rs11886868 and rs4671393 of BCL11A associated with HbF level variation and modulate clinical events among sickle cell anemia patients

Leila Chaouch1, Imen Moumni1, Houyem Ouragini1, Imen Darragi1, Miniar Kalai1, Dorra Chaouachi1, Imen Boudrigua1, Raouf Hafsia2, Salem Abbes1

1Université de Tunis El Manar, Institut Pasteur de Tunis, Laboratoire d’Hématologie Moléculaire et Cellulaire, Tunisia, 2Université de Tunis El Manar, Hopital Aziza Othmena, d’Hématologie Clinique, Tunisia

Aims: Fetal hemoglobin (HbF) modulates the phenotype of sickle cell anemia (SCA) by inhibiting deoxy sickle hemoglobin (HbS) polymerization. HbF genes are genetically regulated, and the level of HbF and its distribution among sickle erythrocytes is highly variable. Herein, we aimed to determine whether two functional polymorphisms of BCL11A are implicated in the variation of HbF and clinical events in SCA Tunisian patients.

Material and methods: The studied population consisted of 148 SCA patients with SS phenotype. The group of patients was divided into two subgroups according to the threshold point of %HbF which is 15%. Genotyping of rs11886868 and rs4671393 was performed using PCR/Sequencing. To test for trait association with the candidate SNPs, genotype and allele frequencies between ‘group who had %HbF < 15’ and ‘group who had %HbF > 15’ (controls) were compared using Pearson’s chi-square test (compare 2, version 1.02). The association of each genotype and the combined genotype with complications was performed by logistic regression test.

Results: Our findings showed that the majority of patients carried genotype CT of rs11886868 and genotypes AG and GG of rs4671393 present HbF level < 15%. RR = 0.08, RR = 0.176, and RR = 0.189, respectively. The results showed a significant association between the alleles T of rs11886868 and G of rs4671393 and %HbF < 15% with P = 0.016; RR = 0.39 and P = 8.9 x 10\(^{-3}\); RR = 0.567, respectively. Interestingly, the C allele of the rs11886868 and the A allele of the rs4671393 were associated with an ameliorated phenotype in patient’s SCA. The combination of the genotypes GG and CT explains more phenotypic variance than the sum of the two BCL11A SNPs taken individually.

Keywords: Sickle cell anemia, HbF, BCL11A gene

Introduction
On the basis of many studies, it is believed that the elevation of HbF and the presence of alpha-thalassemia would improve the clinical course and general well-being of patients with sickle cell anemia (SCA).\(^1,2\) The beneficial effects of high HbF in SCA and in beta-thalassemia, where HbF can substitute for HbA, launched an effort to find drugs capable of increasing HbF levels.\(^3\) In the past few years, the treatment of SCA was based on the elevation of HbF using hydroxyurea.\(^4\) Patients respond to this treatment albeit to very different degrees, and some with very little clinical benefit.\(^5\) Among responders, the increment in HbF is variable, suggesting the need for additional agents capable of inducing HbF and perhaps broadening its cellular distribution. Subsequent studies demonstrated that B-cell lymphoma/leukemia 11A (BCL11A) is a bona fide repressor of HbF expression.\(^7-9\)

Previous genome-wide association studies have reported a significant association between the BCL11A gene and hereditary persistence of fetal hemoglobin (HbF).\(^10-12\)

Moreover, one class of promising agents are histone deacetylase (HDAC) inhibitors whose inhibition is associated with increased expression of HBG. Arginine butyrate, a short-chain fatty acid with HDAC inhibitory activity used as single agent or with hydroxyurea, has been associated with increases
in HbF. High throughput screening studies with follow-up of promising candidates have suggested that strong inhibitors of HDAC1 and HDAC2 were associated with substantial increments in both HBG expression and HbF in vitro. BCL11A has been shown to interact with HDAC1 and HDAC2. Herein, we aimed to determine whether two functional polymorphisms of BCL11A are implicated in the variation of HbF and clinical events in SCA Tunisian patients.

Material
This is a retrospective study which consisted of 148 SCA patients with SS phenotype. All patients had repeated HbF determinations and about whom large numbers of hematological indices and clinical data are available. There were 76 males and 72 females. The ages at entry ranged from 1 to 18 years.

Data laboratory
Diagnosis of sickle cell patient was performed using cation-exchange high-performance liquid chromatography (D10, Bio-Rad) and further confirmation by means of molecular diagnosis by restriction fragment length polymorphism using DdeI as previously described by Bendaoud. Biochemical data were averaged for each patient in steady state (at least three values). We determined total and HbF concentrations (D10, Bio-Rad), reticulocyte count, and other hematological parameters using ABX PENTRA 60 C+.

Clinical parameters
For purposes of analysis, six commonly occurring events that were used as measures of severity were divided into two categories on the basis of the nature of the episode. Clinical events included in our study are: vaso-occlusive crisis (VOC), osteonecrosis, stroke, cholelithiasis.

Determination of threshold level
The chosen HbF groups resulted in the following distribution of sample sizes: 42 patients with 5–10% HbF, 32 patients with 10–15% HbF, 20 patients with 15–20% HbF, and 54 patients with 20–30% HbF. This caused the relatively high incidence rate at this level: 15%. The mean and standard deviation for HbF were 15 ± 7% and the range was 5–33.

Genotyping of rs11886868 and rs4671393
Genomic DNA was isolated from white blood cells of total blood using standard method (phenol/chloroform). rs11886868 and rs4671393 of BCL11A were genotyped by polymerase chain reaction (PCR)/sequencing using two pairs of primers namely: CACTGAACCCCCACCTACCA and R: GCAGCCTGGAGGATGACAAA for the rs11886868 and F: ACCTCCCCATTAGCAGCA, R: CTCCTCTCCCCGTACCTTCC for the rs4671393. PCR was performed in 25 μl reaction volumes containing 100 ng of genomic DNA, 0.2 mmol/l of each dNTP, 50 mmol/l KCl, 15 mmol/l Tris–HCl (pH 8.0), 2.5 mmol/l MgCl₂, 0.5 U AmpliTaq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), and 10 pmol of each forward and reverse primers. The PCR cycling conditions included an initial denaturation of 10 minutes at 94°C followed by 35 cycles of 94°C for 1 minute, annealing at 59°C for 1 minute, and extension at 72°C for 1 minute. The run was ended by a final extension at 72°C for 7 minutes. PCR products were then purified and doubly sequenced (forward and reverse) by ABI PRISM Big Dye Terminator on Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 310 DNA sequencer (PEApplied Biosystems, Foster City, USA).

Data analysis
One hundred and forty-eight patients with SS phenotype were enrolled in the analysis. The sample of SCA patients was divided into two groups according to the threshold level of HbF (15%). We compared demographic and hematological and clinical data between the two groups of patients.

This research is comprised of two sections. The test for trait association with the candidate SNPs, genotype and allele frequencies between two groups according to the threshold level of HbF (15%) were the focus of Section 1. In Section 2, the focus was to determine the risk of clinical events’ occurrence in different genotypes and in combined genotype.

Statistical analysis
The demographic and hematological data are normally distributed, so we used means and standard deviations. For each variable (demographic, hematological, and biochemical) differences between cases and controls were evaluated applying the t-test or the non-parametric Mann–Whitney test as appropriate using SPSS (version 18). The Hardy–Weinberg equilibrium was tested using the software package Arlequin (version 3.01). Genetic differences between the two groups were evaluated by applying exact tests to genotypic or allelic contingency tables using compare 2 (version 1.02). The relationships between genotypes found and clinical events were determined using logistic regression.

Results
The exploration of HbF profile in our population of study showed that the threshold level of HbF is 15%. The two groups of patients stratified accordingly to the HbF% were compared for age, sex ratio, and the hematological parameters. The results showed a
significant difference for the Hb level and mean corpuscular volume between the two groups (Table 1).

The investigation of Section 1 demonstrates that for each polymorphism the samples were found to be in Hardy–Weinberg equilibrium ($P > 0.05$). The results of the genotyping of the rs11886868 in intron 2 of the $BCL11A$ show the presence of three genotypes: CC, CT, and TT. C is the normal allele and T is the mutant one. We observed that the majority of patients with normal genotype CC presented HbF $> 15\%$. The statistical analysis shows that genotype CT and mutant allele T are associated with diminution of HbF level (Table 2). As for the genotyping of the rs4671393 in intron 2 of the $BCL11A$, the results show the presence of three genotypes: AA, AG, and GG. A is the normal allele and G is the mutant one. We observed that the majority of patients with heterozygote genotype AG and mutant genotype GG presented HbF $< 15\%$. The statistical analysis shows that genotypes AG and GG and mutant allele G are associated with diminution of HbF level (Table 2). Interestingly, the combination of the genotypes GG and CT presents strongest association with HbF% variance than the sum of the two $BCL11A$ SNPs taken individually (Table 2).

The investigation of Section 2 demonstrates that the occurrence of each clinical event is variable between the two groups of patients according to the threshold level of HbF. The results show that the risk of occurrence of clinical events is higher in the group who presented genotypes associated with HbF $< 15\%$ (Table 3). In addition, the C allele of the rs11886868 and the A allele of the rs4671393 were associated with an ameliorated phenotype in patient’s SCA. The combination of the genotypes GG and CT explains more phenotypic variance than the sum of the two $BCL11A$ SNPs taken individually (Table 3).

**Discussion**

Menzel et al. have reported that the strongest associations with HbF variation were in a region spanning 14 kb in the second intron of the $BCL11A$ gene. The

---

**Table 1** Hematological, demographic, and clinical data of studied population

|                      | SS patients with HbF < 15% | SS patients with HbF > 15% | $P$  |
|----------------------|-----------------------------|----------------------------|------|
| Age (mean)           | 27 ± 2.9                    | 31 ± 3.6                   | 0.425|
| Sex ratio (M/F)      | 39/37                       | 37/35                      | 0.423|
| Hb (g/dl)            | 7.3 ± 0.9                   | 10 ± 0.6                   | 0.046|
| RBC (1012/l)         | 2.89 ± 1.02                 | 3.69 ± 1.2                 | 0.425|
| MCV (fl)             | 77.2 ± 1.3                  | 80 ± 2.7                   | 0.059|
| MCH (pg)             | 35.7 ± 1.02                 | 37 ± 2                     | 0.132|
| RDW (%)              | 5.29 ± 1.02                 | 5.69 ± 0.1                 | 0.690|
| HbA                  | 0                           | 0                          | 1    |
| HbS (%)              | 86.4 ± 0.4                  | 76 ± 0.1                   | 0.05 |
| HbF (%)              | 10.6 ± 4.3                  | 22 ± 7.3                   | 0.045|
| HbaA2                | 3 ± 0.1                     | 2.7 ± 0.3                  | 0.725|

SS: homozygous of beta-globin gene mutation.
The demographic and hematological values are indicated as mean ± standard deviation.
Hb, hemoglobin; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red blood distribution width.
Statistics for the comparison of demographic and hematological variables between the two groups were performed using the t-test and chi-square test as appropriate (SPSS 16.0).

**Table 2** Distribution of genotypes and allele frequency of polymorphisms studied according to the HbF level in SCA patients

| BCL11A gene | SCA patients with HbF < 15% | SCA patients with HbF > 15% | $P$  | RR CI 95% |
|-------------|-----------------------------|----------------------------|------|-----------|
| Rs 11886868 |                             |                            |      |           |
| CC          | 0                           | 14                         |      |           |
| CT          | 46                          | 14                         | 0.029| 0.08 (0.002–0.885) |
| TT          | 28                          | 28                         |      |           |
| C           | 0.42                        | 0.62                       |      |           |
| T           | 0.70                        | 0.58                       |      |           |
| Rs4671393   |                             |                            |      |           |
| AA          | 0                           | 22                         |      |           |
| AG          | 28                          | 20                         | 0.016| 0.0176 (0.03–1.15) |
| GG          | 46                          | 32                         | 0.021| 0.189 (0.03–1.22) |
| A           | 0.187                       | 0.396                      |      |           |
| G           | 0.812                       | 0.566                      |      | 8.9 × 10$^{-3}$ (0.35–0.93) |
| Combined genotype |                        |                            |      |           |
| GG/CT       | 46                          | 14                         | 0.01 | 0.59 (0.188–0.951) |
| AG/CT       | 28                          | 14                         | 0.05 | 0.89 (0.176–0.853) |

*: reference group; $P$, index of significance; RR, relative risk; CI, interval of confidence.
second association cluster spanned 67 kb in the 3-prime region of the gene downstream of exon 5 of the \textit{BCL11A} gene.

By genome-wide analysis of 4305 Sardinian individuals, Uda \textit{et al}.\textsuperscript{11} found a strongest association between the C allele of an SNP (rs11886868) in intron 2 of the \textit{BCL11A} gene ($P = 10^{-35}$) and variation of HbF. In addition, the C allele was associated with an ameliorated phenotype in patients with beta-thalassemia and SCA, indicating that SNPs in the \textit{BCL11A} gene may modify these phenotypes by augmenting HbF levels.

Among 1275 African Americans and 350 Brazilians with sickle cell disease, Lettre \textit{et al}.\textsuperscript{16} found a significant association between HbF levels and SNPs in the \textit{BCL11A} gene. The most significant association among both groups was with rs4671393 ($P = 2 \times 10^{-42}$) among African Americans, $P = 3 \times 10^{-8}$ among Brazilians). The effect of these SNPs could explain 6.7–14.1% of variance in HbF levels. Our results show that rs11886868 and rs4671393 are associated to the variation of HbF. Moreover, the statistical analysis shows that genotype CT and mutant allele T of rs11886868 are associated with diminution of HbF level. The same result was observed for genotypes AG and GG and mutant allele G of rs4671393. Interestingly, the most significant association among our group was with rs4671393 ($P = 8.9 \times 10^{-3}$). This finding is similar to those observed in African Americans and Brazilians.

Galarneau \textit{et al}.\textsuperscript{12} re-sequenced 175.2 kb from these loci in 190 individuals including the HapMap European CEU and Nigerian YRI founders and 70 African Americans with SCA. The authors discovered 1489 sequence variants, including 910 previously unreported variants. Using this information and data from HapMap, Galarneau \textit{et al}. selected and genotyped 95 SNPs, including 17 at the \textit{BCL11A} locus, in 1032 African Americans with SCA. Consistent with earlier reports, rs4671393 in \textit{BCL11A} intron 2 was the genetic marker most strongly associated with HbF levels ($P = 3.7 \times 10^{-37}$). Stepwise conditional analyses found two other SNPs in \textit{BCL11A} intron 2, rs7599488 and rs10189857, which independently associated with HbF levels. These two SNPs were in weak linkage disequilibrium (LD) with rs4671393 ($r(2) = 0.17$ and $r(2) = 0.15$, respectively) but were in strong LD with each other ($r(2) = 0.96$). These three SNPs form four haplotypes that represent 99.7% of all haplotypes at this locus. These haplotypes were more strongly associated with HbF levels ($P = 4.0 \times 10^{-45}$) than was rs4671393 and explained 18.1% of the phenotypic variation in HbF levels. Thus, these haplotypes explain more phenotypic variance than the sum of the three \textit{BCL11A} SNPs taken individually (14.7%). Galarneau \textit{et al}.\textsuperscript{12} concluded that it is likely that the difference in phenotypic variance explained is due to the presence of HbF-increasing and HbF-decreasing alleles on the same haplotype background, where associated SNPs in LD masked each other’s phenotypic effect. In our study, the C allele of the rs11886868 and the G allele of the rs4671393 and AG, GG and mutant allele G of rs4671393 were associated with an ameliorated phenotype in patient’s SCA. Sheehan \textit{et al}.\textsuperscript{17} have documented that genetic polymorphisms do modify laboratory and clinical phenotypes even in very young patients with SCA. In Tunisian patients, one previous article has reported the implication of polymorphic sequences cis to the beta-globin gene in HbF variation but they do not explore polymorphisms in Trans of the beta-globin gene.\textsuperscript{18} The originality of this study is the association of the mutant genotypes of two SNPs in Trans of the beta-globin gene studied and the phenotypic variability of HbF and clinical events. The results show that the risk of occurrence of clinical events is higher in the group who presented genotypes associated with HbF $< 15\%$. The combination of the genotypes GG and CT explains more phenotypic variance than the sum of the two \textit{BCL11A} SNPs taken individually.

\begin{table}[h]
\centering
\caption{The relationships between genotypes found and clinical events determined using logistic regression}
\begin{tabular}{ ll c c c c c c}
\hline
BCL11A gene & VOC & Stroke & Osteonecrosis & Cholelithiasis & $P$ \\
\hline
rs11886868 & & & & & 0.009 \\
CC & 46 & 10 & 40 & 36 & 0.009 \\
CT & 28 & 6 & 20 & 15 & 0.011 \\
rs4671393 & & & & & 1$^*$ \\
AA & 0 & 0 & 0 & 0 & 1$^*$ \\
AG & 28 & 8 & 20 & 18 & 0.016 \\
GG & 46 & 6 & 36 & 30 & 0.021 \\
Combined genotype & & & & & 8.2 $\times 10^{-3}$ \\
AG/CT & 28 & 8 & 20 & 18 & 0.04 \\
\hline
\end{tabular}
\end{table}
Disclaimer statements
Contributors Manipulation statistics redaction.
Funding LR11IPT07.
Conflicts of interest None.
Ethics approval None.

References
1 Rucknagel D, Ferrucci S, Whitten CF, Sarniak I, Odenheimer D, Sing C, et al. alpha-Thalassemia and HbF concentration in sickle cell anemia. Prog Clin Biol Res. 1984;165:103–20.
2 Sankaran VG, Menne TF, Xu J, Akie TE, Lettre G, Van Handel B, et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. Science 2008; 322(5909):1839–42.
3 Eridani S, Avermaria F, Mosca A. Reactivation of fetal hemoglobin in thalassemia and sickle cell disease. Thalassemia Reports 2014;4:2196.
4 Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. N Engl J Med. 1995;332(20):1317–22.
5 McGann PT, Ware RE. Hydroxyurea for sickle cell anemia: what have we learned and what questions still remain?. Curr Opin Hematol. 2011;18(3):158–65.
6 Brandow AM, Jirovec DL, Panepinto JA. Hydroxyurea in children with sickle cell disease: practice patterns and barriers to utilization. Am J Hematol. 2010;85(8):611–3.
7 Sankaran VG, Menne TF, Xu J, Akie TE, Lettre G, Van Handel B, et al. Developmental and species-divergent globin switching are driven by BCL11A. Nature 2009;460(7259):1093–7.
8 Xu J, Sankaran VG, Ni M, Menne TF, Puran RV, Kim N, et al. Transcriptional silencing of gamma-globin by BCL11A involves long range interactions and cooperation with SOX6. Genes Dev. 2010;24(8):783–98.
9 Jawaid K, Wahlberg K, Thein SL, Best S. Binding patterns of BCL11A in the globin and GATA1 loci and characterization of the BCL11A fetal hemoglobin locus. Blood Cells Mol Dis. 2010;45:140–6.
10 Menzel S, Garner C, Gut I, Matsuda F, Yamaguchi M, Heath S, et al. A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. Nat Genet. 2007; 39:1197–9.
11 Uda M, Galanello R, Sanna S, Lettre G, Sankaran VG, Chen W, et al. Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. Proc Natl Acad Sci USA 2008;105: 1620–5.
12 Galarneau G, Palmer CD, Sankaran VG, Orkin SH, Hirschhorn JN, Letter G. Fine-mapping at three loci known to affect fetal hemoglobin levels explains additional genetic variation. Nat Genet. 2010;42:1049–51.
13 Bradner JE, Maka R, Tanguturia SK, Mazitscheka R, Haggartyta SJ, Rossa K, et al. Chemical genetic strategy identifies histone deacetylase 1 (HDAC1) and HDAC2 as therapeutic targets in sickle cell disease. Proc Natl Acad Sci USA 2010;107: 12617–22.
14 Xu JD, Bauer DE, Kerenyi MA, Voa TD, Hou S, Hsu YJ, et al. Co-repressor-dependent silencing of fetal hemoglobin expression by BCL11A. Proc Natl Acad Sci USA 2013, 110(16):6518–23.
15 Bendaoud B, Hosni I, Mosbahi I, Hafisia R, Prehu C, Abbas S. Three new mutations account for the prevalence of glucose 6 phosphate dehydrogenase (G6PD) deficiency in Tunisia. Pathol Biol (Paris) 2013;61:64–9.
16 Lettre G, Sankaran VG, Bezerra MAC, Araujo AS, Uda M, Sanna S, et al. DNA polymorphisms at the BCL11A; HBS1L-MYB, and beta-globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. Proc Natl Acad Sci USA 2008;105:11869–74.
17 Sheehan VA, Luo Z, Flanagan JM, Howard TA, Thompson BW, Wang WC, et al. Genetic modifiers of sickle cell anemia in the BABY HUG cohort: influence on laboratory and clinical phenotypes. Am J Hematol. 2013;88:571–6.
18 Moumni I, Ben Mustapha M, Ben Mansour I, Zorou A, Douzi K, Sassi S, et al. Fetal hemoglobin in Tunisian sickle cell disease patient: relationship with polymorphic sequences cis to the β-globin gene. Indian J Hematol Blood Transfus. 2015; doi:10.1007/s12288-015-0504-7.