The role of rs1984112_G at CD36 gene in increasing reticulocyte level among sickle cell disease patients

Miniar Kalai*, Marwa Dridi*, Leila Chaouch, Imen Moumni, Houyem Ouragini, Imen Darragi, Imen Boudrigua, Dorra Chaouachi, Fethi Mellouli, Mohamed Bejaoui and Salem Abbes

Université de Tunis El Manar, Institut Pasteur de Tunis, Laboratoire d’Hématologie Moléculaire et Cellulaire, Tunis, Tunisia

ABSTRACT
Aims and background: Mediators of adhesion become a potential new target for pharmacological therapy to struggle the complications of sickle cell disease (SCD). Several mechanisms for increased adherence have been postulated and the well-studied are CD36 and VLA4 which encoded by ITGA4. Herein, we sought to determine whether one polymorphism of CD36 namely: rs1984112 and three exons of ITGA4 (4, 5, and 6) are implicated in hemolytic status and clinical events among SCD Tunisian patients.

Material and methods: This study enrolled 99 unrelated Tunisian subjects (63SS and 36Sβ). All SCD patients are children (less than 16 years old). The rs1984112 and the ITGA4’s exons 4, 5, and 6 were analyzed for all subjects by PCR/sequencing. The association of each genotype found with both clinical complications and hemolytic status was performed using t-test. Clinical events studied included vaso-occlusive crisis (VOC), osteonecrosis, stroke, frequent infection, priapism, and acute syndrome.

Results: The results show that rs1984112_G allele at CD36 gene revealed to be associated with higher levels of reticulocyte count (p < 0.01). The statistical result show a near significance of homozygous mutant GG genotype with VOC (p = 0.051). No association between rs1984112_G allele and the clinical severity of SCD were found. Mutational screening of exon 4, 5, and 6 of ITGA4 gene revealed absence of mutated variant.

Conclusion: Our results are similar to those found in Portuguese population which reported the role of rs1984112_G in increasing reticulocyte level among SCD patients. Consequently, the rs1984112_G of CD36 could be considered as a reliable biomarker for predicting patients at high risk for vascular occlusions and thus, allows earlier and more effective therapeutic management.

KEYWORDS
SCD; CD36; VLA4; reticulocytes

Introduction
The term SCD describes a group of inherited red blood cell disorders. People with sickle cell disease (SCD) have abnormal hemoglobin, called hemoglobin S (Hb S) or sickle hemoglobin, in their red blood cells [1]. SCD is commonly composed by homozygosity for the Hb S mutation, as well as heterozygous states involving Hb S mutation and β thalassemia mutation. While there are multiple types of SCD, they all have similar symptoms at different levels of severity. These include: the vasoocclusion which is the major cause of morbidity and mortality in SCD, frequent infections, stroke, acute chest syndrome, osteonecrosis, splenic sequestration, and priapism [2,3]. The adherence of RBC to the endothelium could obstruct blood flow and facilitate vaso-occlusion occurrence [4–6]. Mediators of adhesion have, therefore, become a potential new target for pharmacological therapy to struggle the complications of SCD. Among these mediators several adhesion molecules such as CD36, VLA4, and ICAM should also play an important role in the adhesion of sickle cells to the blood vessels [7]. Recent studies have aimed to identify the role of adhesion molecules in the interaction between the endothelium and sickle erythrocyte [8]. CD36 and VLA4 are among the most extensively studied adhesion receptors [9]. CD36, or glycoprotein IV, is also an adhesion receptor limited to immature erythroid cells [10]. CD36 can bind to endothelial receptors via von Willebrand factor and extracellular matrix proteins such as thrombospondin (TSP). It can mediate adhesion to αVB3 receptors on the endothelium via TSP. It has also been suggested that the interaction of CD36 with TSP and αVB3 is a potential mechanism by which HbS erythrocyte adhesion may contribute to vaso-occlusion [11,12]. In SCD, in response to anemia ‘the stress reticulocytes’ leave the bone marrow expressing VLA4 thereby allowing the adhesion between red blood cells carry the mutation SS (SSRG) and endothelium [13]. The main ligand of VLA4 is VCAM-1 on the surface of activated endothelial cells. Studies have shown through the use of monoclonal antibodies as VLA4 α4 chain is responsible for the adhesion between VLA4 and VCAM-1 [14,15]. The α4 integrin (CD49d) is encoded by the
ITGA4 gene (geneID3676), located in chromosome 2 at 2q31.3 [16]. The exon 4, 5, and 6 of ITGA4 gene encoded the epitope responsible in the link to VCAM-1. The polymorphisms of these molecules should be an important field of study. With this objective, we analyzed the ITGA4’s exon 4, 5, and 6 and the rs1984112 of CD36 then we sought to determine if they are implicated in hemolytic status and clinical events among SCD Tunisian patients.

Material

Our study concerned 99 SCD patients (63 with SS phenotype and 36 with Sβ° phenotype). All patients are children (less than 16 years old). The demographic and the hematological parameters are summarized in Table 1.

Data laboratory

Diagnosis of sickle cell patient is performed using cation-exchange high-performance liquid chromatography (D10 Biorad) and further confirmation by cation-exchange high-performance liquid chromatography (D10 Biorad) and further confirmation by molecular diagnosis by restriction fragment polymorphism using Ddel as previously described by Bendaoud et al. [17] The HbA2 analysis is considered the gold standard for diagnosing thalassemia. Hemolysis status including reticulocyte count, lactate dehydrogenase (LDH) and total bilirubin (BT) level and other hematologic parameters were measured for each patient using (Cobras Integra, Meylan, France; ABX pentra 60c+).

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 enrolled in this study are: VOC, osteonecrosis, stroke, acute syndrome, frequent infection, priapism, and splenic sequestration.

Genotyping of rs1984112 and exon 4, 5, and 6 of ITGA4

Genomic DNA was isolated from white blood cells of total blood using standard method (phenol/chloroform). The rs1984112 and ITGA4’s exon 4, 5, and 6 were genotyped by PCR/sequencing using The following primers namely: F(CD36) (AAACATTCAGCTCTTTTATGTTATGTT), R (GCGGCCTACTAAGATATCC); F4 (TGAGGATGATGCACAGTGT), R4 (TGCAAAGGTGGGTAGTTAAAGACG); F5 (TCCTATGAAAGAGCAGAGGA), R5(TCTCTTTGCCCTCACTAGAA) and F6 (GAGGTTCGGCTGGCTGAG), R6(CTGGCCATTATACTTTGGAGA). Polymerase chain reaction 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, U.S.A), 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 45 seconds, annealing at (62°C for CD36; 63°C for ITGA4) during 30 seconds and extension at 72°C for 45 seconds. The run was ended by a final extension at 72°C for 10 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, U.S.A) and an ABI 310 DNA sequencer (PEApplied Biosystems, Foster City, U.S.A).

Data analysis

Ninety-nine patients including 63SS and 36Sβ° phenotype were enrolled in the analysis. This research is comprised of two sections. First, we test the association of genetic profile with hemolytic status. Second, we test for trait association with the candidate SNPs, genotype, and allele frequencies between two groups according to the severity of the disease and to other parts to the presence of each complication.

Statistical analysis

The demographic and hematologic 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 18).
3.01). Genetic differences between the two groups were evaluated applying exact tests to genotypic or allelic contingency tables using compare 2 (version 1.02).

**Results**

Statistics for the comparison of demographic and hematological variables between the two groups (SS and Sβ°) were performed using the t-test and chi-square test as appropriate (SPSS 16.0) and no significant difference was found (Table 1). Hence, it was considered that the SS and Sβ° patients are represented in the same sample and it is not necessary to stratify data into SS and Sβ°. The hemolytic status of our patients is assessed by measuring LDH, BT, and reticulocyte.

The study of clinical records of the patients found that the most common clinical complications are: vaso-occlusive crisis (VOC), osteonecrosis, stroke, frequent infection, priapism, and acute syndrome. The severity of the disease was estimated by clinicians by the following criteria: the occurrence of greater than or equal to three VOC/year or reaching by stroke or the occurrence of acute chest syndrome.

For the polymorphism rs1984112 the samples were found to be in Hardy–Weinberg equilibrium (p > 0.05). The results of the genotyping of the rs1984112 show the presence of three genotypes: AA, AG, and GG. A is the normal allele and G is the mutant one. Our findings show that rs1984112_G allele at CD36 gene revealed to be associated with higher levels of reticulocyte count (p < 0.01) (Table 2). Our results show the absence of association between rs1984112_G allele and the clinical severity of SCD (Table 3). When we compared genotypes and alleles within the two groups of patients according to the status of each complication, we found no significant association except for the VOC. The statistical result show a near significance of homozygous mutant GG genotype with VOC (p = 0.051).

Furthermore we looked for a possible correlation between the rs1984112 polymorphism in the CD36 gene with the severity of the disease and with each complication separately but no association was found except for the VOC. The statistical result show a near significance of homozygous mutant GG genotype with VOC (p = 0.051).

On the other hand we were interested in the study of ITGA4 gene, in particular, we made a mutational screening of three exons (4, 5, and 6). Indeed, these exons encodes for the region responsible for the direct link between VLA4–VCAM-1 which is the ligand for VLA4 on the surface of endothelial cells activated by proinflammatory cytokines.

The result of the mutational screening of three exons (4, 5, and 6) of ITGA4 revealed the absence of mutated variant within this region. To the best of our knowledge, this is the first report which explored the exon 4, 5, and 6 of ITGA4 gene among SCD patients.

**Discussion**

Endothelial dysfunction leads to increased vascular adhesion which is heavily involved in CVOs [18]. This adherence concerns mainly red blood cells carrying the HbS and the endothelium [19].

**Table 2.** Association of rs1982112 with hemolysis parameters.

| rs1982112 | n  | Mean of reticulocyte (%) | BT (µM) | LDH (UI/l) | P1   | P2   | P3   |
|-----------|----|--------------------------|---------|------------|------|------|------|
| AA        | 40 | 8.20 ± 3.87              | 35.56 ± 21.40 | 412.99 ± 165.84 | 1*   |      |      |
| AG        | 47 | 10.23 ± 4.15             | 40.20 ± 20.50 | 399.16 ± 155.34 | 0.022 | 0.660 | 0.433 |
| GG        | 12 | 14.63 ± 7.04             | 41.15 ± 11.03 | 410.22 ± 135.56 | 0.000 | 0.520 | 0.416 |

Reticulocyte level is indicated as mean ± standard deviation. BT: total bilirubin; LDH: lactate dehydrogenase.

**Table 3.** Genotypic and allelic repartition of the rs1984112 among patients according to the severity of the disease.

| rs1984112 | SCA (severe) n = 86 | SCA (not severe) n = 13 | p     |
|-----------|---------------------|-------------------------|-------|
| AA        | 34                  | 6                       | 1*    |
| AG        | 42                  | 5                       | 0.775 |
| GG        | 10                  | 2                       | 1     |
| A         | 0.64                | 0.65                    | 1*    |
| G         | 0.36                | 0.35                    | 1     |

p: index of significance; each value <0.05 is considered as significant.

Recent studies have reported the involvement of polymorphisms affecting genes coding for molecules involved in the vascular adhesion [20].

In the Tunisian population, some candidate genes responsible for the modulation of sickle cell syndrome were studied. Indeed, in three previous studies we have investigated the involvement of some cytokines and their receptors in the following complications: CVOs, stroke, and infections [21–23]. Some variants corresponding to the genes which encode for adhesion molecules were associated with the severity of hemolysis in SCD. This includes the example of VCAM-1 and CD36 which are associated with high levels of LDH in these patients [24].

Our findings show that rs1984112_G allele at CD36 gene revealed to be associated with a higher levels of reticulocyte count (p < 0.01). This result is consistent with the literature. In fact Coelho et al. reported in 2013 in a study on sickle Portuguese the correlation between rs1984112 and the increased number of reticulocytes [24].
Conclusion

Interestingly, our result is similar to those found in Portuguese population which reported the role of rs1984112_G in increasing reticulocyte level among SCD patients. Indeed, the hemolysis process tends to increase the reticulocyte level which is boosted at the same time by the presence of the G mutant. This situation is not favorable to the SCD patients since they have a high adhesive strength and high reticulocyte production that may be involved in vascular occlusions. Consequently, the rs1984112_G of CD36 could be considered as a reliable biomarker for predicting patients at high risk for vascular occlusions and thus, allows earlier and more effective therapeutic management.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Institut Pasteur de Tunis (grant number LR11IPT07).

Ethical approval

The study was in accordance with the 1964 Helsinki declaration and its later amendments. This study was approved by the ethics committee of Pasteur Institute of Tunis.

References

[1] Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. Lancet. 2010;376(9757):2018–2031.
[2] Ashley-Koch A, Yang Q, Olney RS. Sickle hemoglobin (HbS) allele and sickle cell disease: a HuGE review. Am J Epidemiol. 2000;151(9):839–845.
[3] Steinberg MH. Pathophysiologically based drug treatment of sickle cell disease. Trends Pharmacol Sci. 2006;27:204–210.
[4] Kaul DK, Nagel RL. Sickle cell vaso-occlusion: many issues: thrombos and some answers. Experientia. 1993;49:5.
[5] Kaul DK, Fabry ME, Nagel RL. Microvascular sites and characteristics of sickle cell adhesion to vascular endothelium in Thomas shear flow conditions: pathophysiological implications. Proc Natl Acad Sci USA. 1989;86:3356.
[6] Fabry ME, Fine E, Rajanayagam V, et al. Demonstration of endothelial adhesion of sickle cell in vivo: a distinct role for deformable sickle cell discocytes. Blood. 1992;79:1602.
[7] Odièvre MH, Bony V, Benkerrou M, et al. Modulation of erythroid adhesion receptor expression by hydroxyurea in children with sickle cell disease. Haematologica. 2008;93:502–510.
[8] Marilyn J. Telen. Role of adhesion molecules and vascular endothelium in the pathogenesis of sickle cell disease. ASH Education Book. 2007;1:84–90.
[9] Trinh-Trang Tan MM, Vilela-Lamego C, Picot J, et al. Intercellular adhesion molecule-4 and CD36 are implicated in the abnormal adhesiveness of sickle cell SAD mouse erythrocytes to endothelium. Haematologica. 2010;95(5):730–737.

[10] Fernández-Ruiz E, Armesilla AL, Sánchez-Madrid F, et al. Gene encoding the collagen type I and thrombospondin receptor CD36 is located on chromosome 7q11.2. Genomics. 1993;17(3):759–761.

[11] Finnegan EM, Barabino GA, Liu XD, et al. Small-molecule cyclic αVβ3 antagonists inhibit sickle red cell adhesion to vascular endothelium and vasoocclusion. Am J Physiol Heart Circul Physiol. 2007;293:H1038–H1045.

[12] Gambero S, Canalli AA, Traina F, et al. Therapy with hydroxyurea is associated with reduced adhesion molecule gene and protein expression in sickle red cells with a concomitant reduction in adhesive properties. Eur J Haematol. 2007;78:144–151.

[13] Brittain JE, Parise LV. The α4β1 integrin in sickle cell disease. Transfus Clin Biol. 2008;15:19–22.

[14] Gee BE, Platt OS. Sickle reticulocytes adhere to VCAM-1. Blood. 1995;85:268–274.

[15] Pulido R, Elices MJ, Campanero MR, et al. Functional evidence for three distinct and independently inhibitable adhesion activities mediated by the human integrin VLA-4. Correlation with distinct alpha 4 epitopes. J Biol Chem. 1991;266:10241–10245.

[16] Yoshikazu Takada, Xiaojing Ye, Scott Simon. The integrins. Genome Biol. 2007;8:215.

[17] Bendaoud B, Hosni I, Mosbah I, et al. Three new mutations account for the prevalence of glucose 6 phosphate dehydrogenase (G6PD) deficiency in Tunisia. Pathol Biol. 2013;61:64–69.

[18] Elion J, Labie D. Drépanocytose et adhérence cellulaire. Hématologie. 1998;4(3):201–211.

[19] Dhananjay K. Kaul, Eileen Finnegan, Gilda A. Barabino. Sickle red cell – endothelium interactions. Microcirculation. 2009;16(1):97–111.

[20] Driss A, Asare KO, Hibbert JM, et al. Sickle cell disease in the post genomic era: a monogenic disease with a polygenic phenotype. Genomics Insights. 2009;2:23–48.

[21] Chaouch L, Kalai M, Jbara BM, et al. Implication of rs1026611 in the MCP-1 gene and V64I of CCR2 in stroke among SCA Tunisian patients. Clon Transgen. 2014;3(126):1–4.

[22] Chaouch L, Kalai M, Jbara BM, et al. Association of MCP1-2518A/G and CCR2-V64I polymorphisms and vasoocclusive crisis among sickle cell anemia Tunisian patients. Microinflammation. 2014;1(10):41–45.

[23] Kalai M, Chaouch L, Mansour I, et al. Frequency of three polymorphisms of the CCL5 gene (rs2107538, rs2280788 and rs2280789) and their implications for the phenotypic expression of sickle cell anemia in Tunisia. Pol J Pathol. 2013;2:84–89.

[24] Coelho A, Dias A, Morais A, et al. Genetic variations in CD36, HBA, NOS3, and VCAM1 is associated to chronic haemolysis level in sickle cell anaemia: a longitudinal study. Eur J Hematol. 2014;92(3):237–243.