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Association of rs1319868, rs1567811 and rs8041224 of *IGF1R* gene with infection among sickle cell anemia Tunisian patients

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ABSTRACT

Background and aim: Sickle cell anemia (SCA) is characterized by variable patterns of clinical expression. Polymorphisms linked to different genes have been associated with specific complications of the disease. Herein, we focused on the study of the association of 4 polymorphisms of Insulin like Growth Factor 1 receptor (*IGF1R*) gene with infections, which are the major cause of death in SCA. **Material and methods:** This study involved 116 sickle cell patients among whom 58 SS have the same confirmed infectious phenotype. Allele-Specific PCR was performed for the study of rs1319868, whereas the PCR/sequencing method was carried out for rs1567811, rs2872060 and rs8041224. **Results:** The results showed that rs1319868 and rs1567811 were associated with a decreased risk of infection among SS patients ($p = 0.038$, RR = 0.54; $p = 0.044$, RR = 0.56, respectively). Interestingly, the combination of different genotypes showed the association of the genotype GT of rs1319868 and the genotype CC of rs8041224 with further decreased infection risk in SCA ($p = 0.028$, RR = 0.04). **Conclusion:** These significant associations of *IGF1R* SNPs with infection suggest that this gene could play an important role in the immune function in SCA.

Introduction

Despite sharing the same β globin mutation, the range of severity in the phenotype of SCA is striking, with patients disabled by many complications. SCA patients are also differently predisposed to several pathological manifestations. Over the last 20 years scientific research has tried to elucidate the role of several factors responsible for this clinical variability [1]. In fact sickle cell phenotype appears

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to be modulated by polymorphisms of genes involved in several biological mechanisms whose understanding will elucidate the pathophysiology of the disease [2]. This modulation has been firstly attributed to the structure of β globin cluster. As a matter of fact the variation in hemoglobin F (HbF) levels is linked with β globin gene cluster haplotypes and other chromosomal sites, significantly influencing the severity of the disease [3]. Additionally, coexistence of α-thalassemia with SCA produces hematological and clinical consequences that are beneficial in some complications but damaging in others [4]. Nevertheless these globin modifier genes of the SCA severity do not seem to be enough to explain all the phenotypic heterogeneity of this disease.

In the last decade, many studies suggest that SCA phenotype is multigenic, including other mutations located in non-globin genes that appear to be involved in the clinical variability of the disease [5]. In fact, some of these genes may interact to modulate several complications simultaneously. Among these the *BMP6* affecting both stroke, leg ulcer and infections, *TGFBR3* involved in vascular necrosis, leg ulcers, priapism and infections [6]. Other genes may be specific to a single complication include the case of *UGT1A1* in cholelithiasis [7–11] and *IGF1R* in infection [12].

Indeed, polymorphisms in a number of genes involved in the immune response have been suggested as contributing factors to increased susceptibility to infection in SCA. These genetic events are an important part of the infectious phenotype and explain the inter-individual differences in spite of all the patients having the same sickle globin gene in the homozygous form. Infection is common in SCA, and severe bacterial infections are the major causes of morbidity and mortality in SCA patients [13]. Particular *HLA II* subtypes have been shown to be predisposing or protecting factors for infectious complications [14] while some single nucleotide polymorphisms in the *IGF1R* and *TGFβ/BMP* pathway genes have been associated with an increased risk of infection [15]. IGF1R is pervasively expressed by almost all cells, regulates major biological processes (angiogenesis, apoptosis, cell differentiation, etc.) through interactions with several signaling pathways [16]. Variation in *IGF1R* gene might modulate the risk of infection by contributing to abnormal signaling in the TGFβ pathway and affect the response of B and T lymphocytes to bacteria [15].

In this paper, we aimed to explore the association of four known polymorphisms of *IGF1R* gene namely: rs1319868, rs1567811, rs2872060 and rs8041224 with an infection-prone phenotype among Tunisian SCA patients.

Material and methods

Material

This study was performed from November 2009 until May 2010, in Pasteur Institute of Tunis, Tunis, Tunisia. The study involved 116 unrelated SCA subjects, all Tunisians, among whom 58 were patients with reported infection cases. Control subjects had no history of infection. All of them have homozygosity for βT/A globin mutation which called SS and they have the same Benin haplotype for microsatellite and restriction haplotype. Extensive patients' clinical information was gathered in order to establish a case file database. Table I summarizes the main characteristics of the studied population.

Clinical events analyzed

Data and clinical events were taken from patient's history via search of the clinical registry. On the other hand all of the patients chosen have developed the same type of infectious events including: pulmonary, meningitis, osteomyelitis and urinary infection.

Laboratory methods

Venous blood samples of 2.5 ml volume were collected from 116 sickle cell subjects enrolled in this study and was

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

	Case SCA patients with infection	Control SCA patients without infection	*p*
Number	58 SS	58 SS	
Age (mean)	27 ± 2.9	31 ± 3.6	0.425
Sex ratio (M/F)	19/39	22/36	0.423
Hb (g/dl)	7.3 ± 0.9	7.9 ± 1.3	0.521
RBC (1012/L)	2.89 ± 1.02	3.29 ± 0.9	0.270
MCV (fl)	77.2 ± 1.3	79.7 ± 0.9	0.560
MCH (pg)	35.7 ± 1.02	34.9 ± 2.1	0.100
RDW (%)	5.29 ± 1.02	4.83 ± 0.5	0.579
HbA	0	0	1
HbS (%)	86.4 ± 0.4	86 ± 0.3	1
HbF(%)	10.6 ± 0.3	11 ± 0.1	1
HbA2	3 ± 0.1	3 ± 0.2	1

The demographic and hematologic values are indicated as mean ± standard deviation.
SS – homozygous of β globin gene mutation; Hb – hemoglobin; RBC – red blood cell; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; RDW – red blood distribution width.
Statistics for the comparison of demographic and hematological variables between the two groups were performed using the *t* test and chi-square test as appropriate (SPSS 16.0).

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. βs-globin gene was performed by restriction fragment length polymorphism (RFLP) as previously described by Romana M et al. 2000 [17]. We determined total and fetal hemoglobin (Hb F) concentrations by HPLC (D10 BioRad).

Molecular methods

116 patients chosen for the molecular methods were selected on the basis of homozygosity for β globin gene. Genomic DNA was extracted from peripheral blood by the standard phenol-chloroform procedure. rs1567811, rs2872060 and rs8041224 genotyping was performed using PCR/sequencing. Whereas rs1319868 genotyping was performed using AS-PCR.

rs1567811, rs2872060 and rs8041224 genotyping

The analysis of rs1567811, rs2872060 and rs8041224 was performed by means of simplex PCR using primers shown in Table II. Each PCR reaction contained 100 ng of template genomic DNA. After an initial denaturing at 94 °C for 10 min, PCR was carried out for 35 cycles each consisting of denaturing at 94 °C for 1 min followed by an annealing step at different temperatures for each primer pair during 1 min and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 10 min. PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase and subsequently doubly sequenced by ABI PRISM Big Dye Terminator Ready Reaction using ABI 310 DNA Sequencer (PE Applied Biosystems, Fosterlity, USA).

rs1319868 genotyping

An allele-specific PCR was performed for rs1319868 using 3 primers, one common (13C) and two allele specific (13W: wild type; 13 M: mutant). The primer sequences are listed in the table below (Tab. II). The amplification consisted of an initial denaturing at 94 °C for 10 min followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s. The final extension step was 72 °C for 10 min. PCR fragments were run out on 2% agarose gel for 1 hour, and the expected 221 pb band was visualized under UV.

Data analysis

The 116 SS patients were divided into two groups according to the presence or absence of the infection. Firstly, we compared demographic, hematological and clinical data between the two groups of patients (case and control). Secondly, we test for trait association with the candidate SNPs, genotype and allele frequencies between two groups according to the presence or the absence of the infection.

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 the author calculated means and standard deviations using SPSS (18.0). Then 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 Beyond compare 2 (version 1.02). Stratification of different combination of genotypes found according to the presence or absence of infection was evaluated by logistic regression model using SPSS (18.0) and statistical significance was defined as $p < 0.05$.

For the haplotype analysis, linkage disequilibrium between SNPs and the presentation of marker haplotypes was investigated with Haploview (version 4.2).

Table II – conditions of PCR

SNP ID	SNP location	Primer set	Primers	Amplicon size pb	Anneal temperature
rs1319868	−5782 5′UTR	13C	5′CACCGCAAATGACAAGTGAAAA3′	221 pb	60 °C
		13W	5′TGGGCCAAGAGTGATAGGCAC3′		
		13M	5′TGGGCCAAGAGTGATAGGCAA3′		
rs1567811	+35496 intron 2	15F	5′GCTTTGAGAGACCGAGACAGGA3′	362 pb	59 °C
		15R	5′AATGACCGGCAAACTGTGAAAT3′		
rs8041224	+51328 intron 2	80F	5′GGGAACCTGAGGATTTGGAAGT3′	377 pb	58 °C
		80R	5′GAACCAAAACTTCCCTCCCTGT3′		
rs2872060	+4203 exon 21	28F	5′CGCTCATGTAAGAGAGGGCTGT3′	400 pb	59 °C
		28R	5′CCTGAAGCCAGATTCCAACAAG3′		

SNP position was set using the reference sequence from NCBI database. C – common primer; W – wild type primer; M – mutant primer; F – forward primer; R – reverse primer.

Results

Demographic, hematological and biochemical analysis

The hemoglobin profiles of the sample of patients show the presence of 1 phenotype which is the homozygous mutant SS patients.

The distribution of each continuous variable was performed using the t-test. The results show that there is no significant difference between the two groups of SCA patient according to the presence or the absence of infection ($p > 0.05$).

Polymorphisms analysis

For each polymorphism the samples were found to be in Hardy-Weinberg equilibrium ($p > 0.05$).

The analysis of the rs1319868 showed the presence of three genotypes namely: GG, GT and TT in both patient groups. The results showed that the mutant allele T was significantly associated with the infection in SCA and that this allele seems to decrease the susceptibility to infection in SCA (Tab. III).

The analysis of the rs1567811 showed the presence of three genotypes namely: CC, CG and GG in both patient groups. The results showed that the mutant allele G was significantly associated with the infection in SCA and that this allele seems to decrease the susceptibility to infection in SCA (Tab. III).

The analysis of the rs8041224 showed the presence of three genotypes namely: TT, TC and CC in both patient groups. The findings showed no significant association between patients and controls according to genotypic and allelic profile (Tab. III).

The analysis of rs2872060 showed the presence of three genotypes namely: GG, GT and TT in both patient groups. The findings showed no significant association between patients and controls according to genotypic and allelic profile (Tab. III).

Significant finding was found in genotypic combinations of different SNPs. Interestingly, stratification of genotypes found using logistic regression according to the presence or absence of infection revealed that the combination of rs1319868 in heterozygous state GT and rs8041224 in mutant homozygous state CC was the most significant (25.86% control and 3.44% case, data not shown) and hence appears to decrease the susceptibility to infection in SCA with a global p value of 0.028 yielded RR of 0.04 (95% CI, 0.001–1.048) (Tab. IV). Next, haplotype probability was performed through deduction of different possibilities of allele combination. The statistical methods assume that all subjects are unrelated and that haplotypes are ambiguous (due to unknown linkage phase of the genetic markers). The genetic markers are supposed to be codominant (one-to-one correspondence between their genotypes and their phenotypes),

Table III – Distribution of polymorphisms genotypes and allele frequency according to the presence or not of Infection in SCA patients

Polymorphisms	Control N = 58	Case N = 58	*p* value	RR CI 95%
rs1319868				
GG	20	32	1*	–
GT	28	20	0.074	–
TT	10	6	0.160	–
G	68	84	1*	–
T	48	32	0.038	0.54 (0.299–0.969)
rs1567811				
CC	6	10	1*	–
CG	26	34	0.898	–
GG	26	14	0.114	–
C	38	54	1*	–
G	78	62	0.044	0.56 (0.317–0.985)
rs8041224				
TT	5	10	1*	1*
TC	23	28	0.608	–
CC	30	20	0.128	–
T	33	48	1*	–
C	83	68	0.054	–
rs2872060				
GG	16	11	1*	1*
GT	29	28	0.628	–
TT	13	29	0.244	–
G	61	50	1*	1*
T	55	66	0.189	–

1* – reference group; P – index of significance; RR – relative risk; CI – interval of confidence.
Case: SCA patients with infection.
Control: SCA patients without infection.

Table IV – Repartition of combinated genotypes according to the presence of infection

Combinated genotypes	Infection	
	p	OR (CI 95%)
(rs1319868/rs1567811)	0.230	–
(rs1319868/rs2872060)	0.120	–
(rs1319868/rs8041224 GT/CC)	0.028	0.04 (0.001–1.048)
(rs1567811/rs2872060)	0.560	–
(rs1567811/rs8041224)	0.740	–

P – index of significance; OR – odds ratio, CI – interval of confidence.

and so we refered to the measurements of genetic markers as genotypes. The findings showed that the haplotype GGTC was major in case (0.387) and control (0.295) and that the haplotype TGGT was minor in all subjects (0.112 in cases and 0.13 in controls). However no significant differences were detected between the two groups of SCA patients (data not shown). For linkage disequilibrium, expressed as r^2, evaluation between pairwise SNPs was determinate. Interestingly, significant findings were found with 2 *IGF1R* markers namely: rs1567811 and rs8041224 in strong linkage disequilibrium with the most significant association ($r^2 = 29$).

Discussion

Previous studies have suggested that genetic heterogeneity influence the susceptibility to infection in SCA [14, 15, 18–20]. Polymorphisms in a number of gene involved in immune response have been suggested as contributing modify this susceptibility [1]. HLA II has been shown to be protecting for infections complications [14] while polymorphisms of the manose-bending lectin [21], Fc receptor [19] and gene of the TGFβ/BMP pathway have been associated with an increased risk of infection. As for the *IGF1R* gene, Adewoye et al. have showed that the mutant allele T of rs1319868 increase the risk of infection with $p = 0.059$ and RR = 1.92 (95% CI, 1.20–3.03). By cons, our results showed that the mutant allele T seems to have a protective role against infection $p = 0.027$; RR = 0.54 (95% CI, 0.317–0.985). This result can be confirmed by the fact that this polymorphism is affecting a binding site for transcription factors at 5′ UTR of *IGF1R* gene.

The same study has reported that the homozygous GG of rs1567811 is not associated with infection ($p = 0.0586$). Whereas our findings showed that the mutant allele G is associated with a protective factor against infection among SCA patients with $p = 0.032$; RR = 0.56 (95% CI, 0.317–0.985). Furthermore, Adewoye et al. have showed that the mutant homozygous genotype CC of rs8041224, appears to increase the risk of infection in SCA with $p = 0.0133$; RR = 1.96 (95% CI, 1.15–3.33) and that the homozygous mutant TT of polymorphism rs2872060 presented a risk factor for infection with $p = 0.0321$; RR = 3.03, (95% CI, 1.10–8.33). Our findings showed that rs2872060 and rs8041224 were not associated with infection in SCA. This result can be explained by the localization of the rs2872060 in STS which be eliminated by splicing activity and therefore it has no effect on expression or activity of the IGF1 receptor and the localization of rs8041224 in intronic region.

The limit of the study reported by Adewoye et al. is that they enrolled in their study different ethnic groups; however our subjects are all Tunisians. Moreover they have enrolled in their study only patients with bacteremia, whereas, this study included pulmonary, meningitis, osteomyelitis and urinary infection.

In addition the additive effect of the mutations studied by different genotypic combinations was tested. A correlation between the combination GT/CC (rs1319868/rs8041224) and infection in SCA suggest a protective role against this complication with $p = 0.028$; RR = 0.04 (95% CI, 0.001–1.048). To the best of our knowledge this result is reported for the first time among SCA patients.

Conclusion

The novelty of this report is that it is the first time that a similar study was made on the SCA Tunisian patients. The results show a clear association of rs1319868, rs1567811 and rs8041224 of *IGF1R* gene with Infection among SCA Tunisian patients. Interestingly the findings show the association of the mutant allele G of rs1567811 with protective effect for infection, association not described previously in other population. The results will require additional validations and functional studies to understand the mechanisms by which IGF1R might play a role in the modulation of this complication.

Authors' contributions/ Wkład autorów

LC – study design, statistical analysis, data interpretation, manuscript preparation. MK – data collection. MBS – data collection and interpretation, statistical analysis, manuscript preparation, literature search, funds collection. IM – statistical analysis. HO – data interpretation. SA – manuscript preparation. FM, RH, IB, DC – funds collection.

Conflict of interest/ Konflikt interesu

None declared.

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Ethics/ Etyka

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to

Biomedical journals. This study was approval by the ethics committee of Pasteur Institute of Tunis.

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