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Implication of rs1026611 in the *MCP-1* Gene and V64I of *CCR2* in Stroke among SCA Tunisian Patients

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Abstract

Stroke is a devastating and potentially fatal complication to sickle cell Anemia. Strokes are difficult to explain on the basis of the central pathological process in SCA, namely the occlusion of small vessels by deformed sickled cells. We examined whether Single Nucleotide Polymorphism (SNP) variants in the MCP-1 or CCR2 genes independently or in combination are associated with occurrence of Cerebrovascular Accidents (AVC) in SCA Tunisian patients.

Material and methods: 100 SCA patients among whom 19 have AVC were enrolled in this study. Clinical diagnosis of stroke was performed by the use of Transcranial Doppler ultrasonography (TCD). The genotyping of rs1026611 in the *MCP-1* gene and V64I of *CCR2* was performed using PCR/RFLP.

Results: Our findings showed no association of the polymorphisms studied with occurrence of AVC in SCA Tunisian patients.

Keywords: MCP-1-2518A/G; CCR2V64I; SCA; AVC

Introduction

Stroke remains one of the important complications of SCA and is especially critical in the care of children with this disorder. The epidemiology of stroke and primary and secondary prevention strategies based on transfusion have recently been established in large multicenter studies, but treatment of acute stroke and a basic understanding of what causes cerebrovascular disease in this hemoglobinopathy have progressed very little in recent years [1]. Interestingly, the hypothesis of modifier gene in SCA can help the researchers to understand this disease [2]. Herein we focused on two chemokine's namely Monocyte chemo attractant protein 1 (*MCP-1*), with its receptor chemokine receptor 2 (*CCR2*). *MCP-1* acting in concert with its receptor *CCR2*, promotes recruitment of macrophages into atherosclerotic plaque [3]. Chemokine's, which play an important role in inflammation, are families of cytokines that are important mediators of leukocyte trafficking [4]. MCP-1 is a member of the C–C beta chemokine family that is produced by macrophages, fibroblasts, and endothelial cells to stimulate chemo taxis of monocyte/macrophages and other inflammatory cells. The human MCP-1 regulates the infiltration of monocytes, memory T cells and macrophages and other inflammatory cells by binding to the membrane CC chemokine receptor 2 (*CCR2*) [5-8]. MCP-1 protein may be regulated by a Single Nucleotide Polymorphism (SNP) occurring at position –2518 of the *MCP-1* gene promoter. The -2518A/G polymorphism (rs1026611) in the *MCP-1* gene can influence plasma *MCP-1* concentration and has been suggested as a risk factor for atherosclerosis [9-14]. Numerous studies have been performed on the association of the -2518 A/G Polymorphisms in the MCP-1 gene with atherosclerosis susceptibility.

In the last few years, genetic determinants have been shown to influence the risk of stroke and many SNPs in different genes have been found to be associated with ischemic stroke (Table 1).

The presence among the risk factors of genes already associated with stroke in the general population, such as SELP, suggests that some genetic factors predisposing to stroke may be shared by both SCA patients and stroke victims in general [15-17].

Material

Our study enrolled 100 sickle cell patients among whom 19 presented confirmed AVC. Patients were selected on the basis of homozygosity for β^s-globin gene. Demographic, hematological and clinical data of subjects studied are summarized in Table 1.

	SCA patients Without AVC N=81	SCA patients With AVC N=19	P
Range of Age	5-25	5-25	1
Sex ratio	41/59	9/10	0.12
Hb (g/dl)	9.7 ± 0.7	9.3 ± 0.5	0.42
RBC (10^{12}L)	3.29 ± 0.9	2.89 ± 1.02	0.08
MCV(fl)	79.7 ± 0.9	74.2 ± 1.3	0.095
MCH(pg)	34.9 ± 2.1	35.7 ± 1.02	0.075
RDW(%)	4.83 ± 0.5	5.29 ± 1.02	0.12
HbA	0	0	1
HbS (%)	86 ± 0.3	86.4 ± 0.4	1

HbF(%)	10 ± 0.1	10.6 ± 0.3	0.82
HbA2	3 ± 0.2	3 ± 0.1	1
Hb: hemoglobin, RBC: red blood cell, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin and RDW: red blood distribution			

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

Methods

Clinical events

Data and clinical events were taken from patient's history via search of the clinical registry. Screen all children with SS ages 2–16 using TCD.

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 60^{c+}). Genomic DNA was extracted from peripheral blood using the standard phenol-chloroform procedure. β^{s}-globin gene was performed by Restriction Fragment Length Polymorphism (RFLP) as previously described by Romana M *et al* 2000 [18]. We determined total and fetal Hemoglobin (Hb F) concentrations by HPLC (D10 BioRad).

Polymorphisms genotyping

A 25 µl PCR mixture contained 1.75 mM $MgCl_2$, 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 ng 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 25µl
-2518 A/G of MCP-1	F : GCTCCGGGCCCAGTATCT R : GGCCATCTCACCTCATCTTCC	689pb	94°C 10 mn 35x(94°C 1 mn 62°C 1 mn,72°C 1 mn) 72°C 10 mn
V64I of CCR2	F : TTGTGGGCAACATGATGG R : TGAAGAAGATTCCGCCAAAA	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 204 pb and 18 pb 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 AVC. 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). Stratification of different combination of genotypes found according to the presence or absence of AVC was evaluated by logistic regression model using SPSS (18.0) and statistical significance was defined as $p<0.05$.

Results

Patients chosen for the molecular methods were selected on the basis of homozygosity for β globin gene. The two groups of patients stratified accordingly to the occurrence of AVC were compared for age, sex ratio and hematological data including HbF. No significant association was found ($p>0.05$) (Table 1).

Polymorphisms analysis

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

The analysis of the rs1026611 in the *MCP-1* gene showed the presence of three genotypes namely: AA, AG and GG among SS patients without AVC. Whereas, GG was absent among SS patients with AVC (Figure 1a).

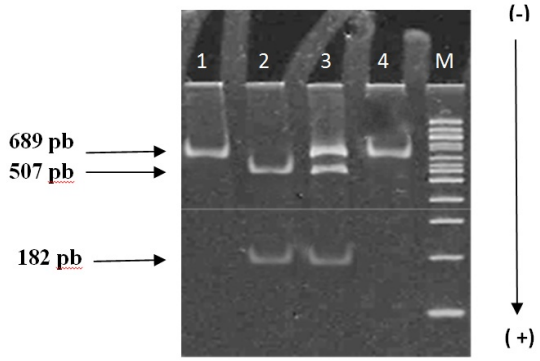

Figure 1a: Polyacrylamide gel electrophoresis for the genotyping of MCP-1-2518A/G after enzymatic digestion (PvuII) 1: PCR product without digestion, 2: mutant homozygote GG, 3: heterozygote AG, 4 normal homozygote AA, M: size marker 100 pb

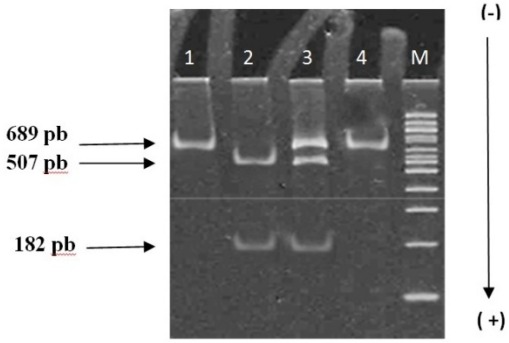

Figure 1b: Polyacrylamide gel electrophoresis for the genotyping of CCR2V64I after enzymatic digestion (BsaB1); 1: mutant homozygote AA, 2: heterozygote AG, 3: normal homozygote GG, 4: PCR product without digestion, M: size marker 100 pb

The analysis of the V64I *CCR2* showed the presence of three genotypes namely: GG, GA and AA in both patient groups (Figure 1b). Our findings showed no significant association between patients and controls according to genotypic and allelic profile of the two polymorphisms studied (Table 3).

	AVC Absence N=81	Presence N=19	P
MCP1-2518A/G			
A	0.802	0.789	1*
G	0.198	0.211	1
AA	54	11	1*
AG	22	8	0.408
GG	5	0	1
CCR2-V64I			
G	0.0.901	0.789	1*
A	0.099	0.211	0.512
GG	68	13	1*
AG	10	4	0.269
GG	3	2	0.208

Table 3: Genotypic and allelic distribution of studied polymorphisms according to the presence or the absence of AVC

Discussion

Previous studies have suggested that genetic heterogeneity influence the susceptibility to AVC in SCA [14-15,18-22]. Some studies attempt to suggest the role of TGF-beta signaling s pathway in increasing risk of stroke. They have showed the association of variants in TGFBR3 and in beta receptor II (TGFBR2), which have essential, non-redundant roles in TGF-beta signaling. Interestingly, BMP6 is part of the TGF-beta super-family, and three previous have reported that variants in BMP6 are associated with increased risk of stroke. This conjecture is further supported by the association of stroke with Colony Stimulating Factor 2 (CSF2), a protein necessary for the survival, proliferation and differentiation of leukocyte progenitors. Other genes involved according to this study are ADCY9, chemokine (C-C motif) ligand 2 (CCL2), endothelin converting enzyme 1 (ECE1), v-ets erythroblastosis virus E26 oncogene homolog (ERG), hepatocyte growth factor receptor (MET) and TEK tyrosine kinase (TEK). As for the polymorphism MCP1-2518A/G, this is the first report on the association of this polymorphism and occurrence of AVC in SCA. Our results show the lack of significant association among our studied population. Whereas for the *CCR2* V64I polymorphism, only one previous study on American SCA patients have reported no association between the latter polymorphism and AVC. Herein, we found the same results.

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 showed no significant association between patients and controls according to genotypic and allelic profile of the two polymorphisms studied. To further define the genetic basis of stroke, more SNPs in candidate genes of different functional classes might be examined in our population with the likelihood of having a stroke.

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