

## Association of MCP1-2518A/G and CCR2 –V64I Polymorphisms and Vaso-occlusive Crisis among Sickle Cell Anemia Tunisian Patients

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### Abstract

**Objective:** To further define the genetic basis of clinical variability in sickle cell anemia (SCA), we focus on the known functional polymorphisms of MCP-1 and CCR2 and we also discuss their associations with complications of SCA including vaso-occlusive crisis (VOC) and infection.

**Methods:** 100 SCA patients were enrolled in this investigation. The sample of patients was divided into two groups according to the presence or the absence of each complication. Polymorphisms studied namely: MCP-1-2518 A/G and CCR2 –V64I were genotyped for all subjects using PCR/RFLP. To test for trait association with the candidate SNPs, genotype and allele frequencies between cases (group with complication) and controls (group without complication) were compared using Pearson's chi\_square test with a significance threshold of  $p < 0.05$  (compare 2, version 1.02).

**Results:** Interestingly, we found a significant associations between MCP1-2518 A/G and V64I of CCR2 and occurrence of vaso-occlusive crisis among SCA patients  $p < 0.05$ . These associations have not been described previously.

**Conclusion:** The novelty of this report is that it is the first time that a similar study was made on the Tunisian sickle cell population and that the results show a novel association between functional polymorphisms namely: MCP1-2518 A/G and V64I of CCR2 and vaso-occlusive crisis among SCA Tunisian patients.

**Keywords** Sickle cell anemia; MCP1-2518A/G; CCR2 –V64I; Vaso-occlusive Crisis

### Abbreviations

CI: Confidence Interval, RR: Relative Risk; p: Index of Significance; SCA: Sickle Cell Anemia; VOC: Vaso-occlusive Crisis; RANTES: Regulated upon Activation Normal T cell-Expressed and Secreted; DARC: Duffy Antigen Receptor Chemokine; MCP-1: Monocyte Chemo-attractant Protein-1; CCL2: Chemokine Ligand2; CCR2: Chemokine Receptor 2

### Introduction

SCA is a single-gene mutation genetic disease caused by change of Glu6Val at the hemoglobin beta chain gene and characterized by high variable clinical complications [1]. This inter individual variability is due to the influence of globin and non-globin genes so called modifier genes [2]. Polymorphisms linked to various modifier genes have been studied in previous populations in order to determine any associations with SCA complications [3]. The UGT1A gene promoter (TA) repeat polymorphism has been confirmed in different populations, making this gene a potentially reliable biomarker that can be used as a diagnostic predictor [4-8]. On the other hand, polymorphisms situated

in some chemokine and chemokine-receptors were the subject of several studies [9,10]. Chemokine which are chemo-attractive cytokines, are one of many proteins playing an important role in the inflammation process and could be involved in the context of pro-inflammatory sickle cell disease. Some chemokine polymorphisms were found associated with clinical complications in sickle cell anemia (SCA) disease in some studies but not others [11-15]. It has been demonstrated that 2 functional polymorphisms in the proximal promoter region and in intron1 of the RANTES gene (-28C/G, -403G/A and INT1T/C). INT1TC has been described as associated with infectious complications in SCA in Paris or Cotonou patients [11] We have been interesting previously in the implication of RANTES in SCA Tunisian patients and our results have showed no association between -28C/G, -403G/A and INT1T/C and SCA [14] CCL2 (Chemokine ligand2) also known as Monocyte Chemo-attractant Protein 1 (MCP-1), is produced by lymphocyte and is thought to be responsible for monocyte and T-lymphocytes recruitment in acute inflammatory conditions and may be an important mediator in chronic inflammation [12,16]. The G allele of -2518 A>G MCP1 polymorphism [dbSNP: rs1024611] was found to increase MCP-1 expression. In fact, this polymorphism is located in the distal regulatory region of the MCP-1 gene relative to the major transcriptional start site of the gene [17]. MCP-1 is the major ligand for CCR2 (chemokine receptor2). CCR2 gene has 4 single nucleotide

polymorphisms (SNPs) identified in exon3 of which the major functional polymorphism V64I and its underlying G to A non-synonymous mutation at position 190, is the most common in the Caucasian population [18]. Furthermore, V64I polymorphism appears to result in a reduced binding of MCP-1 [19].

In this paper, we intend to study the impact of MCP-1-2518 A/G and CCR2 –V64I on the occurrence of VOC and infection for the first time among SCA Tunisian children. SCA is the second sickle cell hemoglobinopathy after b-thalassemia in Tunisia, representing a real public health problem. The average frequency of the trait in our country is 1.89% [20].

## Subjects and Methods

### Subjects

100 unrelated SCA Tunisian individuals were enrolled in this study. All sickle cell cases included in this study were selected on the basis of

the homozygosis  $\beta$ s-gene (HBB) locus. The characteristics of all subjects are summarized in Table1. Ethical approval was obtained from the patients. The study protocol is in accordance with the ethical standards laid down in the 1964 and 1975 Declarations of Helsinki including the latest modifications of 2008 (<http://www.wma.net/en/10home/index.html>).

### Methods

**Definition of clinical events:** Data and clinical events were taken from patient's history via search of the clinical registry. The patients chosen in this investigation have developed more than 20 VOC since birth. On the other hand all patients chosen have developed the same type of infectious events included: pulmonary, HCV (Hepatitis C Virus), meningitis, osteomyelitis and urinary infection.

	SCA patient Without VOC N=16(SS)	SCA Patient with VOC N=84(SS)	p	SCA Patient without infection N=66(SS)	SCA Patient with infection N=34(SS)	p
Age	29 ± 5	30 ± 5	0.45	30 ± 5	28 ± 5	0.33
Sex ratio M/F	6-Oct	51/33	0.08	30/36	15/19	0.12
Hb (g/dl)	9.1 ± 0.5	8.7 ± 0.5	0.22	8.9 ± 0.5	8.6 ± 0.5	0.65
RBC (1012 L)	3.29 ± 0.5	3.29 ± 0.9	1	2.89 ± 0.3	3.12 ± 0.2	0.54
WBC/mm3	10.3 ± 0.2	10.5 ± 0.1	0.65	11 ± 0.2	11 ± 0.6	0.46
MCV (fl)	79.7 ± 0.8	79.7 ± 0.9	1	79.7 ± 0.9	79.7 ± 0.9	1
MCH (pg)	34.9 ± 3.2	34.9 ± 2.1	1	35.8 ± 2.1	34.7 ± 2.1	1
RDW(%)	4.83 ± 0.4	4.80 ± 0.5	1	4.83 ± 0.5	5.10 ± 0.1	0.62
HbA	0	0	1	0	0	1
HbS (%)	86 ± 0.4	86 ± 0.3	1	86 ± 0.3	86 ± 0.3	1
HbF(%)	11 ± 0.3	11 ± 0.1	1	11 ± 0.1	11 ± 0.1	1
HbA2	3 ± 0.1	3 ± 0.2	1	3 ± 0.2	3 ± 0.2	1

Hb: hemoglobin; RBC: red blood cell; WBC: white blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; RDW: red blood distribution; VOC: vaso occlusive-crisis; P: index of significance; SS: homozygous of  $\beta$ s- globin gene mutation; The hematologic values are indicated as mean ± standard deviation.

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

**Laboratory methods:** Venous blood samples of 2.5 ml volume were drawn from the study subjects and were collected in K2-EDTA anticoagulant containers. SCA was diagnosed on the basis of cation-exchange high performance liquid chromatography (HPLC) (D10, Biorad) and further confirmation by means of DNA studies. The complete blood counts including counts of red blood cells (RBC), white blood cells (WBC), and the measurement of hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW) were performed using an automated cell counter (ABX pentra 60c+). Genomic DNA was extracted from peripheral blood using the standard phenol-

chloroform procedure.  $\beta$ s-globin gene was performed by restriction fragment length polymorphism (RFLP) as previously described by Romana M et al. [21]. We determined total and fetal hemoglobin (Hb F) concentrations by HPLC (D10 BioRad).

**Polymorphisms genotyping:** A 25  $\mu$ l PCR mixture contained 1.75 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.25 mM of each primer, 1XPCR buffer and 1.5 U Taq polymerase (Invitrogen, life technologies, Carlsbad, CA, USA) and 150 mg of total DNA as template. The PCR amplification was performed using a Biometra thermal cycler (TPersonal, Germany). The primers, the size of PCR product and the PCR working protocol cycle of each polymorphism were summarized in Table 2.

Polymorphisms	Primers (5'-3')	Product length	Cycling conditioned for 25 µl
-2518 A/G of MCP-1	F : GCTCCGGGCCAGTATCT R : GGCCATCTCACCTCATCTTCC	689pb	94°C 10 mn 35x(94°C 1 mn 62°C 1 mn,72°C 1 mn) 72°C10 mn
V64I of CCR2	F : TTGTGGGCAACATGATGG R : TGAAGAAGATTCGCCAAAA	222pb	95°C 10 mn 38x(95°C 30s 57°C 30s,72°C 1 mn)72°C 10 mn

**Table 2:** PCR conditions of studied polymorphisms

Detection of polymorphism MCP1-2518A/G was performed by PCR/RFLP. The PCR products were digested by PvuII (New England Biolabs, U.K.) which yields 507 pb and 182 pb when G is at position -2518. The products were separated on polyacrylamide gel, stained with ethidium bromide.

CCR2 -V64I was analyzed by PCR/RFLP. The PCR products were digested by BsaBI (New England Biolabs, U.K.) which yields 204pb and 18pb when mutant allele A is found. The products were separated on polyacrylamide gel, stained with ethidium bromide.

**Statistical analysis:** The sample of patients was divided into two groups according to the presence or absence of each complication. The demographic and hematologic data were normally distributed, so we calculated means and standard deviations using SPSS (18.0). We compared demographic and hematological and clinical data between the two groups of patients applying the t test. All SNPs were tested for deviation from the Hardy-Weinberg equilibrium using the software package Arlequin (version 3.01). Chi Square test or fisher test was used to determine genetic differences between patients using compare 2 (version 1.02).

## Results

The exploration of hemoglobin profile showed that the patients have homozygous β-S mutation and they haven't α thalassemia. The sample of patients was divided into two groups according to the presence or absence of each complication.

The two groups of patients stratified accordingly to the occurrence of VOC and infection were compared for age, sex ratio and

hematological data including HbF. No significant association was found (p>0.05) (Table 1).

For each polymorphism the samples were found to be in Hardy-Weinberg equilibrium (p>0.05). The genotyping of MCP-1 -2518A/G polymorphism shows the presence of three genotypes namely: AA, AG and GG (Table 3). Our findings show that patients with VOC presented 69.04% of genotype AA, 27.38% of genotype AG and 3.57% of genotype GG. Moreover, patients with infection presented 61.76% of genotype AA, 38.23% of genotype AG and no one with genotype GG. The genotypic and allelic distribution between patients according to the presence or not of each complication using Fisher's exact test and Chi square test showed a significant association between genotype AG and genotype GG and VOC among SCA patients (Table 3). Interestingly, the latter genotype appears to present a protective factor to the occurrence of VOC among SCA patients (Table 3). These results showed that the -2518 MCP-1 polymorphism is related to the susceptibility of VOC. Individuals with the AA genotype were at higher risk of this complication than subjects with the AG and the GG genotype. The genotyping of CCR2-V64I polymorphism show the presence of three genotypes namely: GG, GA and AA (Table 3). Our findings showed that patients with VOC presented 65.47% of genotype GG, 30.95% of genotype GA and 3.57% of genotype AA. Moreover, patients with infection presented 85.29% of genotype GG, 11.76% of genotype GA and 2.94% of genotype AA. Statistical analysis showed a significant association between genotype AA and allele A with VOC. Genotype AA and allele a present a risk factor for VOC (Table 3).

	VOC		P RR (CI95%)	Infection		P RR (CI95%)
	Presence N=84	Absence N=16	Presence N=34	Absence N=66		
MCP12518A/G						
AA	58	3	1*	21	44	1*
AG	23	10	1.210-3 0.12 (0.020-0.530)	13	17	0.417
GG	3	3	7.510-3 0.05 (0.05-0.603)	0	5	0.313

A	0.82	0.50	1*	0.81	0.794	1*
G	0.17	0.50	2.910-4 (0.087-0.506)	0.18	0.206	0.300
CCR2-V64I						
GG	55	6	1*	29	52	1*
GA	26	6	0.372	4	10	0.538
AA	3	4	7.110-3 12.32 (1.551-98.474)	1	4	0.659
G	0.80	0.56	1*	0.92	0.86	1*
A	0.19	0.43	4.910-3 1.269(1.04-1.55)	0.07	0.14	0.425
P: index of significance; RR: Relative-Risk; 1*: reference group; VOC: vaso-occlusive crisis.						

**Table 3:** Genotypic and allelic distribution of studied chemokine's polymorphisms

## Discussion

VOC and Infection are common in SCA and severe bacterial infections are the major causes of morbidity and mortality in SCA [19]. The pro-inflammatory context of the sickle cell disease promotes the liberation of cytokines. Cytokine polymorphisms were found associated with clinical complications in SCA in some studies but not others [11-14]. Interestingly, the investigation of polymorphism MCP1-2518 A/G show that patients with the AA genotype were at higher risk of VOC than subjects with the AG and GG genotype. Allele G has been identified to increase MCP1 expression. While allele A decrease the MCP1 expression [17]. The association between MCP1-2518 and SCA has never been described to date. Our findings highlighted a novel relationship between MCP1-2518 and VOC among SCA patients. However, [9] have reported an association between rs4586 of MCP-1 and stroke in SCA. As for CCR2V64I, our findings show that genotype AA and allele A present a risk factor for VOC. However, Hoppe et al, 2004 have not reported any association between V64I and stroke in SCA. In our study we were interested in the effect of combined genotypes explored in VOC and in infection nevertheless statistical analysis showed lack of significant association.

## Conclusion

Studies on chemokine polymorphisms have provided new possibilities for developing targeted drug therapy for many diseases. Our data provide evidence that MCP1-2518A/G and V64I of CCR2 were associated with clinical complication of SCA. A future treatment offering protection by inducing production or expression of the MCP1 and CCR2 protein may be beneficial for patients with SCA.

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