients [*N* = 100]	SS [*N* = 66]	Sβ [*N* = 34]	*p* value**
Age [years (mean ± SD)]	13.68 ± 8.91	14.27 ± 8.54	12.53 ± 9.62	0.244
Sex [*N* (%)]				
Male	47 (47%)	31 (46.9%)	16 (47.1%)	0.661
Female	53 (53%)	35 (53%)	18 (52.9%)	
Consanguinity [*N* (%)]	44 (44%)	33 (50%)	11 (32.4%)	0.092
Other SCD patient in family [*N* (%)]	61 (61%)	41 (62.1%)	20 (58.8%)	0.749
G6PD deficiency [*N* (%)]	14 (14%)	11 (16.7%)	3 (8.8%)	0.371
Duration of illness [years (mean ± SD)]	± 8.12	12.09 ± 7.69	9.91 ± 8.83	0.65
Clinical data				
1. VOC history				
VOC/year [N (%)]				
0	1 (1%)	0%	1 (2.9%)	0.282
< 10	51 (51%)	34 (51.5%)	17 (50%)	
10–20	47 (47%)	32 (48.5%)	15 (44.1%)	
> 20	1 (1%)	0%	1 (2.9%)	
Severe VOC per lifetime [N (%)]				
0	22 (22%)	11 (16.7%)	11 (32.4%)	0.201
>3	6 (6%)	4 (6.1%)	2 (5.9%)	
<3	72 (72%)	51 (77.3%)	21 (61.8%)	
2. Transfusion history [*N* (%)]				
Transfusion per lifetime	95 (95%)	63 (95.5%)	32 (94.1%)	1
Transfusion frequency per lifetime				
< 10	68 (68%)	44 (66.70%)	24 (70.6%)	0.690
10–20	32 (32%)	22 (33.3%)	10 (29.4%)	
> 20	0 (0%)	0 (0%)	0 (0%)	
3. Frequent hospitalization (3 times/year over last year) [N (%)]	18 (18%)	13 (19.7%)	5 (14.7%)	0.1
4. Disease-related complications [*N* (%)]				
Acute chest syndrome	4 (4%)	2 (3%)	2 (5.9%)	0.603
Elevated pulmonary artery pressure	17 (17%)	13 (19.7%)	4 (11.8%)	0.317
Osteomyelitis	6 (6%)	5 (7.6%)	1 (2.9%)	0.661
Avascular bone necrosis	4 (4%)	2 (3%)	2 (5.9%)	0.603
Hand and foot syndrome	18 (18%)	13 (19.7%)	5 (14.7%)	0.538
Priapism	2 (2%)	1 (1.5%)	1 (2.9%)	1
Stroke or TIA	5 (5%)	4 (6.1%)	1 (2.9%)	0.659
Fractures	6 (6%)	2 (3%)	4 (11.8%)	0.176
Leg ulcers	3 (3%)	2 (3%)	1 (2.9%)	1
5. Splenic status [*N* (%)]				
Splenomegaly	34 (34%)	19 (28.8%)	15 (44.1%)	0.282
Splenectomy	20 (20%)	15 (22.7%)	5 (14.7%)	
Laboratory investigations				
Baseline Hb [g/dl (mean ± SD)]	8.11 ± 1.79	7.86 ± 1.66	8.60 ± 1.97	0.014*
Baseline MCV [fl (mean ± SD)]	78.37 ± 13.03	83.79 ± 10.24	67.74 ± 11.11	< 0.001*
Baseline MCH [pg (mean ± SD)]	25.25 ± 3.89	26.45 ± 3.91	22.94 ± 2.63	< 0.001*
Baseline TLC [× 10^9/L (mean ± SD)]	12.24 ± 14.43	12.54 ± 17.03	11.65 ± 7.27	0.98
Steady-state Hb [g/dl (mean ± SD)]	8.40 ± 1.36	8.24 ± 1.36	8.76 ± 1.33	0.065
Steady-state reticulocytic count [% (mean ± SD)]	5.96 ± 4.47	6.51 ± 4.84	4.90 ± 3.49	0.123
Steady-state MCV [fl (mean ± SD)]	82.07 ± 12.47	87.18 ± 9.89	72.16 ± 10.99	< 0.001*
Steady-state MCH [pg (mean ± SD)]	26.82 ± 5.21	28.47 ± 5.18	23.63 ± 3.54	< 0.001*
Steady-state TLC [×10^9/L (mean ± SD)]	10.60 ± 5.04	11.27 ± 5.53	9.30 ± 3.64	0.071
Steady-state platelets [×10^9/L (mean ± SD)]	327.55 ± 173.26	335.59 ± 182.12	311.94 ± 156.05	0.51
Steady-state AST [U/L (mean ± SD)]	47.71 ± 35.46	52.97 ± 41.71	37.50 ± 13.55	0.028*
Steady-state ALT [U/L (mean ± SD)]	32.12 ± 33.53	35.52 ± 38.38	25.53 ± 20.09	0.212
Steady-state total bilirubin [mg/dl (mean ± SD)]	2.40 ± 1.96	2.82 ± 2.25	1.59 ± 0.73	0.006*
Steady-state direct bilirubin [mg/dl (mean ± SD)]	0.48 ± 0.41	0.57 ± 0.47	0.31 ± 0.16	0.003*
Steady-state ferritin [ng/ml (mean ± SD)]	1308.46 ± 1130.39	1374.89 ± 1188.13	1185.66 ± 1021.17	0.401
Steady-state LDH [U/L (mean ± SD)]	448.08 ± 229.39	476.55 ± 263.71	392.82 ± 126.93	0.038*
Baseline HbS [% (mean ± SD)]	68.11 ± 16.31	68.72 ± 16.84	66.93 ± 15.40	0.469
Baseline HbF [% (mean ± SD)]	11.52 ± 10.29	10.87 ± 9.96	10.94 ± 12.6	0.351
Steady-state HbS [% (mean ± SD)]	61.33 ± 17.23	66.36 ± 14.85	58.70 ± 17.90	0.032*
Steady-state HbF [% (mean ± SD)]	± 11.01	11.73 ± 11.44	15.55 ± 9.81	0.075
Treatment received				
Hydroxyurea therapy	99 (99%)	66 (100%)	33 (97%)	
Compliance to Hydroxyurea therapy	54 (54%)	31 (47%)	23 (69.7%)	
Dose of Hydroxyurea [(mean ± SD)]	20.98 ± 5.69	21.12 ± 5.6	20.59 ± 5.94	0.032*
Responder to Hydroxyurea	85 (85%)	57 (85%)	28 (87.5%)	0.557
Non-responder	99 (99%)	10 (15.15%)	4 (11.8%)	1

N number of positive cases; *VOC* vaso-occlusive crisis; *Hb* hemoglobin; *TLC* total leucocytic count; *MCV* mean corpuscular volume; *MCH* MEAN corpuscular hemoglobin; *HbF* fetal hemoglobin; *SD* standard deviation

p value** comparison of the Clinical and laboratory data between sickle homozygous (SS) and sickle β thalassemia (Sβ) patient groups

SCD patients were receiving HU except one patient who did not start yet at time of data collection. Ninety-nine (99.0%) of them were responders to HU as evidenced by decreased frequency of VOC, hospitalization, and blood transfusion.

Comparison between Group I and II showed no statistically significant difference in demographic or clinical data. Baseline Hb level was significantly higher in group I (SCD-Sβ) ($p = 0.014$), while baseline MCV and MCH, steady-state MCV, MCH, AST, total bilirubin, direct bilirubin, LDH, HbA1, and HbS levels were significantly higher in group I (SCD-SS). No statistically significant difference was noted between both groups regarding their response to HU therapy (Table 2).

BCL11A, HBS1L-MYB, and Xmn1 γ^G 158 polymorphisms in SCD and controls

Comparing the distribution of the genotypes of the studied SNPs between SCD patients and controls revealed no statistically significant difference except for the hetero-mutant genotype of BCL11A (CT) which was significantly higher in SCD patients (whether SS or Sβ) compared with controls. There was no statistically significant different distribution of studied SNPs between group I and II (Table 3).

For each SNP, patients having the polymorphic genotypes were compared with those with the wild-type genotypes as regards their clinical and laboratory data, in group I and II. For BCL11A, in group I, attacks of hand and foot syndrome were significantly more frequent in those carrying wild genotype (CC) while more frequent attacks of VOCs (10–20/year) were significantly higher in those harboring the polymorphic genotype (CT). In group II, hospitalization frequency (> 3 times/year) was significantly higher in those harboring the wild genotype (CC) and response to HU was significantly higher is those harboring the polymorphic genotypes (CT). For HSB1L, in group I, osteomyelitis and stroke/TIA were significantly higher in patients carrying the polymorphic genotype (CT), while in group II, steady-state HbF level was significantly higher in those patients carrying the polymorphic genotypes (AG + GG). For Xmn1, baseline HbF levels were found to be comparable in either SS or Sβ patient groups harboring the wild or the polymorphic genotypes of the studied SNPs.

Combined genotype analysis in SCD patients (SS + Sβ) revealed that baseline HbF level were significantly higher in those with co-inheritance of polymorphic genotypes of BCL11A + HSB1L-MYB and BCL11A + Xmn1 (Table 4). Baseline HbF level was recorded at presentation, while HbF level during follow-up visits was referred to as the steady-state HbF. Steady-state HbF level was expressed as a percentage and fold increase (10% increment above the baseline level). We found that steady-state HbF level was significantly higher in Sβ patients harboring the HSB1L polymorphic genotypes compared with those carrying the wild genotypes. The fold change of HbF did not differ in either SS or Sβ patient groups harboring the wild or the polymorphic genotypes of the studied SNPs.

Discussion

The clinical heterogeneity of SCD presents a challenge in patients' management. The clinical presentation of SCD ranges from a totally benign one, in which patients may not even be aware of their disease status especially with very high HbF to a marked form with frequent and severe pain episodes, acute clinical events, and early mortality [18, 19]. These observations were followed by several studies in order to understand the natural history of SCD, such as The Cooperative Study of Sickle Cell Disease (CSSCD). CSSCD was a multi-institutional natural history study [20, 21] that identified HbF as a modifier of mortality and a risk factor for early death in African American SCD patients [22]. These findings have been supported by numerous studies of European, Indian, Middle Eastern, and African SCD patients [23], highlighting the importance of genetic regulators of HbF production [21].

Three QTL have been demonstrated to be associated with increased HbF levels and consequently influence the clinical course of SCD and account for the inter-individual HbF variability [12, 13]. Variants within the β-globin gene cluster that affect the expression of HBG1 and HBG genes coding for the γ-chain of HbF were determined, such as rs7482144 (also termed Xmn1 γ^G-158 C/T), a polymorphism within the HBG2 promotor [24]. BCL11A gene, located within the locus control region (LCR) and intergenic regions of the β-globin locus, encodes a zinc finger transcription factor that represses HbF synthesis (γ-globin expression) and HbF production [25, 26]. MYB is a proto-oncogene encoding the c-MYB transcription factor, which is essential in erythroid differentiation [27]. Disruption of the HBSL1-MYB intergenic region in murine model leads to elevated expression of embryonic globins and increased HbF [28]. The present cross-sectional study aimed to investigate the prevalence of a representative SNPs for three HbF QTLs (BCL11A, HMIP-2, and the β-globin gene cluster), the BCL11A (rs1886868 C/T), HBS1L-MYB (rs9389268 A/G), and Xmn1 γ^G158 (rs7842144 C/T), in a sample of the pediatric Egyptian SCD patients. We evaluated the frequency of HbF-enhancer haplotypes at these loci to obtain a first overview of the genetic situation of SCD patients in Egypt. Furthermore, to clarify the possible influence of these SNPs on HbF levels and subsequently disease-associated morbidity.

Baseline HbF level was recorded at presentation, while HbF level during follow-up visits was referred to

Table 3 Genotype distributions of BCL11A, HBS1L-MYB and Xmn1 γ^G 158 polymorphism in SCD, (SS), (Sβ) patient groups and controls

	Control [$N = 100$]	SCD [$N = 100$]	SS [$N = 66$]	Sβ [$N = 34$]	p value
BCL11A rs1886868 gene polymorphism					
CC genotype	29 (29%)	21 (21%)	13 (19.7%)	8 (23.5%)	1 (Reference)
CT genotype	50 (50%)	79 (79%)	53 (80.3%)	26 (76.47%)	< 0.001[a] < 0.001[b] 0.005[c] 0.66[d]
TT genotype	21 (21%)	0%	0%	0%	–
CT + TT	71 (71%)	79 (79%)	53 (80.3%)	26 (76.47%)	0.19[a] 0.18[b] 0.54[c] 0.66[d]
C allele	0.54 (54%)	0.605 (60.5%)	0.598 (59.8%)	0.617 (61.8%)	1 (reference)
T allele	0.46 (46%)	0.395 (39.5%)	0.401 (40.2%)	0.382 (38.2%)	0.39[a] 0.39[b] 0.25[c] 0.77[d]
HBS1L-MYB (rs9389268 A/G)					
AA genotype	49 (49%)	48 (48%)	30 (45.5%)	18 (52.9%)	1 (Reference)
AG genotype	47 (47%)	49 (49%)	34 (51.5%)	15 (44.1%)	0.94[a] 0.74[b] 0.78[c] 0.98[d]
GG genotype	4 (4%)	3 (3%)	2 (3.0%)	1 (2.9%)	0.7[a] 0.85[b] 0.94[c] 0.79[d]
AG + GG	51 (51%)	52 (52%)	36 (54.54%)	16 (47%)	0.89[a] 0.65[b] 0.69[c] 0.48[d]
A allele	0.725 (72.5%)	0.725 (72.5%)	0.712 (71.2%)	0.75 (75%)	1 (reference)
G allele	0.275 (27.5%)	0.275 (27.5%)	0.287 (28.8%)	0.25 (25%)	1[a] 0.8[8b] 0.63[c] 0.52[d]
Xmn1 γ^G 158 (rs7842144 C/T)					
CC genotype	94 (94%)	94 (94%)	61 (92.4%)	33 (97.1%)	1 (Reference)
CT genotype	6 (6%)	6 (6%)	5 (7.6%)	1 (2.9%)	1[a] 0.76[b] 0.68[c] 0.66[d]
TT genotype	0%	0%	0%	0%	–
CT + TT	6 (6%)	6 (6%)	5 (7.6%)	1 (2.9%)	1[a] 0.69[b] 0.68[c] 0.43[d]
C allele	0.97 (97%)	0.97 (97%)	0.962 (96.2%)	0.985 (98.5%)	1 (reference)
T allele	0.03 (3%)	0.03 (3%)	0.037 (3.8%)	0.014 (1.5%)	1[a] 0.7[b] 0.31[c] 0.17[d]

Comparison of genotype distributions of BCL11A, HBS1L-MYB, and Xmn1 γ^G 158 polymorphism between the following groups:

[a] SCD (SS + Sβ) patients and controls

[b] (SS) patient group and controls

[c] (Sβ) patient group and controls

[d] (SS) and (Sβ) patients

as the steady-state HbF. Steady-state HbF was expressed as a percentage and fold increase (10% increment above baseline levels) reflecting the conjoint effect of the existing genetic polymorphism and HU therapy. Steady-state HbF levels were used as an indicator of disease severity.

Menzel et al. [29] have demonstrated that the strongest association with HbF variability was located in intron 2 of the BCL11A gene. The second association cluster is located at the 3′ of the gene downstream of exon 5 of the BCL11A gene. The BCL11A locus is represented by SNP (rs11886868) where C is the wild (common) allele and T is the polymorphic (minor) one. A statistically significant different genotype distribution was observed in SCD, SS (group I) and Sβ (group II) patient groups compared with controls (Table 3). In our Egyptian SCD patients, the variant allele (T) frequency was 0.395 while the wild allele (C) frequency was 0.605. In SS and Sβ patient groups, the baseline HbF levels were comparable between patients harboring polymorphic and wild genotypes. The association of genotypes of the *BCL11A* gene with increased HbF was not observed in this study; this can be attributed to restricted sample size. To clarify the possible impact of the studied BCL11A SNP on the clinicopathological features of the disease, statistical comparison revealed that the annual frequency of VOCs was significantly higher in those harboring the polymorphic genotypes (CT) of BCL11A. This could be attributed to the presence of the (T) allele which was reported to be associated with low HBF production. This goes in accordance with the findings reported by Makani et al. [30] who studied BCL11A (rs11886868) in 1045 Tanzanian patients (SS) and 151 African British patients (146 SS + S/β^0). They demonstrated a marked reduction of mean HbF levels for the genotypes containing the minor allele (T) together with a high prevalence of the minor allele (T) (at a frequency of 0.26). On the contrary, by genome-wide analysis of 4305 Sardinian individuals, Uda et al. [31] found the strongest association between the (C) allele of (rs11886868) in intron 2 of the BCL11A gene ($P = 10(-35)$) and HbF variability. In addition, the C allele was associated with an ameliorated phenotype in patients with β-thalassemia and SCD, indicating that SNPs in the BCL11A gene may modify these clinical phenotypes by increasing HbF levels. Moreover, BCL11A (rs11886868) strongly correlates with HbF levels in CSSCD, where the high-HbF (and protective) allele is "C" in all the studied populations so far [30–33].

The minor allele frequency (MAF) of HBS1L-MYB (rs9382268); G allele was 0.275. This is close to that reported for the African Americans (CSSCD) and Brazilians [8] where the MAFs were 0.19 and 0.20, respectively. The genotypes containing the minor allele (AG + GG) showed higher baseline HbF levels, but the genetic association of the SNP and baseline HbF levels did not reach a statistically significant level in (Sβ) patient group (in order to get it, we would probably have to increase the number of patients studied). HMIP (HBS1L-MYB intergenic polymorphisms) are distributed in three disequilibrium blocks: HMIP blocks 1, 2, and 3. Common alleles within the three blocks are associated with HbF production, with HMIP-2 accounting for the majority of the variation [34]. Eleven SNPs were detected within HMIP-2, all of which exhibited a strong correlation with HbF levels in Europe, but only some of them displayed a significant association in SCD African ancestry patients [35, 36]. This finding is a clear example of the insights gained by using populations of different ethnicities to replicate and detect genetic association. The association of rs28384513 and rs9399137 with HbF levels was first described in northern European descendants [35] and, subsequently, the association of rs4895441 was reported in non-anemic Sardinian patients [29]. Later studies demonstrated that association of these three SNPs with HbF levels in other populations: African American and Brazilian [8], African British [30, 36], and Tanzanian [30].

One of the genetic determinants that are thought to increase HbF levels is the C → T substitution at − 158 of the γ^G globin gene (Xmn1 γ^G-158 C/T gene polymorphism) [37]. For the HBB gene-like cluster, the HbS β-globin gene is found on 4 or 5 common haplotypes reflecting its regions of origin in Africa, the Middle East, and the Indian subcontinent [37–39]. Bantu haplotype showed the lowest HbF levels while Senegal or Saudi-Indian haplotype showed the highest; Benin haplotype showed intermediate HbF levels [13, 40, 41]. Carriers of these SCD haplotypes include rs7482144, Xmn1 γ^G-158 C/T gene polymorphism with considerable HbF variability [42]. Our study revealed that XMN1 (rs7482144 C/T) showed no statistically significant different distribution among SCD cases and controls (Table 3); we could not test this association in the Egyptian SCD cohort because rs7482144 was monomorphic as the frequency of the C allele was much higher than T allele (0.97 vs 0.03, respectively).

We further investigated whether the multiple variants at the three studied loci represented an independent genetic association with HbF levels. Our combined genotype analysis revealed a significant association between baseline HbF levels and co-inheritance of polymorphic genotypes of the studied SNPs. For our SCD patients (SS + SB), baseline HbF level was significantly higher in those with co-inheritance of polymorphic genotypes of BCL11A + HSB1L-MYB and BCL11A + Xmn1 (Table 4). Our findings were in accordance with a study on Tanzanian SCD patients where SNPs in HBS1L-MYB and BCL11A were also found to have a significant impact on HbF levels [30].

In our study, the combination of the T allele of (rs11886868) and the mutant allele G of (rs9389268) and the co-incidence of T allele of (rs11886868) and the T allele of (rs7482144) were associated with an ameliorated phenotype in SCD patients. Sheehan et al. [43] have documented clinical and laboratory phenotypes are modified by these genetic polymorphisms. Our study also demonstrated the association of the mutant genotypes of three SNPs studied and the phenotypic variability of HbF and clinical events.

Previous analyses of the CSSCD database revealed that increased HbF levels correlate with less severe complications, fewer pain episodes [21], and improved survival [22]. Platt et al. [21] originally demonstrated that steady-state HbF levels

Table 4 Comparison of baseline HbF levels in patients harboring BCL11A, HBS1L - MYB and Xmn1 - HBG2 genetic polymorphism in SCD patients (SS + Sβ) [*N* = 100]

Combined genotype analysis in SCD patients (SS + SB) [*N* = 100]			
BCL11A	HBS1L-MYB	Xmn-HBG2	*p* value
+	+		0.031
+		+	0.025
	+	+	0.306

+ Polymorphic genotypes

- Wild genotypes

are a strong indicator of the VOCs frequency, Lettre et al.'s [8] initial observation that HbF-associated SNPs provide predictive information for pain episodes beyond their impact on single measurements of steady-state HbF levels—due to the fact that these variants will influence HbF levels over the lifetime of a patient [44, 45]—suggests that genotyping of these variants may be potentially useful to stratify SCD patients according to severity risk, and to adjust therapeutic modalities accordingly. Therefore, we studied the relationship between the genotypes of the SNPs studied and the annual frequency of VOCs. In our study, for BCL11A SNP, the steady-state HbF levels were comparable in either SS or Sβ harboring the wild or polymorphic genotypes. The annual frequency of VOCs (10–20/year) was significantly higher in SS patients harboring the polymorphic genotypes (CT) due to the presence of the (T) allele that had been reported to be associated with a low HbF levels. So the polymorphic allele (T) of BCL11A was significantly associated with the rate of crisis which reflects the disease severity.

In conclusion, this is a preliminary study to validate SNPs that have been well elucidated in SCD patients with predominantly African or European ancestry, in a sample of the Egyptian pediatric SCD population. Genetic association of these SNPs with baseline HbF levels did not reach a statistically significant level. The variants frequency reflected the specific ethnic make-up of our patients. Moreover, we might report that the enhancer haplotypes of the studied SNPs, BCL11A, HSB1L, and Xmn1, had no positive impact on HbF level solely on the studied SCD cohort, but had if coexisted. Identification of the genetic regulators of HbF production can provide crucial genetic information for patient stratification and prediction of the disease severity. Such information could provide more personalized patient care according to the expected phenotype.

Recommendations

We recommend performing more studies on a larger cohort of patients to gain greater insight into potential impact of the QTL polymorphisms on HbF levels. With their unique genetic background, Egyptian patients are expected to yield a distinctive insight into the effect of modifier loci in SCD. Basic research to understand the molecular mechanisms by which these genetic variants alter HbF expression is important, as it may lead to better-targeted therapeutic modalities.

Authors' contribution All authors were involved in choosing the topic, study design, following the practical part of the paper, revision of the statistical analysis and formulating the results, writing and editing the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate Informed consent was obtained from all subjects' legal guardians in accordance with the 1964 Helsinki Declaration.

Ethical approval The research protocol was approved by the Research Ethics Committee of Pediatrics and Clinical Pathology Departments, Faculty of Medicine, Cairo University.

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