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ORIGINAL ARTICLE

Detection of BCL11A and HBS1L-MYB Genotypes in Sickle Cell Anemia

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Abstract Sickle Cell Anemia (SCA) is one of the most common monogenic disorders worldwide. Molecular modifiers of clinical symptoms play an essential role in the amelioration of the effects of the disease. Single Nucleotide Polymorphisms (SNPs) of the BCL11A gene and within the HBS1L-MYB intergenic region, which are located outside the β-globin locus on chromosome 11, are considered to be genetic modifiers that are associated with elevated levels of foetal haemoglobin HbF, and thus they reduce the clinical impact of sickle haemoglobin, HbS. The work reported here aimed to detect the most common SNPs of BCL11A and HBS1L-MYB related to HbF in SCA patients and to estimate the frequency of occurrence of these genotypes. A total of 132 SCA patients whose condition was stable were recruited from Jeddah city, Saudi Arabia. SNPs at site locus rs4671393 on BCL11A, and at loci rs28384513 and rs9399137 on HBS1L-MYB were identified using TaqMan genotyping assay. Haematological parameters were analysed based on complete blood count and haemoglobin separation using the capillary electrophoresis technique. Highly significant differences in the diagnostic haematological parameters, including all blood-cell types and HbF, were observed between the study cohort and control groups. We also found that BCL11A rs4671393 genotypes of GG and AG were more likely to show increases in HbF levels than other genotypes. In addition, a strong relationship was found between HBS1L-MYB rs9399137 and rs28384513 genotypes in the cohort, whereas no significant association was observed between BCL11A rs4671393 variant and other variants. Our study highlights the importance of investigating genetic determinants that play roles in the amelioration of the severity of clinical symptoms and complications of SCA.

Keywords Sickle cell anemia · Single Nucleotide Polymorphisms · HbF

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Introduction

Sickle Cell Anaemia (SCA) is among the world's most common monogenic disorders. In Saudi Arabia, it is estimated that 4.2% of the population exhibits the heterozygous form while 0.26% has the homozygous form. The highest prevalence is reported in the Eastern Province [1]. Consanguineous marriage is a common traditional habit in Arabian Gulf countries, so the

inherited disorder spreads widely. The clinical manifestation of SCA varies from mild to severe haemolytic anaemia based on several factors such as the mode of inheritance, sickle haemoglobin (HbS) genotype, β-globin cluster haplotypes, α-globin numbers and foetal haemoglobin (HbF) expression [2]. Patients with severe forms may suffer from acute chest syndrome, stroke, splenic and renal dysfunction, pain crises and bacterial infections. Amelioration effects and genetic modification to reduce clinical severity have been widely addressed through many studies [3–5]. Among these, determination and identification of molecular factors that regulate HbF expression have been under research focus for many years, since HbF is considered a natural, protective molecule that interferes with the deterioration effect of HbS polymerisation. Normally, HbF constitutes the major type of haemoglobin present at birth and gradually decreases to 1–2% of all haemoglobin in the body by the first year of life [6].

However, HbF levels may vary considerably in SCA patients due to their levels of genetic determinants such as Single Nucleotide Polymorphisms (SNPs). These SNPs are considered the main factors that affect production of HbF and thus can increase interference with HbS synthesis. Therefore, increased levels of HbF and the number of erythrocytes that have HbF can ameliorate the severity of the SCA as well as of β-thalassaemia [7]. Several SNPs have been identified at several loci that are associated with the presence of various amounts of HbF in patients with SCA or β-thalassaemia and in healthy adults. The presence of these SNPs at their loci is responsible for 20–50% of the HbF trait variance in patients with SCA or β-thalassaemia, and in healthy Europeans [5, 8–10]. Genetic variation at the β-globin (HBB) cluster on chromosome 11p, at the BCL11A gene on chromosome 2p and at the HBS1L-MYB region on chromosome 6q have been widely investigated in several populations and shown to influence HbF levels and disease severity of haemoglobinopathies that involve HBB, particularly SCA and β-thalassaemia. Such loci express variable phenotypic effects through modulation of expression of the γ-globin gene (HBG) resulting in variability in HbF production. These loci thus are considered Quantitative Trait Loci (QTL) [11]. Presence of SNPs at these loci causes an upregulation of HBG or of transcription factors that have direct positive effects on the synthesis of HbF instead of HbS, resulting in reductions in the severity of the disease.

To our knowledge, there is no clear picture of the prevalence of these SNPs in SCA patients of the western region of Saudi Arabia. Therefore we conducted this work in order to detect the most common SNPs related to HbF in SCA patients in that region and estimate their genotype frequencies. These SNPs included BCL11A, rs4671393; and HBS1L-MYB, rs28384513 and rs9399137.

Materials and Methods

Ethical Approval

The study was approved by the Research Ethics Committee at King Abdulaziz University Hospital, Ref. No. (HA-02-J-008).

Subjects Studied

The subjects of this study were 132 patients diagnosed as having SCA and whose condition was in a steady state. Blood samples were collected after consent was gained from patients at King Abdulaziz University Hospital. Diagnostic investigation involved Complete Blood Count (CBC) performed on a Sysmex XE-2100 analyser (Sysmex Corporation, Kobe, Japan), and estimation of HbS and HbF levels through use of a capillary electrophoresis instrument (Sebia, Issy-les-Moulineaux, France). Samples with high HbF levels (> 2%) were included in the study for subsequent molecular analysis.

Molecular investigation involved DNA extraction using a Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. Briefly, 300 µl of whole blood were added to a double volume of cell lysis solution. Vigorous mixing was followed by incubation for 10 min at 65 °C and centrifugation at high speed for one minute. The supernatant was mixed thoroughly with 100 µl of protein precipitation solution and then centrifuged at high speed for three minutes. This was followed by the addition of 500 µl of isopropanol to the generated supernatant and centrifugation for DNA precipitation. Removal of excess salt was performed using 70% ethanol prior to DNA rehydration with 100 µl of tris-ethylenediaminetetraacetic acid (TE) buffer.

After this, DNA samples were genotyped for BCL11A rs4671393, HBS1L-MYB rs28384513 and HBS1L-MYB rs9399137 using TaqMan SNP genotyping assay (Applied BioSystems, Foster City, CA, USA) following the manufacturer's procedure. Briefly, about 10 ng of gDNA was amplified using 0.5 µl of diluted 1:2 40 × primer-TaqMan probe mix and 5 µl of 2X TaqMan Fast Advanced master mix. Variant calling was determined using an allelic discrimination plot performed on an Applied BioSystem StepOne reverse transcriptase polymerase chain reaction (RT-PCR) system.

Statistical Analysis

Data analysis was conducted using SPSS Inc. Software, version 22.0. The frequencies of occurrence of genotypes and alleles were estimated within our cohort population to determine any significant differences in these frequencies between sickle-cell disease patients and control groups. To study the association of HbF levels with sickle-cell disease and to determine the effect of BCL11A variant rs4671393 and HBS1L-MYB variants rs28384513 and rs9399137 on HbF levels within the SCA group, binary logistic regression was performed. Odds ratios (ORs) were calculated to determine the effects of the genotypes on HbF levels among the study groups.

Results

In a cohort of 132 sickle-cell patients and 25 controls, highly significant differences were observed between both groups in age and haematological parameters: White Blood Cell (WBC), Red Blood Cell (RBC), Hb, Haematocrit (Htc), Mean Corpuscular Haemoglobin Concentration (MCHC), Red Cell Distribution Width (RDW), Platelet (PLT) and HbF levels ($P = < 0.001$) (Table 1). This was expected as RBC levels are usually found to be significantly lower in SCA patients than in healthy individuals. Notably, the patient group was characterised by raised HbF levels. However, no HbS level differences were tested between patient and control groups because all control cases had HbS levels of zero.

Table 1 Hematological parameters in sickle cell anemia versus control groups

Parameters	SCA	Control	*P* value
Age	19.8 ± 11.7	34 ± 20.6	–
WBC	11.4 ± 5.7	8.7 ± 4.1	*< 0.001*
RBC	3 ± 0.8	4.7 ± 0.8	*< 0.001*
Hb	8.4 ± 1.6	13.5 ± 2.8	*< 0.001*
Hematocrit (Hct)	24.9 ± 4.6	39.3 ± 8.8	*< 0.001*
MCV	82.7 ± 10	82.9 ± 10.4	0.457
MCH	27.9 ± 3.8	28.6 ± 3.4	0.469
MCHC	33.7 ± 1.3	34.5 ± 1.2	0.102
RDW	19 ± 3.6	13.5 ± 2	*< 0.001*
Platelets (Plt)	382.7 ± 150.2	264.4 ± 67.1	*< 0.001*
HbF	10.2 ± 7.3	0.56 ± 1	*< 0.001*
HbS	64.7 ± 21.7	–	–

Italic values indicate statistically significant of *P* value ($P < 0.05$)

Quantitative data are presented as (mean ± SD)

P values are based on *t* test for normal continuous variables

Mann–Whitney test for not normal continuous variables

Table 2 Frequencies of BCL11A and HBS1L-MYB genotypes in sickle cell anemia

SNP	SCA	Control	*P* value
Genotype frequency (*BCL11A rs4671393)*			0.563
A/A	14 (10.61%)	1 (4%)	
A/G	48 (36.36%) 36.36	9 (36%)	
G/G	70 (53.03%)	15 (60%)	
Total	132	25	
Allele frequency			
A	76 (28.79%)	11 (55%)	
G	188 (71.21%)	9 (45%)	
Total	264	20	
Genotype frequency (*HBS1L-MYB rs9399137)*			< 0.001
C/T	26 (19.70%)	0 (0%)	
T/T	106 (80.30%)	9 (100%)	
Total	132	9	
Allele frequency			
C	26 (9.85%)	0 (0%)	
T	238 (90.15%)	18 (100%)	
Total	264	18	
Genotype frequency (*HBS1L-MYB rs28384513)*			0.002
G/G	0 (0%)	2 (9.09%)	
G/T	43 (33.08%)	6 (27.27%)	
T/T	87 (66.92%)	14 (63.64%)	
	130	22	
Allele frequency			
G	43 (16.54%)	10 (27.78%)	
T	217 (83.46%)	26 (72.22%)	
Total	260	36	

Qualitative data presented as frequencies

P values are based on Chi square test for categorical variables

To determine the relationship between SCA and each genotype of BCL11A rs4671393 and HBS1L-MYB rs9399137, and HBS1L-MYB rs28384513, Minor Allele Frequencies (MAFs) were calculated and they are shown in Table 2. For BCL11A rs4671393 locus G > A, and HBS1L-MYB rs9399137 T > C locus and rs28384513 locus T > G, MAFs were 28.79%, 9.85% and 16.54% respectively. Highly statistically significant relationships between HBS1L-MYB rs9399137 and rs28384513 genotypes and the SCA group were detected, $P = < 0.001$, $P = 0.002$ respectively. In contrast, no significant association was observed between the BCL11A rs4671393 variant and SCA, $P = 0.563$. It is noteworthy to mention that we started initially with 25 control samples which were genotyped. However, for this particular SNP (HSB1L-MYB rs9399137), only data from nine samples were available for statistical analysis. Samples with

Table 3 Association signal of Fetal hemoglobin level (HbF) with sickle cell anemia for BCL11A rs4671393 and HBS1L- MYB rs9399137 and rs28384513 loci

Variable	B	*P* value	OR	95% CI for OR	
HbF	1.376	*P = < 0.001*	3.96	2.092	7.492
BCL11A rs4671393 genotype					
A/A	0.965	0.46	2.63	0.20	34.52
A/G	0.916	0.005	2.50	1.33	4.70
G/G	2.614	0.004	13.66	2.316	80.532
HBS1L-MYB rs9399137					
T/T	1.54	0.006	4.68	1.55	14.10
HBS1L-MYB rs28384513 Genotype					
G/T	85.39	0.96	1.212E+37	0.000	–
T/T	1.78	0.004	5.90	1.79	19.52

OR: estimated Odd Ratio. CI: 95% Confidence Interval

B: slope of the line between the predictor variable and the dependent variable

undetermined genotype calling were excluded and missing information was considered during the analysis.

Logistic regression analysis revealed a strong association between SCA and an increase in foetal haemoglobin level; $P = < 0.001$ (Table 3). The data indicate that the SCA patients had HbF levels 3.96 times those of the control group. Further investigation was performed to study the impact of the BCL11A rs4671393 locus on the HbF level. It was found that a very strong effect was reported for AG and GG genotypes; $P = 0.005$ and $P = 0.004$ respectively. It can be inferred that SCA patients with AG or GG genotypes are more likely to have elevated levels of HbF; the odds ratios were calculated respectively as 2.50 and 13.66. Similar significant impacts on HbF level in SCA patients were identified among those with the HBS1L-MYB rs9399137 TT genotype ($P = 0.004$, $OR = 4.68$) and those with the HBS1L-MYB rs28384513 TT genotype ($P = 0.004$, $OR = 5.90$). These findings indicate that patients with the TT genotype of both SNPs are likely to exhibit HbF levels elevated by 4.68 and 5.90 times compared with their healthy counterparts. Apparently, the T allele on HBS1L-MYB rs2838451 and HBS1L-MYB rs9399137, and the G allele on BCL11A rs4671393, were the potential genetic factors in the homozygous form that had the most effect on the elevation of HbF levels in our SCA cohort.

Discussion

Sickle cell anaemia is a common monogenic disorder, and in some regions of Saudi Arabia it presents in high frequency due to consanguineous marriage [12, 13]. The disease is characterised by a variable phenotypic severity that is influenced by several factors such as gender, co-inheritance of other globin anomalies, presence of genetic modifiers such as HbF, environmental factors, and behavioural and health conditions [11, 14, 15]. This variability in clinical severity affects the haematological diagnostic parameters accordingly. In this study, we investigated three SNPs related to HbF levels in SCA patients and estimated their genotype frequencies. These SNPs were BCL11A rs4671393; and HBS1L-MYB rs28384513 and rs9399137. We selected these three polymorphisms based on their frequeny of investigation performed by other studies [3, 16, 17]. We also examined changes in haematological parameters among SCA patients compared with healthy controls.

We found some significant differences in the haematological parameters among the studied cohort. These changes can act as markers for clinical complications in these patients. With regards to WBC and platelet counts, we found significant increase in the total corrected WBC and platelets which are not unusual in SCA status. The majority of studies performed on SCA patients have shown significant increases in the total counts since the disease is characterised by active inflammation and haemolysis, which trigger continuous production of WBC and platelets resulting in high peripheral counts [18–24]. A previous study performed on SCA patients to evaluate the laboratory markers during steady states of the disease found that increased emergency department utilisation was significantly associated with elevated steady-state counts of WBC and platelets ($p < 0.001$). This suggested that the patients were in an inflammatory state even at their baseline steady states [18]. Okpala [19] states that in severe forms of sickle-cell disease, leukocytosis, particularly neutrophilia, contributes to the pathophysiological progress of the disease through various events such as infection and involvement of other vital organs such as kidneys. This is supported by a recent study performed on patients with Sickle Cell Disorder (SCD) in the eastern region of Saudi Arabia, in which it was found that the majority of abnormal leucocyte counts represented high WBC and PLT counts [22]. Another study performed recently in Ghanaian patients with sickle-cell disease found that WBC and PLT counts were significantly higher in both female and male patients with SCD than in their healthy counterparts ($P < 0.05$) [20]. Our result is consistent with the above-mentioned studies, since we observed significant abnormality in term of increasing counts of WBCs and platelets. On the other hand, RBC parameters were observed to be significantly low compared with those of the control group. This was expected, since the whole marker of SCA in patients is the presence of anaemia, which reflects active

haemolysis of RBCs. This finding is similar to those of other works performed on RBC parameters [20, 25–27].

BCL11A rs4671393 and HBS1L-MYB rs28384513 and rs9399137 are quantitative trait loci (QTL) found in intergenic regions, and they can act as potential sites for re-activation of HbF. Thus it is important to analyse them in the Saudi population. Our patient cohort was recruited from Jeddah city in the western region of Saudi Arabia, where HBS1L-MYB has been found to be statistically and significantly associated with SCA.

On the other hand, our analysis showed no statistically significant association between the genotypes of BCL11A rs4671393 at this specific locus and SCA, but instead discovered a strong association with HbF production. A recent study made the same finding of no significant differences in allele frequencies of SNPs in the BCL11A region between patients and controls [28].The study was performed among patients with β-thalassaemia, while our work was done in patients with SCA, but we can infer the same finding since both disorders affect the HBB gene. Our results were expected as SCA results from a point mutation in the HBB gene but not from BCL11A. The BCLA11A is a transcription factor in the zinc-finger protein family that has a role in the suppression of HbF production. Mutation of BCL11A results in re-activation of HbF, causing an amelioration of SCA severity [29]. The effect of BCL11A rs4671393 genotypes was observed to be more probably associated with genotypes GG and AG, which is consistent with a previous study performed in Tunisian SCA patients [30].

In addition, we found a strong statistical association between the presence of HBS1L-MYB variants and SCA, which indicated a possibility of involvement of these variants in the pathogenesis of SCA. A deep insight investigation is required to reveal this linkage. Mikobi et al. [31] compared the observed frequencies of eight SNPs present in Congolese SCA patients with those found in healthy patients. At the SNP HBS1L-MYB rs9399137, our result showed that the frequency of genotype TT was significantly higher than that of the genotype CT. In addition, the frequency of HBS1L-MYB, rs28384513 genotypes was observed to be significantly higher in TT than GT. These results are consistent with those of other previous studies [8, 32, 33].

Since the studied SNPs are known to be involved in the elevation of HbF levels, we studied their impact on HbF level in our cohort. The BCL11A, rs4671393 genotypes for AG and GG were found to be strongly associated with elevated HbF levels. It can therefore be inferred that SCA patients who have AG or GG genotypes are more likely to have elevated HbF levels, with odds ratios calculated respectively as 2.50 and 13.66. Our results showed a consistency with other studies performed in SCD and β-thalassaemia [5, 28, 30]. For instance, a previous study was performed in two independent SCD cohorts: the cohort that took part in the African American Cooperative Study of Sickle Cell Disease (CSSCD) and an SCD cohort from Brazil. The aim of the study was to analyse linkage between presence of BCL11A SNPs, HBS1L-MYB SNPs and elevated HbF levels. Both types of SNP were strongly associated with HbF level variation. The study concluded that BCL11A at rs4671393 locus could be considered one of the best signals of association with HbF levels in SCA patients [5]. This association was also investigated among Saudi patients from the southwestern region. It was found that BCL11A was the sole QTL associated with elevated HbF levels; HBS1L-MYB SNPs showed no similar association [33]. However, our results showed partial agreement with the conclusion of the previous Saudi study with regard to the BCL11A SNP but not with other SNPs. This variation may be due to the involvement of other external factors and conditions that have not been accounted for.

In conclusion, our study demonstrates the importance of examining genetic determinants that play important roles in ameliorating the severity of clinical symptoms and complications of SCD by targeting genes involved in upregulation of HbF. Stimulation of HbF production can be used as an option to treat SCA patients.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest associated with this publication.

Ethical Approval The study is approved by the Research Ethics Committee at King Abdulaziz university Hospital, Ref. No. (HA-02-J-008).

Informed Consent Informed consent was obtained from all individual participants included in the study.

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