HbF-promoting polymorphisms may specifically reduce the residual risk of cerebral vasculopathy in SCA children with alpha-thalassemia

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Key words: SCA, HbF quantitative trait loci, *XmnI*, *BCL11A*, *HBS1L-MYB*, cerebral vasculopathy, alpha-globin genotype.

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Abbreviations:

SCA: Sickle cell anemia

Hb: hemoglobin

HbF QTL: HbF quantitative trait loci

VOC: vaso-occlusive crisis

HMIP: HBS1L-MYB intergenic region

AF: allelic frequency

SNP: single nucleotide polymorphism

Abstract (115 words)

Sickle cell anemia (SCA) is a disease characterized by abnormal red blood cell rheology. Because of their effects on HbS polymerization and red blood cell deformability, alpha-thalassemia and the residual HbF level are known genetic modifiers of the disease. The aim of our study was to determine if the number of HbF quantitative trait loci (QTL) would also favor a specific sub-phenotype of SCA as it is the case for alpha-thalassemia. Our results confirmed that alpha-thalassemia protected from cerebral vasculopathy but increased the risk for frequent painful vaso-occlusive crises. We also showed that more HbF-QTL may provide an additional and specific protection against cerebral vasculopathy but only for children with alpha-thalassemia (-α/$\alpha\alpha$ or -α/-α genotypes).

Introduction

Sickle cell anemia (SCA) is a severe monogenic hemoglobinopathy characterized by the production of an abnormal hemoglobin S (HbS). When deoxygenated, HbS polymerizes and causes the sickling of red blood cells (RBCs). Sickle RBCs are more fragile and rigid than healthy RBCs, leading to chronic hemolytic anemia and frequent vaso-occlusive crisis (VOC), respectively. However, the clinical expression of SCA is highly variable from one patient to another and the identification of genetic modulators could be helpful in anticipating appropriate clinical management (1).

Hemoglobin F (HbF) plays a major role in the reduction of SCA mortality and morbidity (2-4). The residual HbF level is genetically determined and three major HbF quantitative trait loci (HbF QTL) have been identified on the human genome, located in the promoter of *HBG2* (11p15), in the *HBS1L-MYB* (HMIP) intergenic region (6q23) and in *BCL11A* (2p16) introns (5). The Thalassemia Severity Score (TSS), which considers sex, alpha/beta-globin genotypes and four HbF inducer polymorphisms, has been shown to positively correlate with the age of the first transfusion in homozygous beta-thalassemia patients (6). In SCA, patients with increased HbF level have less severe anemia and increased RBC deformability (7, 8). However, it is not known whether the number of HbF QTL could be specifically associated with a particular SCD clinical presentation as it is the case for alpha-thalassemia which favors the viscous vaso-occlusive sub-phenotype but prevents from the hemolytic one (9, 10). This study aimed to determine whether the number of HbF QTL, in association or not with alpha-thalassemia, could allow predicting the clinical sub-phenotype of SCA, as does the TSS in homozygous beta-thalassemia.

Materiel and methods

A cohort of 314 unrelated HbSS children with SCA (median 9.6 years; F/M sex ratio 167/147) regularly followed in 4 French SCA Reference centers (Lyon n = 79, Marseille n = 9, Creteil n = 191 and Mayotte n = 35) was constituted. These children had been selected by their local specialized physician so that they could undoubtedly fall into one of the following clinical categories: **(i) Vaso-occlusive crisis group (VOC ; n = 127)** included children that were put on hydroxyurea (HU) therapy for at least 3 hospitalized VOC a year or 2 ACS since birth, without any sign of cerebral vasculopathy; **(ii) Vasculopathy group (VASC ; n = 112)** included children attending a regular exchange transfusion program for stroke or confirmed abnormal transcranial Doppler exam (time-averaged mean of maximum velocities, TAMMX, ≥ 2.0 m.s-1 for the middle, internal, carotid or anterior cerebral arteries) without previous history of HU medication for frequent VOC; **(iii) Anemia group (ANEMIA ; n = 29)** included children with a steady-state Hb level below 70 g/L without cerebral vasculopathy or frequent VOC and **(iv) Complication-free group (FREE ; n = 46)** included children who never received chronic therapy (transfusion or HU) for SCA complication and whose steady-state Hb level was 80 g/L or higher. All children received the same long-term clinical care including an annual transcranial Doppler exam since the age of 4 years. Those whose clinical phenotype was not as clear-cut (conditional TCD velocity, for example) were not enrolled.

All patients or legal caregivers gave their consent for genetic analysis. Alpha-thalassemia status was characterized by multiplex gap-PCR to detect the five common alpha-thalassemia deletions (11). The allelic frequency (AF) of alpha-thalassemia was compared between groups with a Chi square test. For a subset of 158 children, a previously described (2, 12) HbF-QTL score ranging from 0 to 6 and corresponding to the number of HbF-

promoting alleles was calculated. A representative tag-SNP for each loci was employed, namely rs7482144 C>T (*XmnI*) for the β-globin gene cluster (chromosome 11), rs1427407 G>T for *BCL11A* (chromosome 2) and rs28384513 A>C for HMIP (chromosome 6). These SNPs were genotyped using a commercial reverse dot-blot kit (Beta-Thal Modifier StripAssay®, Vienna Lab, Vienna, Austria), Sanger direct sequencing or dedicated real-time PCR methods (12, 13). For statistical analyses, two HbF-QTL groups were considered: patients with a score of 0 or 1 versus others.

Chi-square and ANOVA/LSD post-hoc tests were used to compare sex and age repartition between the four clinical groups. For each group, the distribution of patients in the two HbF-QTL categories was compared with the distribution in the whole cohort using Fisher exact test. The proportions of patients with high HbF-QTL score in each clinical group were also compared side-by-side using Fisher exact test. All calculations were done separately in presence or absence of alpha-thalassemia, either at the heterozygous (-α/αα) or homozygous state (-α/-α). The Statistical Package for the Social Sciences version 20.0 (SPSS Inc., Chicago, IL, USA) was used and significance was defined as $p < 0.05$.

Results

No difference in the F/M sex ratio was observed between groups ($p = 0.47$; data not shown). The mean ± SD ages of the four groups at the time of inclusion were as follows: VOC 12.5 ± 4.3 years, VASC 12.3 ± 3.1, ANEMIA 11.4 ± 4.0 and FREE 10.3 ± 3.4. Among the 314 SCA children, only the -3.7 Kb alpha-thalassemia deletion was identified. The global AF was 0.27 (109 heterozygotes -α/αα and 29 homozygotes -α/-α) with significant differences according to the clinical groups ($p < 0.001$; Chi square test). The AF of the -3.7 Kb deletion

was significantly higher in the VOC group (0.34; $p < 0.001$) and significantly lower in the VASC group (0.17; $p < 0.001$) compared to the ANEMIA and FREE groups (0.26 and 0.28, respectively).

The genotyping results of the HbF QTL and the corresponding scores are given in table I, according to the clinical group and the alpha-thalassemia status. Global AF of *BCL11A* and HMIP SNPs (0.26 and 0.30, respectively) were higher than *XmnI* (0.08), reflecting a low prevalence of Senegal and Arab-Indian β^S haplotypes. As a consequence, most of the patients (110 out of 158) had a score of 0 or 1. Very interestingly, in presence of alpha-thalassemia at the heterozygous or homozygous state, no patient belonging to the higher HbF category fall into the VASC group ($p = 0.02$). Consequently, the proportion of patients with a HbF-QTLs score ≥ 2 was significantly lower compare to the three other groups (Figure 1B), which was absolutely not the case for patients with normal alpha-globin genotype (Figure 1A). No other unbalanced repartition was observed.

Discussion

The protective effect of alpha-thalassemia against SCA cerebral vasculopathy is very well known. However, this protection is not absolute, as reflected by our cohort where 12 out of 70 patients with alpha-thalassemia belonged to the vasculopathy group. There is thus a room for improvement which seems to be given by the additional presence of at least two favorable HbF QTLs. This observation is not very surprising per se since we already observed a differential effect of the HbF QTL according to the alpha-globin genotype for VOC occurrence and for some biological markers of severity (leucocytes and CRP) in a pediatric Senegalese cohort (12). In that study, we could not focus on cerebral vasculopathy due to

the absence of regular transcranial Doppler exam in Senegal. Moreover, the Senegalese children were all HU-free and none of them attended a regular exchange-transfusion program. Since it was not the case in our study, we classified children according to their natural clinical evolution and not according to recent clinical and biological data which could obviously be influenced by HU or regular transfusions. The reliability of our clinical classification was ascertained by the fact that the well-known modulating effects of alpha-thalassemia (protection from cerebral vasculopathy but higher risk of frequent VOC) clearly appeared, thus confirming previous studies (14, 15).

The protecting effects of alpha-thalassemia on cerebral vasculopathy are thought to result from its effects on red blood cell deformability and hemolysis (16). However, the lower hemolysis in SCA patients with alpha-thalassemia causes a rise in blood viscosity, which would explain the higher frequency of VOC-like events compared to patients without alpha-thalassemia (14). In addition, it has been demonstrated that SCA patients with alpha-thalassemia had higher red blood cell aggregation than those without, which could further increase the risk for VOC (17). Given the relatively small number of children in each phenotypic category, a replication study on another independent SCA cohort (ideally enriched in Senegal haplotypes for a higher *XmnI* AF) is obviously needed to support our conclusion. To increase the number of children in the VASC group, it could be envisaged to consider those with a conditional TCD (TAMMX between 1.7 and 2m.s^{-1}) and silent cerebral infracts (small cortical or subcortical or larger subcortical) at magnetic resonance imaging (MRI) testing. In the same way, a replication cohort enriched in patients with the β^S Senegal haplotype (*XmnI* positive) may allow to obtain patients with an HbF QTL-score of 5 or 6.

Friedrisch *et al.* already found that BCL11A SNPs were associated with higher HbF

induction under HU medication (18). Our work shows another potential use of the HbF QTL for the clinical management of patients with SCA. A few different HbF genetic combined variables have been described in the literature and they will undoubtedly add significant value in the next future for both clinical studies and patient stratification in therapeutic purpose. They all rely on the same three genetic loci (*XmnI*, *BCL11A* and HMIP) but they vary each other by the chosen tag-SNP and/or its relative weight in the model. In our HbF QTL-score, there is one SNP per loci and all three loci have equal ponderation. Conversely, in the 4-variant model recently described by Gardner *et al.* (19), the highest ponderation is given to the *BCL11A* locus for which two SNP are genotyped (rs1427407 and rs6545816). A different SNP is also employed for HMIP (rs66650371 instead of rs28384513 in our study) despite its very low AF in African populations without significant European admixture (2). It would be very interesting to test whether a higher Gardner score is also associated with a lower prevalence of cerebral vasculopathy in SCA children with alpha-thalassemia. From a technical point of view, the Beta Thal Modifier Strip Assay kit represents a very simple, reliable and time-efficient alternative for the genotyping of the main HbF QTLs (but not BCL11A rs6545816) compared to the use of dedicated Sanger / real-time PCR methods. Next-Generation Sequencing would be another possible alternative but care should be taken for *XmnI* which is located in a region of high homology between the *HBG2* and *HBG1* gene promoters.

In conclusion, our work suggests a specific protective effect of the HbF-QTL on SCA cerebral vasculopathy but in presence of alpha-thalassemia only (either at the homozygous or heterozygous state). Apart from a necessary confirmation on replication cohorts, this result would also require to be functionally ascertained by rheological measurements. Theoretically, in presence of alpha thalassemia, SCA patients with at least two HbF QTL

would be expected to have a higher deformability (but not necessarily a higher viscosity) than patients with 0 or 1 HbF-QTL only. On the contrary, the number of HbF QTL would not have any influence on the rheological parameters in SCA patients without alpha-thalassemia.

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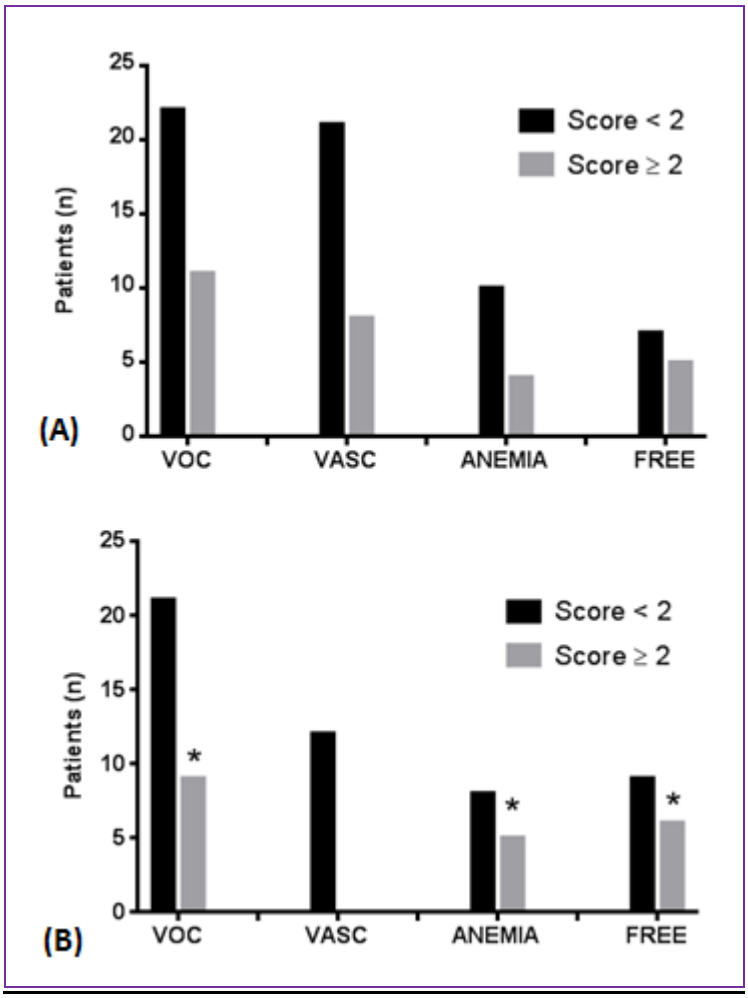

<u>Figure 1:</u> Distribution of SCA patients with HbF QTL score < 2 or ≥ 2 according to the clinical sub-group, in absence (A) or presence (B) of alpha-thalassemia (-α/αα or -α/-α genotypes). * p < 0.05 at Fisher exact test versus the VASC group. No side-by-side comparison was significant.

Table I: Genotyping results of the HbF-QTLs according to the alpha-globin genotype in 158 patients with SCA

		SCA patients with normal alpha-globin genotype (αα/αα) - n = 88				SCA patients with alpha-thalassemia (-α/αα or -α/-α) - n = 70			
		VOC (n = 33)	VASC (n = 29)	ANEMIA (n = 14)	FREE (n = 12)	VOC (n = 30)	VASC (n = 12)	ANEMIA (n = 13)	FREE (n = 15)
*XmnI (*AF)		0.09	0.05	0.07	0	0.12	0	0.23	0.08
BCL11A rs1427407 (AF)		0.26	0.22	0.14	0.33	0.22	0.08	0.19	0.21
HMIP rs28384513 (AF)		0.24	0.33	0.32	0.33	0.25	0.13	0.15	0.46
HbF-QTL score	0 or 1 (n)	22	21	10	7	21	12	8	9
	≥ 2 (n)	11	8	4	5	9	0	5	6
	p*	0.84	0.83	1	0.52	1	**0.02**	0.54	0.56

SCA: sickle cell anemia; HbF-QTL: quantitative trait loci of hemoglobin F; AF: Allelic frequency; *: Fisher exact test versus the whole cohort.