Polymorphism and Association of the E-selectin and ICAM-1-K469E Genes with Sickle Cell Disease in Congo

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Abstract

Introduction: E-selectin and ICAM-1 are cellular adhesion molecules that play important roles in the pathogenesis of Sickle Cell Disease (SCD), especially in the vaso-occlusion process. Numerous studies reported that single nucleotide polymorphisms in E-selectin and ICAM-1 genes may be associated with the clinical expression of several diseases. However, no evidence exists regarding the association with SCD.

Objectives: Investigate the association of E-selectins (S128R and S149R) and ICAM-1-K469E polymorphisms with SCD in Congolese patients.

Methodology: This case-control study included 110 SCD patients in vaso-occlusive pain crisis and 120 controls (healthy non-sickle cell subjects). Polymorphisms were genotyped by PCR-RFLP method. The differences in genotype and allele frequencies between both groups were analyzed with the Fisher’s exact test. A P level of <0.05 was considered significant.

Results: The frequency of the ICAM-1-K469E EE genotype was significantly higher in SCD patients compared to controls (8.2% vs 0.8%; P=0.008). The odd ratio value (OR=11.89; 95% CI = 1.48-96.53; P=0.01) indicated a high association with SCD. Furthermore, the E allele was also significantly associated with SCD (OR=1.72; 95% CI=1.10-2.68; P=0.008). By contrast, no statistically significant differences were found between SCD patients and controls regarding the allele and genotype frequencies of E-selectin S128R and S149R polymorphisms.

Conclusion: Our findings suggest that the ICAM-1-K469E polymorphism may be associated with SCD among Congolese patients, possibly with the vaso-occlusive crisis. The EE genotype and the E allele may be genetic risk factors. However, because of the small sample size, this report should be considered as exploratory and further studies are required to confirm these genetic associations with SCD.

Keywords: Sickle cell disease; E-selectin S128R; E-selectin S149R; ICAM-1; Polymorphism; Vaso-occlusive crisis

Introduction

Sickle cell disease (SCD) is a group of inherited red blood cell disorder affecting millions worldwide. It is associated with extensive multi-organ morbidity and an increased risk of early mortality [1]. Globally, more than 300,000 infants are born annually worldwide with SCD; the majority living in sub-Saharan Africa and India [2]. Furthermore, it is estimated that approximately 52,000 Europeans and 100,000 Americans have SCD, but the number of patients with SCD in both continents are steadily increasing due to migration [3,4].

SCD is caused by the substitution of glutamic acid by valine at the sixth position of the beta globin chain of the hemoglobin molecule [5]. This results in a defective Hemoglobin molecule (HbS) which is unable to carry enough oxygen. People who inherit this abnormal gene from both parents (homozygous HbSS) have SCA, and are highly susceptible to the lethal effects of infections such as malaria [6]. Under hypoxia, HbS polymerizes inside the red cell, resulting in the formation of sickle-shaped red blood cells that aggregate and occlude the micro vasculature. The damage and rigidity of these red blood cells could lead to hemolysis, vaso-occlusion and ischemia-reperfusion injury [7].

Clinical manifestations of SCD are variable, ranging from acute complications (e.g., hand-foot syndrome, vaso-occlusive pain crisis, stroke, priapism and acute infections) to chronic complications (e.g., silent cerebral hypertension, renal failure, heart failure, leg ulcers and osteonecrosis) [8]. The Vaso-Occlusive Crisis (VOC) is the main complication and cause of hospitalization related to SCD [9]. This occurs when red blood cells, leukocytes, platelets as well as endothelial cells aggregating in endothelial venules and block blood flow [10]. As

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a result, the tissues become deprived of oxygen, causing severe pain in muscles and bones [7]. During VOC, the endothelial cells over express cellular adhesion molecules, including Intercellular Adhesion Molecule-1 (ICAM-1) and E-selectin, which promote the vaso-occlusion [11]. ICAM-1 (CD54) is a member of the immunoglobulin superfamily, involved in the recruitment and activation of leukocytes at the site of inflammation [12]. E-selectin (CD62E) is a group of selectins that are expressed on vascular endothelial cells after activation by proinflammatory cytokines. It binds to sialylated or fucosylated molecules on neutrophils, monocytes and lymphocytes, mediating their firm adhesion to endothelial cells [13]. Blum A, et al. [14] reported that levels of ICAM-1 and E-selectin are significantly increased in SCD patients during VOC.

Although SCD is considered as a monogenic disorder, other host genetic variants might affect specific clinical expression among patients [15]. For example, the Single Nucleotide Polymorphisms (SNP) in the encoding gene of bone morphogenetic protein 6 (BMP6), Klotho (KL), or annexin A2 (ANXA2) have been associated with osteonecrosis in SCD patients [16]. SNPs in the Beta-1 Adrenergic Receptor (ADRB1) gene have been associated with the increased risk of pulmonary hypertension [17]. On the other hand, SNP in the gene of Tumor Necrosis Factor-Alpha (TNF-a) or Human Leukocyte Antigen (HLA) have been associated with the increased risk of stroke [18]. Despite the importance of E-selectin and ICAM-1 in the pathogenesis of SCD, there is currently insufficient information about their genetic polymorphisms in sickle cell patients. Based on these grounds, the present study aimed to investigate the genetic polymorphism of E-selectins (S128R and S149R) and ICAM-1 K469E among sickle cell patients in Congo. We also examined the possible association of these polymorphisms with SCD.

Methodology

Study populations and ethical considerations

This was a case-control study including sickle cell patients with the vaso-occlusive pain crisis (SCD patients) and 120 controls (healthy non-sickle cell subjects), carried out between March 2020 and January 2021 in Brazzaville. The latter is the capital and the most populous city of the Republic of Congo, with about 2 million (nearly 50% of the total population) according to the national household census. SCD patients were recruited during their hospitalization at the National Reference Center for Sickle Cell Disease (CNRD), which represents the largest medical center for the diagnosis, hospitalization and follow-up of sickle cell patients in the country. VOC was clinically defined as pain in the extremities, back and abdomen without any other explanations. The SCD group included patients with homozygous SCD (HbSS) diagnosed before this study and recorded at the CNRD. Patients transfused three months before this study were excluded. Those with diabetes mellitus, hypertension, or renal failure were also excluded. The control group consisted of blood donors from the National Blood Transfusion Centre (CNTS) of Brazzaville. A total of 110 SCD patients and 120 controls were included in this study. The study was approved by the ethics Committee of the Ministry of Scientific Research of Congo (reference number: 259/MIRSIT/IRSSA/CERSSA). Informed consent was obtained from the patients or guardians before inclusion in the study and collecting blood samples.

Sampling and genotyping

Peripheral blood samples (5 ml) were collected into Ethylenediamine-Tetraacetic Acid (EDTA) containing tubes from participants, and stored at -20°C until use. Genomic DNA was extracted using the Zymo Research DNA extraction kit, according to the manufacturer instructions (Zymo Research, Irvine, CA, USA). DNA was eluted in 100µl of DNase-free sterile water and stored at -20°C. Genotyping of genes under study was performed by the PCR-Restriction Fragment Length Polymorphism (RFLP) assay. To amplify the E-selectin S128R gene, the following oligonucleotide primers were used: Forward, 5’-ATG GCA CCT TGT AGG ACT GCT-3’ and reverse, 5’-GTC TCA GCT CAC GAT CAT CAC CAT-3’ [19]. For the E-selectin S149R gene, the primers were: Forward, 5’-TCT ATG GCA CTC TGT AGG AC-3’ and reverse: 5’-AGA ACC AGA CITT ACT TTG CTC-3’[20]. For the ICAM-1 K469E gene, the forward primer was 5’-GGA ACC CAT TGC CGG AGC-3’ and the reverse primer 5’-GGT GAG GAT TGCC ATT AGG TC-3’ [21]. The PCR reactions were done in a volume of 25 µl containing 12.5µl 2X Dream Taq Hot Start Green (Thermo Fisher Scientific, Massachusetts, USA), 1µl of each primer (10 mM), 2 µl DNA (100ng/ µl) and 8.5µl DNase-free water. The PCR amplifications were carried out using the thermal cycler Bio-Rad T100™. Main cycling parameters were 95°C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 45 s and extension at 72°C for 60 s with final extension at 72°C for 10 min. The E-selectin (S128R and S149R) and ICAM-1 K469E PCR products (10 µl) were digested with 5U of PstI and BstUI (Thermo Fisher Scientific, Massachusetts, USA), respectively, in a final volume of 25 µl, at 37°C for two hours. Thereafter, the products were analyzed by agarose gel electrophoresis using a 3% agarose gel, 1× TBE buffer and 1µg/ml ethidium bromide, and visualized under Ultraviolet (UV) light.

Statistical analysis

The deviation of genotypes from the Hardy-Weinberg equilibrium in the SCD patient group and the control group was assessed by Chi-squared (χ²) test using the online testing platform (https://wpcalc.com). Allelic frequencies were estimated by gene counting. Differences in genotypic and allelic frequencies between SCD patient and controls were analyzed with Fisher’s exact test using the statistical software program Epi-info version 7.2. Odds ratios (OR) and corresponding 95% confidence interval were calculated using the online testing platform (https://www.gigacalculator.com) to examine the association with SCD. A P-value<0.05 was considered statistically significant.

Results

Among 110 SCD patients enrolled in the study, 55.4% (n=61) were females and 44.6% (n=49) males. The majority were under 18 (72.7%); the mean age was 21.9 ± 14.4 years (range 5 to 60 years). In the control group (N=120), 58.3% were males and 41.7% females; the mean age was 32.9 ± 10.6 years.

As expected, the length of the PCR product from the E-selectin S128R gene was 357 bp. The digestion with PstI generated a fragment of 357 bp for the RR genotype (homozygous), three fragments (357, 219 and 138 bp) for the SR genotype (heterozygous) and two fragments (219 and 138 bp) for the SS genotype (homozygous) (Figure 1A). For E-selectin S149R, the expected length of the PCR product was 240 bp, and the PstI digestion products were 240, 142 and 98 bp for the SR genotype, 142 and 98 bp for the SS genotype and 240 bp for the RR genotype (Figure 1B). For the ICAM-1 K469E gene, the length of the PCR product was 223 bp. The digestion with BstUI resulted in an undigested fragment of 223 bp for the KK genotype, 223, 136 and 87 bp for the KE genotype and 136 and 87 bp for the EE genotype (Figure 1C).

Table 1 summarizes the allele and genotype frequencies of E-selectin S128R and S149R polymorphisms in SCD patients and controls.
Table 1: Allele and genotype frequencies of the E-selectin S128R and S149R polymorphisms in SCD patients and controls.

<table>
<thead>
<tr>
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<th>E-selectin S128R</th>
<th>E-selectin S149R</th>
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<tbody>
<tr>
<td></td>
<td>SCD Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>Genotype, n (%)</td>
<td>(N=110)</td>
<td>(N=120)</td>
</tr>
<tr>
<td>SS</td>
<td>95 (86.4)</td>
<td>100 (83.3)</td>
</tr>
<tr>
<td>SR</td>
<td>14 (12.7)</td>
<td>17 (14.2)</td>
</tr>
<tr>
<td>RR</td>
<td>1 (0.9)</td>
<td>3 (2.5)</td>
</tr>
<tr>
<td>Dominant: SS vs SR+RR</td>
<td>95 vs 15</td>
<td>100 vs 20</td>
</tr>
<tr>
<td>Recessive: RR vs S+SR</td>
<td>1 vs 109</td>
<td>3 vs 117</td>
</tr>
<tr>
<td>Allele, n (%)</td>
<td>S 204 (92.7)</td>
<td>217 (90.4)</td>
</tr>
<tr>
<td></td>
<td>R 16 (7.3)</td>
<td>23 (9.6)</td>
</tr>
<tr>
<td>HWE (x2(P value))</td>
<td>0.349 (0.554)</td>
<td>3.998 (0.046)</td>
</tr>
</tbody>
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SCD, sickle cell disease; OR, odds ratio; 95% CI, 95% confidence interval; HWE, Hardy-Weinberg equilibrium. (a), P value resulting from Fisher’s exact test; (b), P value of the odd ratio.

SCD patients and 9.6% and 14.6% among controls, respectively. There was also no significant difference between patients and controls regarding the frequency of the R allele of both polymorphisms (P=0.406 and 0.497). Whether for codominant models (RS and SS) dominant model (SR+RR) or recessive model (SS+SR), the Odd Ratio (OR) values were inferior or near to 1. Taken together, our results indicate that E-selectin S128R and S149R polymorphisms may not be considered genetic risk factors for SCD complications.

Table 2 shows the allele and genotype frequencies of the ICAM-1-K469E polymorphism in SCD patients and controls. Out of 110 SCD patients.
patients, 53.6% were homozygous KK, 38.2% heterozygous KE and 8.2% were homozygous EE. Among controls (N=120), 65% were KK, 34.2% KE genotype and only 0.83% were EE. The chi-squared (χ2) test indicated that the genotype distribution in both groups was consistent with the Hardy-Weinberg equilibrium (P=0.05). The Fisher's exact tests comparing the frequency of the KK and KE genotypes did not reveal a significant difference between patients and controls (P=0.083 and 0.583, respectively). By contrast, with regard of the EE genotype, there was statistical evidence of difference between SCD patients and controls (P=0.008). The odd ratio value (OR=11.89; 95% CI=1.32-85.13; P =0.01) showed that this genotype may be a genetic risk factor for SCD complications. The recessive model (KK+KE) was associated with SCD (OR=10.60; 95%CI=1.32-85.13; P =0.013). The frequency of the K allele resulted 72.7% among SC patients and 81.2% in controls and that of the minor E allele resulted 27.3% among SCD patients and 19.8% in the control group. The frequency of the E allele was significantly higher in patients compared to controls (P=0.019), and the odd ratio value (OR=1.72; 95% CI=1.10-2.68; P=0.008) suggesting its association with SCD.

### Discussion

Sickle Cell Disease (SCD) is one of the greatest public health concerns, especially in sub-Saharan Africa. In the Republic of Congo, the overall prevalence of this disease is 1.25%, with estimated sickle cell trait of between 22 to 25% [22]. SCD is characterized by heterogeneity of clinical complications among patients [5]. Indeed, some patients have no severe symptoms while others experience frequent complications such as VOC, stroke, priapism, pulmonary hypertension and acute infections. Identifying the genetic factors that cause specific clinical complications in sickle cell patients has value in providing risk information and facilitating behavioral changes to avoid clinical complications. Thus, the present study investigated the polymorphism of the E-selectin gene and the ICAM-1 gene in sickle cell patients, and their possible association with SCD.

In humans, the E-selectin protein is encoded by the SELE gene for which several SNPs have been described, including E-selectin S128R and S149R [23]. In these SNPs, the serine is substituted by arginine in exon 4 at position 128 or 149 in the Epidermal Growth Factor (EGF) domain of the protein, respectively. Das S, et al. [24] reported that this substitution in E-selectin S128R affects its ligand binding affinity and increases the adhesiveness of leukocytes to the endothelium. In our case-control study, we examined the association between these two SNPs and SCD in the Congolese population, using the PCR-RLFP method for the genotyping. This technique is used in a wide range of screening applications to characterize single nucleotide polymorphisms (SNPs), as it offers a reliable and rapid way to genotype polymorphisms [25].

Our study included 110 SCD-patients in VOC and 120 healthy controls. The genotyping analysis of the E-selectin S128R and S149R polymorphisms showed that the S5 was the most prevalent and the RR the least common genotype. This trend was also observed in other ethnic populations as reported elsewhere [20,26]. When we compared the frequencies of genotypes and alleles in SCD patients versus controls, we found no statistically significant difference. In addition, the odd ratio values were not significant, indicating no association between the E-selectin S128R and S149R polymorphisms and SCD. Yet, these SNPs have been found to be associated with other pathologies also involving the endothelial dysfunction. For example, it has been reported that the R allele and the RS genotype in E-selectinS128R may be associated with the increased risk of ischemic stroke in Chinese, Asian, African, and Caucasian populations [26,27]. Wang X, et al. [28] suggested that theS128R polymorphism may be associated with the increased risk of coronary artery disease. On the other hand, the SR genotype of the E-selectin S149R gene may be associated with hypertension in African-Americans [29]. To the best of our knowledge, our study is the first to investigate the possible association between E-selectin polymorphisms and SCD. One reason for the lack of the association observed in our study may be the small size of study populations. Therefore, we cannot totally exclude the possibility that either of these SELE polymorphisms may influence the pathogenesis of SCD.

ICAM-1 is a molecule involved in the recruitment and activation of leukocytes at the site of inflammation. It is produced primarily on endothelial cells, but also on epithelial and immune cells in response to inflammatory cytokines [12]. ICAM-1 can also be found as a secreted...
molecule (sICAM-1), and elevated levels of sICAM-1 are related to the pathogenesis of numerous clinical diseases such as chronic obstructive pulmonary disease, asthma, multiple sclerosis, rheinitis and cancer [12]. Abnormally high levels of sICAM-1 are also observed in sickle cell patients with VOC [30]. However, the question remains whether elevated sICAM levels are related to polymorphisms in the encoding gene. Several variants of the ICAM-1 gene, including ICAM-1-E469K (rs5498), ICAM-1-R241G (rs1799969) and rs281432 (C/G) have been identified [31]. ICAM-1-E469K is the most investigated, and numerous studies have reported the association of its polymorphisms with various diseases. For example, a meta-analysis reported that the E allele of ICAM-1-E469K may be associated with susceptibility to Crohn's disease, but not to ulcerative colitis, in Europeans [32]. Another meta-analysis suggested that the ICAM-1-E469K polymorphism is associated with the risk of coronary heart disease risk amongst Chinese and Caucasians populations, and K allele is a risk factor [33]. Nepal G, et al. [34] the K469E polymorphism of the ICAM-1 gene is associated with ischemic stroke in the Caucasian population. However, there would be no association with rheumatoid arthritis [35].

Our study is the first to examine the association between the ICAM-1-E469K polymorphism and SCD. The genotype distribution showed that the EE genotype was the least frequent in both SCD patients (8.2%) and controls (9.3%), but was significantly associated with SCD (OR=1.19, 95% CI=1.08-2.36; P =0.01). Moreover, the E allele was also associated with SCD (OR=1.21, 95% CI=1.01-2.01; P=0.01). Taken together, our findings indicate that the EE genotype and the E allele of ICAM-1-E469K polymorphism may be used as genetic markers in SCD. However, from these results, we cannot claim the association with the risk of VOC. So, further studies, comparing SCD patients with VOC to those who have not yet experienced a VOC or depending on the age, number and frequency of VOC, are required.

One of the limitations of our study is the sample size of case and control groups which were not large enough. This could reduce the power of statistical tests and increase the likelihood of errors. Our results may also be affected by sources of bias that are common in the case-control studies.

Conclusion
This study provides preliminary evidence that the ICAM-1 K469E polymorphism but not the E-selectin polymorphism may be associated with SCD, possibly with VOC complication. However, further studies with large sample sizes and in other ethnic populations should be conducted to confirm our findings.

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Conflicts of Interest
The authors declare no conflicts of interest.

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