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

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The pro-inflammatory context of sickle cell disease promotes the liberation of cytokines such as CCL5, encoded by a gene located on chromosome 17. Herein, the occurrence of three variations of CCL5 in sickle cell anemia (SCA) and their relations to two major complications – painful crisis and presence of infections – were investigated.
100 SCA Tunisian patients and 100 healthy subjects were included in the case control study. Then the sample of patients was divided into two groups according to the presence or absence of each complication. The polymorphisms, namely g.-403G>A, g.-28C>G and g.In1.+1T>C, were analyzed by PCR/sequencing.
Our findings show the presence of eight genotypes, namely GG, GA and AA of g.-403G>A, CC, CG and GG of g.-28C>G, and TT and TC of g.In1.+1T>C. The frequencies of studied single nucleotide polymorphisms (SNPs) and haplotypes in SCA patients do not differ significantly from healthy control group results. There is also no significant association between the analyzed polymorphisms and complications as for painful crisis and presence of infections ($p > 0.05$).
Altogether, our data support the conclusion that the three polymorphisms of CCL5, namely g.-403G>A, g.-28C>G and g.In1.+1T>C, do not seem to be involved in the clinical variability of SCA in Tunisia.

Key words: sickle cell anemia, RANTES, polymorphisms, painful crisis, infections.

Introduction

Sickle cell anemia (SCA) is an autosomal recessive disease, due to the substitution of an adenine by a thymine on nucleotide 17 in exon 1 of the β-globin gene (βs). This substitution is responsible for the synthesis of abnormal hemoglobin called HbS [1]. Sickle cell anemia is characterized by several complications such as painful crisis, stroke, infections, splenic sequestration and other degenerative organs. The clinical phenotypes of homozygous HbS are extremely variable among patients [2, 3]. This inter-individual variability suggests a multifactorial genetic intervention, out of morbid gene (βs) of sickle cell anemia [4]. Polymorphisms linked to various modifier genes (globin and non-globin) have been studied in previous populations in order to determine any associations with SCA complications such as C677T of MTHFR, which has been described as associated with avascular necrosis. SNP

rs1042713 in ADRB2 and SNP rs3730070 of ADCY6 polymorphisms were associated with elevated adhesion. 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 of cholelithiasis in SCA [4]. The pro-inflammatory context of SCA may favor the liberation of pro-inflammatory cytokines. Cytokines seem to be involved in several possible mechanisms in the pathogenesis of vaso-occlusive phenomena in SCA: vascular endothelial activation, induction of red-cell adhesiveness to vascular endothelium, induction of neutrophil adhesiveness to endothelium, development of vascular intimal hyperplasia, platelet activation, endothelin-1 production, and dysregulation of endothelial apoptosis. Furthermore, cytokines are responsible for monocyte and T-lymphocytes recruitment in acute inflammatory conditions and may be an important mediator in chronic inflammation [5]. Regulated upon activation, normal T cell-expressed and secreted (RANTES), also called CCL5 [chemokine (C-C motif) ligand 5], is a member of the CC chemokine family which is involved in the chemotaxis of leukocytes into inflammatory sites. In addition, RANTES activates the Gardos channel via its erythrocyte receptor called Duffy antigen receptor for chemokines (DARC). The activation of this channel causes a massive loss of potassium and water, leading to the dehydration of red cells [6, 7]. The human CCL5 gene spans 8.8 kb on chromosome 17 (q11.2-q12) and consists of a promoter, three exons and two introns. This gene is home to various polymorphisms, three of which, namely g.-403G>A and g.-28C>G in the promoter and g.In1.+1T>C in the first intron, have been suggested to affect the expression of CCL5 [8, 9]. Indeed, the substitutions of G by A at position -403 and of C by G at position -28 have been reported to up-regulate CCL5 transcription [10, 11]. Conversely, the substitution of T by C in intron 1 is associated with reduced CCL5 transcription [8]. Several studies have shown the association of CCL5 gene variants with infectious diseases including AIDS [8, 12] and tuberculosis [13, 14] and with several chronic inflammatory diseases such as atopic dermatitis [15] and asthma [16]. Among these three polymorphisms only the substitution of T by C in intron 1 has been found in correlation with sickle cell disease, by Dossou-Yovo *et al.* (2009) [9].

Herein, we aimed to study the association of functional polymorphisms, namely g-403G>A and g.-28C>G in the promoter and g.In1.+1T>C in the first intron, with SCA then with two complications of the disease, i.e. painful crisis and presence of infections. The choice of these complications was based on the implication of CCL5 in the pathophysiology of SCA. Indeed, the activation of the Gardos channel by CCL5 causes the dehydration of red cells and therefore may aggravate the occurrence of vaso-occlusive crisis (VOC) [6, 7]. Furthermore, CCL5 plays an important role in the immune response to infection. CCL5 has a predominant chemotactic action on monocytes-macrophages and hence in attraction and sequestration of mononuclear-phagocytes and dendritic cells [8, 12].

Material and methods

Population studied

The study enrolled 200 subjects including 100 SCA patients and 100 healthy subjects were used as controls. SCA patients were selected on the basis of homozygosity for the βs-globin gene. All these patients were followed up at the laboratory of Clinical Hematology in Aziza Othmana Hospital. In our study, we selected SCA patients in whom the fetal hemoglobin (HbF) level varied from 10% to 15%. The healthy subjects were chosen from the blood bank on the basis of their normal hematologic data. Informed consent was obtained from all patients. Hematological and clinical data of subjects studied are summarized in Table I.

Methods

Diagnosis and laboratory parameters of SCA patients

Hematologic data were obtained with an automated cell counter (ABX pentra 60c+). Diagnosis of sickle cell anemia was performed using cation-exchange high performance liquid chromatography (HPLC) (D10 BIO-RAD) by specific elution windows defined for abnormal hemoglobins such as Hb S, Hb D, Hb E and Hb C and further confirmed by means of molecular

Table I. Hematological, demographic and clinical data of studied population

	SCA patients	Control group
Number	100 SS	100 AA
Age (mean)	30 ±5	30 ±5
range	25-35	25-35
Sex ratio (M/F)	36/64	50/50
Hb (g/dl)	7.6 ±1.3	12.2 ±0.9
RBC (10^{12}/l)	2.38±0.1	4.5 ±0.6
WBC (10^3/mm^3)	15.8 ±0.9	4.82 ±0.2
MCV (fl)	96.5 ±0.9	86 ±1.1
MCH (pg)	28.7 ±2.1	35 ±1.3
RDW (%)	8 ±1.8	2 ±0.1
HbA	0	97 ±0.3
HbS (%)	86.8 ±0.7	0
HbF (%)	13.2 ±1.6	0
HbA2	0	3 ±0.1
Pain crisis	84	0
Infection	34	0

The hematological values are indicated as mean ± standard deviation.

methods. Mutation at codon 6 of the β^S globin gene was determined by restriction fragment length polymorphism (PCR-RFLP) using DdeI [17]. Biochemical data were averaged for each patient in steady state (at least three values). Total and fetal hemoglobin concentrations, reticulocyte count and other hematological parameters were determined.

Clinical events analyzed

The current work is a retrospective study. Past history of painful crisis and infection was investigated for each patient from their records. The infectious events included pulmonary infection, HCV (hepatitis C virus), meningitis, osteomyelitis and urinary infection.

g.-403G>A, g.-28C>G and g.In1.+1T>C of CCL5 genotyping

Genomic DNA was extracted from peripheral blood by the standard phenol-chloroform procedure. Polymorphisms g.-403G>A, g.-28C>G and g.In1.+1T>C were genotyped by polymerase chain reaction (PCR) using the pair of primers CCL5F (5'-CAGAGGACCCTCCTCAATAAAAC-3') and CCL5R (5'-CTCCCCAACATGAGTCCACAC-3'). PCR reaction amplified a sequence of 899 pb. 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 $MgCl_2$, 0.5 U Amplitaq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) and 10 pmol of each forward and reverse primer. The PCR cycling conditions included an initial denaturation step of 10 min at 94°C followed by 35 cycles of 94°C for 60 s, annealing at 62°C for 60 s and extension at 72°C for 1 min. The run was ended by a final extension at 72°C for 10 min.

PCR products were then purified and doubly sequenced (forward and reverse) by ABI PRISM Big Dye Termination ready reaction kit and an ABI 310 DNA sequencer (Applied Biosystems, Foster City, USA). The products obtained were translated into chromatograms using "Bio-Edit" software (Bio Edit Sequence Alignment Editor, V7.0.5).

Statistical analysis

Departures from Hardy-Weinberg equilibrium were tested using the software package Arlequin (version 3.01). χ^2 test using compare 2 (version 1.02) was performed to demonstrate the association of different genotypes with SCA. Genetic differences between groups of patients with or without complications were evaluated by exact tests for genotypic or allelic contingency tables using compare 2 (version 1.02). The p value was calculated for all tests and OR (odds ratio) was estimated for each significant p value. ORs were calculated with 95% confidence intervals (CI) at the 0.05 significant level using the homozygous wild-type genotype as a reference. Regression test using SPSS (16.0) was performed to demonstrate the association of different haplotypes with each complication of SCA.

Table II. Genotypes and alleles of the RANTES polymorphism distribution between patient group and healthy group

GENETIC PROFILE	SCA PATIENTS (N = 100)	CONTROL SUBJECTS (N = 100)	P
-403G/A (genotypes)			
GG	68	69	1*
GA	25	28	0.88
AA	7	3	0.21
-403G/A (alleles)			
G	0.80	0.83	1*
A	0.19	0.17	0.44
-28C/G (genotypes)			
CC	99	100	1*
CG	1	0	1
GG	0	0	1
-28C/G (alleles)			
C	0.99	1	1*
G	0.00	0	1
INT1T/C (genotypes)			
TT	79	79	1*
TC	16	18	0.75
CC	5	3	0.72
INT1T/C (alleles)			
T	0.87	0.88	1*
C	0.13	0.12	0.76

1 – reference group*

Results

Case-control study

For each polymorphism the samples were found to be in Hardy-Weinberg equilibrium ($p > 0.05$). The analysis of polymorphism g.-403G>A showed the presence of three genotypes, namely GG, GA and AA, in both the patient group and the control group. There was no significant association between patients and controls according to genotypic and allelic profile (Table II).

The analysis of polymorphism g.-28C>G showed the presence of 99 patients with genotype CC (wild) and only 1 patient with genotype CG. For the control group all subjects had genotype CC. There was no significant association between patients and controls according to genotypic and allelic profile (Table II). The analysis of polymorphism g.In1.+1T>C showed the

presence of three genotypes, namely TT, TC and CC, in both the patient group and the control group. The comparison of patients and controls according to genotypic and allelic profile showed no significant association with SCA (Table II).

Polymorphism/phenotype correlation

Assessment of hematological, biochemical and clinical data was performed for 200 subjects studied including SCA patients and normal subjects (Table I). We classified the sample of patients according to the presence or absence of each complication. The two groups of patients stratified according to the occurrence of VOC and infection were compared for age, sex ratio and hematological data including HbF. No significant association was found ($p > 0.05$).

We investigated the association between the CCL5 polymorphisms and clinical phenotypes observed in patients with sickle cell disease such as painful crisis and presence of infections. The group of patients was divided into two sub-groups according to the presence or absence of each complication. The distribution of genotypes and alleles was analyzed for the two subgroups. For the polymorphism g.-403G>A, 83.82% of GG, 76.00% of GA and 100.00% of AA carriers had developed a painful crisis at least once in their life. For the polymorphism g.-28C/G, 83.83% of CC and a single GC carrier presented painful crisis while nobody with sickle cell disease presented the genotype GG. As regards the polymorphism g.In1.+1T>C, 84.81% of TT, 75.00% of TC and 100.00% of CC carriers had developed a painful crisis. Among patients who presented infections, genotypes emanating from the three polymorphisms above showed that in all cases the normal genotypes -403GG, -28CC and In1.+1 TT are not associated with the presence of infection. Some patients with mutant genotypes presented an infection, involving in particular 48.00% of -403GA, 28.57% of -403AA, 100.00% of -28CG, 43.75% of In1.+1TC and 40.00% of In1.+1CC carriers. Statistical analysis showed no significant association between CCL5 polymorphisms and either painful crisis or presence of infections (Table III).

Table III. Distribution of genotype and allele frequency in SCA patients according to the presence or absence of complications

	SCA PATIENTS WITH PAINFUL CRISIS (N = 84)	SCA PATIENTS WITHOUT PAINFUL CRISIS (N = 16)	P	SCA PATIENTS WITH INFECTION (N = 34)	SCA PATIENTS WITHOUT INFECTION (N = 66)	P
-403G/A						
GG	57	11	1*	20	48	1*
GA	20	5	0.38	12	13	0.06
AA	7	0	0.58	2	5	1
-28C/G						
CC	83	16	1*	33	66	1*
CG	1	0	1	1	0	1
GG	0	0	1	0	0	1
INT1T/C						
TT	67	12	1*	25	54	1*
TC	12	4	0.46	7	9	0.29
CC	5	0	1	2	3	0.64
Allele frequency						
-403G/A						
G	0.79	0.84	1*	0.76	0.82	1*
A	0.20	0.15	0.54	0.23	0.17	0.30
-28C/G						
C	0.99	1	1*	0.98	1	1*
G	0.005	0	1	0.01	0	1
INT1T/C						
T	0.86	0.87	1*	0.83	0.88	1*
C	0.13	0.12	1	0.16	0.11	0.33

1 – reference group*

Haplotype/phenotype correlation

For the sample of patients, the study of three CCL5 polymorphisms (g.-403G>A (rs2107538), g.-28C>G (rs2280788) and g.In1.+1T>C (rs2280789)) revealed the presence of three haplotypes obtained by homozygosity: rs2107538*G/rs2280788*C/rs2280789*T (N = 65), rs2107538*A/rs2280788*C/rs2280789*T (N = 2) and rs2107538*A/rs2280788*C/rs2280789*C (N = 3). We used logistic regression analysis to determine a possible correlation between different haplotypes and each complication studied, namely vasoocclusive crises and infections. Our results show no significant association between haplotypes found and each complication ($p > 0.05$).

Discussion

The pro-inflammatory context of sickle cell disease promotes the liberation of cytokines. Cytokine polymorphisms were found associated with clinical complications in SCA in some studies but not others. The substitution of T by C in intron 1 of CCL5 was found in correlation with sickle cell disease by Dossou-Yovo *et al.* (2009) [9]. Sebastini *et al.* (2005) reported an association between rs4586 of MCP-1 and stroke in SCA [18]. Hoppe *et al.* (2004) did not report any association between V64I of CCR2 and stroke in SCA [19]. CCL5 is involved in the red cell dehydration process and in inflammatory reactions [7]. The gene encoding this cytokine is home to various polymorphisms; three of them, namely g.-403G>A and g.-28C>G in the promoter and g.In1.+1T>C in the first intron, have been suggested to affect the expression of CCL5 [8]. Among these three polymorphisms only the substitution of T by C in intron 1 has been found in correlation with sickle cell disease, by Dossou-Yovo *et al.* (2009) [9]. CCL5 in a prospective study during 5 years. The study enrolled 115 SS patients composed of 87 Africans from Benin and 28 from "Paris". This study showed that the frequency of the mutant allele -403A is 45.5% in Benin SS patients and is 57.5% in Paris SS patients. In contrast, our results showed that the frequency is much lower, of the order of 19.5%. Concerning the mutant allele -28G, its frequency is 0.5% for sickle cell anemia in Tunisia whereas it is invalid in sickle cell Afro-Europeans. For the allele In1.1+C, we found a frequency of 13%. This has been reported also in the case of Afro-Europeans [9]. These results show that there is inter-ethnic variability in allele frequencies in promoter polymorphisms (g.-403G>A and g.-28C>G) and similarity in the intronic polymorphism allele frequencies (g.In1.+1T>C). Furthermore, our case-control study does not show any significant association between the latter polymorphisms and SCA ($p > 0.05$). In a second step, we aimed to establish a possible association between polymorphisms and painful crisis and infections. Our findings show no significant association between genetic profile including genotype, allele and haplotype with each complication. The study reported by Dossou-Yovo *et al.* [9] did not include an investigation of haplotypes and their implication in each complication but they reported similar results concerning the absence of a correlation between the three polymorphisms and painful crisis within the Afro-European population. Nevertheless, the same study reported the presence of a significant association between RANTES polymorphisms and infection, particularly in patients carrying the mutated allele In1.+1C, suggesting its protective effect against bacterial infection [9].

In conclusion, our data showed that three polymorphisms of CCL5, namely g.-403G>A (rs2107538), g.-28C>G (rs2280788) and g.In1.+1T>C (rs2280789), do not seem to be involved in the clinical variability of SCA in Tunisia.

The authors declare no conflict of interest.

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