ASSOCIATION OF CHEMOKINE AND CHEMOKINE RECEPTOR POLYMORPHISMS WITH ACTIVITY DEGREE OF IBD IN TUNISIAN PATIENTS

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ABSTRACT

La maladie de Crohn (MC) et la rectocolite ulcéro-hémorragique (RCH) sont caractérisées par un terrain génétique très complexe impliquant plus d'un locus de susceptibilité. Dans le but de détecter une éventuelle association entre les polymorphismes fonctionnels des gènes des récepteurs des chémokines CCR5, CCR2 et MCP-1et la susceptibilité à la MC et à la RC, les polymorphismes CCR5-Δ32, CCR5-59029-A/G, CCR2-V64I et MCP-1-2518-G/A ont été analysés par PCR-RFLP et PCR-SSP, chez 194 patients Tunisiens atteints de maladie inflammatoire chronique de l'intestin (MICI:126 MC et 68 RCH) et 169 sujets sains donneurs de sang. Les fréquences génotypiques et alléliques ne révélaient pas de différence statistiquement significative ni entre les patients et les témoins ni entre les patients atteints de MC comparativement à ceux atteints de RCH. Néanmoins, l'analyse des patients avec MC qui ne sont pas de genotype homozygote G/G étaient plus fréquemment en rémission par rapport à ceux porteurs de ce genotype (OR: 0.4; 95%CI: [0.174-0.928]; p=0.03). De même, la fréquence de l'allèle muté CCR2 64I était statistiquement plus élevée chez les patients atteints de la MC en rémission par rapport à ceux en forme active de la maladie (OR: 0.267; 95%CI: [0.09-0.78] ; p=0.01). L'étude multivariée confirmait ces résultants et révélait que les genotypes A/G CCR5-59029 et V64I CCR2 étaient associés à la MC en remission (OR: 2.63; 95%CI: [1.01-6.80]; p=0.047 and OR: 4.64; 95%CI: [1.01-21.31]; p=0.049, respectivement). En conclusion, dans la population Tunisienne les polymorphismes des gènes des récepteurs CCR5 et CCR2 des chémokines seraient impliqués dans le degré d'activité des MICI.

Mots clés : *Maladie inflammatoire chronique de l'intestin; polymorphismes des gènes, chémokines, récepteurs des chémokines.*

RESUME

Crohn's disease (CD) and ulcerative colitis (UC) have complex genetic background that is characterised by more than one susceptibility locus.To detect a possible association between the functional polymorphisms of the chemokine receptors CCR5, CCR2 and MCP-1 genes and susceptibility to CD and UC in Tunisian population, polymorphisms of CCR5-Δ32, CCR5-59029-A/G, CCR2-V64I and MCP-1-2518- G/A were analysed in 194 Inflammatory bowel disease (IBD) patients and 169 healthy blood donors using PCR-RFLP and PCR-SSP methods. The patients were classified in 126 patients with CD and 68 patients with UC. The genotypic and allelic frequencies of all polymorphisms studied, did not reveal significant differences between patients and controls, and among CD and UC patients. However, analysis of CD patients revealed that those without homozygosous G/G genotype are more frequently in remission compared to those with this genotype (OR: 0.4; 95%CI: [0.174-0.928]; p=0.03). Also, the frequency of the CCR2-64I muted allele was statistically higher in CD patients in remission disease than those in active form (OR: 0.267; 95%CI: [0.09-0.78]; p=0.01). Adjustment for known covariates factors (age, gender and immunosuppressive regimen) confirmed these univariate findings and revealed that the CCR5-59029-A/G and CCR2-V64I genotype were associated to remission form of CD (OR: 2.63; 95%CI: [1.01-6.80]; p=0.047 and OR: 4.64; 95%CI: [1.01-21.31]; p=0.049 respectively). In conclusion, the present study supports the involvement of chemokine receptor (CCR2 and CCR5) polymorphisms in activity degree of the IBD disease in Tunisian patients.

Key words : *Inflammatory bowel diseases; gene polymorphisms, chemokines and chemokine receptors.*

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder caused by dysregulated immune responses in a genetically predisposed individual. Obseved IBD familial clustering and increased monozygotic twin concordance has led to the hypothesis that genetic loci containing IBD susceptibility genes can be identified by whole genome linkage mapping approaches [1]. Recent data including genome-wide association studies have identified more than 50 distinct genetic loci that confer susceptibility [2]. In particular, IBD was genetically related to chromosome 3p [3]. This 3p region includes several candidate genes among whom those of the chemokine receptor cluster and interleukine 5α receptor [4, 5]. Chemokines and their corresponding receptors likely play a central role in directing mononuclear cells to the acute and chronic inflammatory sites. Chemokines stimule leukocyte migration and activity through seven-transmembrane-segment G-protein-coupled receptrors [6] and they are divided into two subfamilies, the α- or CXC-chemokines and β- or CC-chemokines, depending on the positioning of the first two (out of four) conserved cysteines. Among the five characterized β-chemokines, CCR2 binds MCP-1 and MCP-3 [7] and CCR5 responds to MIP-1α, MIP-1β and Rantes [8]. These two CC-chemokine receptors are characterized by polymorphisms which alter their function. The chemokine MCP-1 is thought to be important for the recruitment of mononuclear cells and the maintenance of inflammation in IBD and its concentration is also high in the cases of IBD [9]. The degree of intestinal inflammation in Crohn's disease is associated with MCP-1 tissue levels [10]. Furthermore, Herfarth et al. showed that the G/A and G/G genotypes of MCP-1- promoter polymorphism were significantly decreased in patients with a later onset of the disease and both genotypes presented also less frequently with a fistulizing form [11].

In this study, the possible association between functional polymorphisms of CCR5-Δ32, CCR5-59029-A/G, CCR2-V64I and MCP-1-2518-G/A genes and the susceptibility to IBD's disease and/or its phenotype, was analysed in Tunisian population.

MATERIALS AND METHODS

Patients

Blood samples were obtained from one hundred and ninety four subjects with IBD. These patients were classified into 126 patients with CD (63 men, 63 women) with a mean age of 35 ± 11.63 years, and 68 patients with UC (19 men, 49 women) with a mean age of 39 ± 12.12 years.

All subjects were unrelated Tunisians treated, between the period going of year 2006 to 2008, at the Department of Gastroenterology of Charles Nicolle and La Rabta Hospitals in Tunis. The diagnosis of CD and UC was based on clinical, radiologic, endoscopic and/or and histologic examinations in accordance with the Lennard-Jones criteria [12] provided by International Organization for the study of IBD. Only patients with UC or CD for a duration > 6 months were included in the study and those patients with infections and other recognized causes of inflammation were excluded. As shwon in table I, data obtained from each patient included age, disease location and extent at diagnosis, extra-intestinal manifestations (EIM) which were used to group the patients according to the Vienna classification [13] and treatments. UC patients were classified as follows: proctitis, left-sided colitis, or extensive colitis and the severity based on Rachmilewitz endoscopic index.

The clinical activity of CD patients was measured according to the Crohn's Disease Activity Index (CDAI). CD patients with a CDAI >150 and UC patients with a Rachmilewitz index ≥4 were considered as active disease patients at diagnosis [14, 15]. On the other hand, a favourable outcome was defined as no disease activity flares (characterized by the absence of intestinal symptoms that resulted in increased use of medications or led to surgery) during the first 5 years after diagnosis.

In CD group, 47 sera out of 126 (37.3%) were tested positive for anti-Saccharomyces cerevisae antibodies (ASCA). In UC group, 15 sera out 68 (22.1%) exhibited perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA).

Controls

A total of 169 unrelated healthy Tunisian subjects matched for age and sex were used as control population. None of the healthy controls had any clinical and biological evidence of autoimmune diseases such as inflammatory bowel disease, diabetes or other autoimmune diseases.

All patients and controls gave informed consent to participate in this study which was approved by the Ethics Committee of Charles Nicolle Hospital in Tunis.

Table I: Clinical characteristics of study subjects

	CD (n=126)	*UC (n=68)*
Sex Ratio (Male/Female)	63/63	19/49
Age average (years)	35 ± 11.63	39 ± 12.12
Surgical history: n (%)	28 (22.22)	15 (22.05)
Activity of disease (n)		
▪ In remission	95	53
▪ Active	31	15
Location of lesions at diagnosis: (n)	Ileum (35) Colon (28) Ileocolon (63)	Proctitis (23) Left-sided-colitis (30) Extensive colitis (15)
Forms: n (%)	Stenosing: 26 (20.63) Fistula: 17 (13.5) Stenosing + Fistula: 21(16.7)	
Extra-intestinal manifestation: n (%) (EIM)	38 (30.16)[a]	6 (8.82)[b]
Outcome: n (%)		
▪ Favourable [e]	108 (85.7)	65 (95.6)
▪ Cortico-dependency	14 (11.1)	2 (3)
▪ Cortico-resistance	4 (3.2)	1(1.4)
Production of pANCA: n (%)	-	15 (22.06)
Propduction of ASCA: n (%)	47 (37.3)	3 (4.41)
Treatments: n (%)		
▪ Medical [c]	Glucocorticoid: 68 (53.97) 5-ASA: 11 (8.73) Glucocorticoid + 5-ASA: 7 (5.55)	10 (14.7) 51 (75) 5 (7.35)
▪ Surgical	35 (27.77) In start: 19 After 5-ASA: 13 After Glucocorticoid: 1 After Glucocorticoid + Azathioprine [d]: 2	Anastomosis ileoanal (AIA) after corticoïde: 2 (2.9)

[a]: in CD subjects the EIM were as follows: 19 sacroilitis, 2 sacroilitis+uveitis, 1 sacroilitis+osteoporosis, 1sacroilitis+uveitis+osteoporosis, 6 erythema nodosum, 1erythema nodosum+uveitis, 1 erythema nodosum+sacroilitis, 2 osteoporosis, 3 buccal aphtosis and 2 ankylosing spondolytis. [b]: in UC patients the EIM were: 2 primary sclerosing cholangitis, 1 sacroilitis, 1osteopenia, 1erythema nodosum and 1 pseudofolliculitis. [c]: The glucocorticoid treatment was started with peroral prednisolone (1 mg/kg per day). The venous blood samples were drawn before the treatment was started and at the clinical follow-up visit at 2-4 weeks after starting the glucocorticoid. [d]: The maintenance medication dose of azathioprine was: 2- 2.5 mg/kg per day. [e]: was defined as no disease activity flares during the first 5 years after diagnosis. n: number; pANCA: perinuclear anti-neutrophil cytoplasmic antibodies; ASCA: anti-Saccharomyces cerevisae antibodies.

Methods

Investigation of CCR5-Δ32

CCR5-Δ35 genotype was determined by sizing PCR amplicons that include the entire region, as previously published [16]. The 15 μl PCR contained 50 ng DNA, 5 pmol of each primers, 0.5U of Taq polymerase (Promega Corporation, Madison, WI, USA), 1.5 mM MgCl2 and 0,175 mM of each deoxynucleotide triphosphates (dNTPs) were amplified.

The following primers were used : forward (5'-TgTTTgCgTCTCTCCCAg-3') and reverse (5'-CACAgCCCTgTgCCTCTT-3'). Thermo cycling was performed with an initial denaturation at 94°C for 4min followed by 35 cycles of 94°C for 30s, 60°C for 45s and 72°C for 1min, and a final extension at 72°C for 7min. Amplicons were visualized by ultraviolet in 4% agarose gel with ethidium bromide, which result in 233-bp product for the wild-type amplicon (CCR5 +/+) and 201-bp for the deletion product (CCR5- Δ32).

CCR5-59029-G/A polymorphism

Genotyping was performed using the restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) method [16]. The forward primer, 5'-CCCgTgAgCCCATAgTTAAAACTC-3' and reverse primer 5'-TACCAgggCTTTTCAACAgTAAgg-3' were used with PCR conditions identical to those for CCR5-Δ32 except for an annealing temperature of 66°C. A total of 5 μl of PCR product was digested with 3 units of *Bsp12861* (Promega, USA) at 37°C overnight. The presence of the G nucleotide at position 59029 of the CCR5 gene creates a recognition site for the *Bsp12861* enzyme. Cut amplicons from homozygous patients for 59029G appear as a single: 130bp band in agarose gel electrophoresis at 2%, homozygous for 59029A appear as a 258 bp band and heterozygous have both bands.

MCP-1- A/G promoter polymorphism

The identification of this polymorphism was carried out using PCR-RFLP assay as described by Steinmetz et al [17]. The regulatory region of the MCP-1 gene (-1817 to -2746) was amplified by PCR, resulting in 930 bp fragment. Genomic DNA (100ng) was added to 20 μl of amplification buffer containing: 2 mM of $MgCl_2$, 0,2 mM of dNTPs, 5 pmol of each primers, and 0.5U of Taq polymerase (Promega, USA). Primers used were: forward 5'-CCgAgATgTTCCCAgCACAg-3' and reverse 5'-CTgCTTTgCTTgTgCCTCTT-3'. PCR was run for 40 cycles using the following temperature profile: denaturation at 94°C for 60s, annealing at 60°C for 60s extension at 72°C for 1min 30s, followed by a single final extension at 72°C for 10min. Ten microlitres of the PCR products were digested with 5 units of *PvuII* (Promega, USA) in 10x buffer and H2O up to a final volume of 20 μl at 37°C for overnight. The resulting products were separated by gel electrophoresis in 1.5% agarose gel containing ethidium bromide. Samples showing only a 930 bp band were assigned as A/A, samples showing two bands of 708 and 222 bp were considered G/G and samples showing three bands at 930, 708 and 222 bp were typed A/G.

CCR2-V64I polymorphism

CCR2 wild-type (CCR2 +/+) and mutant (CCR2-64I) alleles were typed by polymerase chain reaction using sequence-specific primers (PCR-SSP) as previously described [18]. The primer sequences were: 5'-gTgggCAACATgCTggTCA-3' primer for wild-type allele, 5'-gTgggCAACATgCTggTCG-3' primer for mutant allele and 5'-CCCAAAgACCCACTCATTTg-3' for common primer. For characterization of the CCR2 polymorphism, two amplification reactions were used: the first with specific primers for the wild-type allele sequence, the second with specific primers for the sequence of the mutant allele. In the case of homozygous wild-type individual, the product (173bp band) was observed only in the first reaction; in the case of homozygous mutant individual the product was detected only in the second reaction, and in the case of heterozygous individual, the products were detected in both reactions. Genomic DNA (100ng) was added to 15 μl of amplification buffer containing: 2 mM of MgCl2, 0,2 mM of dNTPs, 5 pmol of each primers and 0.25U of Taq polymerase (Promega) and was amplified under the following conditions : a preheating step to active the Taq polymerase and to denature the DNA at 96°C for 60s, an initial 5 cycles (96°C 25s, 70°C 45s, 72°C 45s) followed by 21 cycles (96°C 25s, 65°C 50s, 72°C 45s) and a final 4 cycles (96°C 25s, 55°C 60s, 72°C 2min). The PCR products were resolved in 2% agarose gel stained with 0.5 μg/ml ethidium bromide.

Statistical analysis

Allelic and genotypic frequencies were evaluated by direct counting. Comparisons of percentages between patients and controls were done through κ^2 test using the Statcalc program (Epi Info version 2010; Centers for Disease Control and Prevention, Atlanta, GA, USA). The odds ratio (OR) and 95% confidence interval were calculated using the same software to measure the strength of the association observed. Fisher exact test was used when an expected cell value was less than 5. $P < 0.05$ was considered significant. Hardy Weinberg equilibrium was tested by calculating the κ^2 for reliability of fit. Calculations were made by using Internet programs from www.ihg2.helmholtz-muenchen.de/cgi. Logistic regression models were used with remission as the response variable to evaluate the relationships with the different factors, (including confounders) and to estimate adjusted OR.

RESULTATS

Data from each patient included gender, age, clinical and biological manifestations of the disease were obtained retrospectively (Table I). The criteria of severity of the disease were estimated for all

patients and defined by the need for surgery (35 cases in CD group and 2 in UC subjects) or the presence of extra-intestinal manifestations (EIM), particularly peripheral arthritis, ankylosing spondylitis in CD disease and primary sclerosing cholangitis in UC.

As shwon in table II, all analysed allele frequencies and genotype distributions were in Hardy-Weinberg equilibrium both in patients and controls. Comparisons between these frequencies among total IBD patients and controls did not reveal significant differences. However, the CCR5-Δ32 muted allele was lower among UC patients (0.007) than in controls and in CD subjects (0.024). Also, knowing that the homozygous CCR5- Δ32/Δ32 genotype is absent among the controls and patients, the distribution of the +/Δ32 CCR5 genotype was lesser in UC patients (1.5%) than in controls (4.7%) and in CD patients (4.8%). These differences were, however, not statistically significant.

The analytical study showed no statistically significant association between these different polymorphisms and the gender or the severity of disease in any of the IBD patients. Moreover, no correlation was found between the polymorphisms studied and the production of pANCA in UC and of ASCA in CD.

Subsequently, we sought to investigate whether the polymorphisms of studied chemokine genes could be linked to a particular clinical phenotype. When stratifying IBD patients according to the Vienna classification, as shown in table III, we found a statistically significant association between CCR5-59029A allele and the pancolitis localization of the lesions in UC group (OR: 2.63; 95%CI: [1.13-6.08]; $p=0.02$). Also, when comparing CD patients with the UC group, with in remission form of the disease (CDAI<150), the frequency of the CCR5-Δ32 muted allele was lower among UC patients (0.010) than in CD subjects (0.021). This difference was, however, not statistically significant. Furthermore, any relationship of such polymorphism with both localization and form of lesions or clinical outcome was found.

Table II: Genotype and allele frequencies of chemokines and their receptors studied in controls and IBD patients

		Genotype Frequency: n (%)			*Allele frequency*	
CCR2-V64I	**n**	**+/+**	**+/64I**	**64I/64I**	**64V**	**64I**
Controls	169	124 (73.4)	41(24.2)	4 (2.4)	0.855	0.145
Patients	194	140 (72.2)	43(22.2)	11(5.6)	0.832	0.168
CD	126	92 (73)	25(19.8)	9 (7.2)	0.829	0.171
UC	68	48 (70.5)	18 (26.5)	2 (3)	0,838	0.162
CCR5-Δ32	**n**	**+/+**	**+/Δ32**	**Δ32/Δ32**	**Wild allele**	**Mutant allele**
Controls	169	161 (95.3)	8 (4.7)	-	0.976	0.024
Patients	194	187 (96.4)	7 (3.6)	-	0.982	0.018
CD	126	120 (95.2)	6 (4.8)	-	0.976	0.024
UC	68	67 (98.5)	1 (1.5)	-	0.993	0.007
CCR5-59029	**n**	**G/G**	**G/A**	**A/A**	**G**	**A**
Controls	169	47 (27.8)	88 (52)	34 (20.2)	0.538	0.461
Patients	194	62 (32)	87 (44.8)	45 (23.2)	0.544	0.456
CD	126	41 (32.5)	54 (42.9)	31 (24.6)	0.540	0.460
UC	68	21 (30.9)	33 (48.5)	14 (20.6)	0.551	0.449
			A/G			
MCP-1	**n**	**A/A**	**A/G**	**G/G**	**A**	**G**
Controls	169	90 (53.3)	67 (39.7)	12 (7)	0.730	0.270
Patients	194	115 (59.3)	73 (37.6)	6 (3.1)	0.781	0.219
CD	126	75 (59.5)	47 (37.3)	4 (3.2)	0.782	0.218
UC	68	40 (58.8)	26 (38.2)	2 (3)	0.779	0.221

n: number; CD: Crohn's disease; UC: ulcerative colitis

Table III. Associations between studied polymorphisms of genes and clinical characteristics of UC patients.

	UC patients (n=68)															
	MCP-1				CCR5-59029				CCR5-Δ32				CCR2-V64I			
	Genotype (%)			allele	Genotype (%)			allele	Genotype (%)			allele	Genotype (%)			allele
	A/A	A/G	G/G		A/A	A/G	G/G		+/+	+/Δ32	Δ32		+/+	+/64I	64I	
Sex																
F (49)	27	21	1	A:0.765	11	23	15	A:0.459	48	1	-	+:0.990	34	15	-	+:0.847
	(55)	(43)	(2)	G:0.235	(22)	(47)	(31)	G:0.541	(98)	(2)		Δ32:0.010	(69)	(31)		64I:0.153
M (19)	13	5	1	A:0.816	3	10	6	A:0.421	19	-	-	-	14	3	2	+:0.816
	(68)	(26)	(5)	G:0.184	(16)	(53)	(32)	G:0.579				-	(74)	(16)	(10)	64I:0.184
Activity																
Remission (53)	28	23	2	A:0.745	11	26	16	A:0.453	52	1	-	+:0.990	36	16	1	+:0.830
	(53)	(43)	(4)	G:0.255	(21)	(49)	(30)	G:0.547	(98)	(2)		Δ32:0.010	(68)	(30)	(2)	64I:0.170
Active (15)	12	3	-	A:0.900	3	4	5	A:0.433	15	-	-		12	2	1	+:0.867
	(80)	(20)		G:0.100	(20)	(47)	(33)	G:0.567				-	(80)	(13)	(7)	64I:0.133
Localization[b]																
Proctitis (23)	13	10	-	A:0.783	3	12	8	A:0.391	23	-	-	-	16	7	-	+:0.848
	(56)	(44)		G:0.217	(13)	(52)	(35)	G:0.609					(70)	(30)		64I:0.152
Left-colitis (30)	17	11	2	A:0.750	6	12	12	A:0.400	30	-	-	-	21	9	-	+:0.850
	(57)	(37)	(6)	G:0.250	(20)	(40)	(40)	G:0.600					(70)	(30)		64I:0.150
Pancolitis (15)	10	5	-	A:0.833	5	9	1	**A:0.633**[a]	14	1	-	+:0.966	11	2	2	+:0.800
	(67)	(33)		G:0.167	(33)	(60)	(7)	G:0.367	(93)	(7)		Δ32:0.034	(74)	(13)	(13)	64I:0.200
Outcome																
Favourable (65)	39	24	2	A:0.785	13	31	21	A:0.438	64	1	-	+:0.992	46	17	2	+: 0.838
	(60)	(37)	(3)	G:0.215	(20)	(48)	(32	G:0.562	(98)	(2)		Δ32:0.007	(71)	(26)	(3)	64I:0.162
Unfavourable (3)	1	2	-	A:0.667	1	2	-	A:0.667	3	-	-	-	2	1	-	+: 0.833
				G:0.333				G:0.333				-				64I:0.167
EIM positive (6)	6	-	-		1	2	3	A:0.333	6	-	-	-	5	-	1	+:0.917
					(17)	(33)	(50)	G:0.667					(83)		(17)	64I:0.083
EIM negative (62)	34	26	2	A:0.758	13	31	18	A:0.460	61	1	-	-	43	18	1	+:0.839
	(55)	(42)	(3)	G:0.242	(21)	(50)	(29)	G:0540					(70)	(29)	(1)	64I:0.161

[a]: The A allele CCR5-59029 frequency was statistically higher in pancolitis localization of the lesions in UC group than in distal or left-sided-colitis:(OR:2.63;CI:1.13-6.08;p=0.02). [b]: The localization of UC at diagnosis.

Table IV: Associations between studied polymorphisms of genes and clinical characteristics of CD patients.

	CD patients (n=126)															
	MCP-1				CCR5-59029				CCR5-D32				CCR2-V64I			
	Genotype (%)			allele	Genotype (%)			allele	Genotype (%)			allele	Genotype (%)			aallele
	A/A	A/G	G/G		A/A	A/G	G/G		+/+	+/D32	D32		+/+	+/64I	64I	
Sex																
F (63)	42	18	3	A:0.810	15	27	21	A:0.452	59	4	-	+:0.968	48	12	3	+:0.857
	(67)	(28)	(5)	G:0.190	(24)	(43)	(33)	G:0548	(94)	(6)		Δ32:0.032	(76)	(19)	(5)	64I:0.143
M (63)	33	29	1	A:0.754	16	27	20	A:0.468	61	2	-	+:0.984	44	13	6	+:0.802
	(52)	(46)	(2)	G:0.246	(25)	(43)	(32)	G:0532	(97)	(3)		Δ32:0.016	(70)	(21)	(9)	64I:0.198
Activity																
remission (95)	57	35	3	A:0.784	25	44	26	**A:0.495**[a]	91	4	-	+:0.979	64	23	8	**+:0.795**
	(60)	(37)	(3)	G:0.216	(21)	(49)	(30)	**G:0.505**	(96)	(4)		Δ32:0.021	(67)	(24)	(8)	**64I:0.205**[b]
Active (31)	18	12	1	A:0.774	6	10	15	**A:0.355**	29	2	-	+:0.968	28	2	1	**+:0.935**
	(58)	(39)	(3)	G:0.226	(20)	(32)	(48)	**G:0.645**	(94)	(6)		Δ32:0.032	(90)	(6)	(3)	**64I:0.065**
Localization[c]																
Ileum (35)	19	16	-	A:0.771	8	15	12	A:0.443	34	1	-	+:0.986	27	5	3	+:0.843
	(54)	(46)		G:0.229	(23)	(43)	(34)	G:0557	(97)	(3)		Δ32:0.014	(77)	(14)	(9)	64I:0.157
Colon (28)	18	9	1	A:0.804	7	11	10	A:0.446	26	2	-	+:0. 964	23	4	1	+:0.893
	(64)	(32)	(4)	G:0.196	(25)	(39)	()36	G:0.554	(93)	(7)		Δ32:0.036	(82)	(14)	(4)	64I:0.107
Ileocolon (63)	38	22	3	A:0.778	16	28	19	A:0.476	60	3	-	+:0.976	42	16	5	+:0.794
	(60)	(35)	(5)	G:0.222	(25)	(45)	(30)	G:0524	(95)	(5)		Δ32:0.024	(67)	(25)	(8))	64I:0.206
Outcome																
Favourable(108)	65	40	3	A:0.787	27	48	33	A:0.472	105	3	-	+:0.986	75	24	9	+: 0.806
	(60)	(37)	(3)	G:0.213	(25)	(44)	(31)	G:0528	(97)	(3)		Δ32:0.014	(70)	(22)	(8)	64I:0.194
Unfavourable(18)	10	7	1	A:0.750	4	6	8	A:0.389	15	3	-	+:0.917	17	1	-	+: 0972
	(55)	(39)	(6)	G:0.250	(22)	(33)	(45)	G:0.611	(83)	(17)		Δ32:0.083	(94)	(6)		64I:0.028
EIM positive (38)	28	9	1	A:0.855	12	18	8	A:0553	35	3	-	+:0.960	26	10	2	+:0.816
	(73)	(24)	(3)	G:0.144	(32)	(47)	(21)	G:0.447	(92)	(8)		Δ32:0.040	(69)	(26)	(5)	64I:0.184
EIM negative (88)	47	38	3	A:0.750	19	36	33	A:0.420	85	3	-	+:0.983	66	15	7	+:0.835
	(53)	(43)	(4)	G:0.250	(22)	(41)	(37)	G:0580	(97)	(3)		Δ32:0.017	(75)	(17)	(8)	64I:0.165
Forms																
Stenosis+Fistula(64)	36	26	2	A:0.766	14	29	21	A:0.445	61	3	-	+:0.977	46	13	5	+:0.820
	(57)	(41)	(2)	G:0.234	(23)	(45)	(32)	G:0555	(95)	(5)		Δ32:0.023	(72)	(20)	(8)	64I:0.180
Not Stenosis+Fistula(62)	39	21	2	A:0.798	17	25	20	A:0.476	59	3	-	+:0.976	46	12	4	+:0.839
	(63)	(34)	(3)	G:0.202	(27)	(40)	(33)	G:0524	(95)	(5)		Δ32:0.024	(74)	(19)	(7)	64I:0.161

[a]: CD patients carrying A/A and/or A/G CCR5-59029 genotypes compared to those with G/G genotype, are more frequent in remission than in period of activity of disease (OR: 0.4; CI: 0.174-0.928 ; p=0.03).
[b]: The frequency of the 64I CCR2 muted allele was statistically higher in CD patients in remission disease than those in active form (OR: 0.267 ; CI: 0.09-0.78 ; p=0.01). [c]*: The localization of CD at diagnosis*

In addition, analysis of CD patients according to clinical behavior revealed that those without homozygosous G/G genotype, are more frequently in remission than those with G/G genotype (OR: 0.4; 95%CI: [0.174-0.928]; $p=0.03$). Also, the frequency of the CCR2-64I muted allele was statistically higher in CD patients in remission disease (0.205) than those in active form (0.065), (OR: 0.267; 95%CI: [0.09-0.78]; $p=0.01$) (Table IV). In multivariate analysis, CCR5-59029-G/A (OR: 2.63; 95%CI: [1.01-6.80]; $p=0.047$) and CCR2-V64I genotypes (OR: 4.64; 95%CI: [1.01-21.31]; $p=0.049$) remained significantly associated with remission form of CD after adjustement for age, gender and immunosuppressive therapy. However, this polymorphism was not associated to the clinical course of the disease.

The allelic distribution of the CC-chemokine MCP-1-2518G in combination with the corresponding chemokine receptor CCR2-64I did not statistically significant differ in patients compared to controls and in both CD and UC group. Indeed we found only one CD patient with MCP-1-2518 G/G associated at CCR2-64I/64I. In controls group this association is absent. However, the homozygous A/A CCR5-59029 genotype associated with the homozygous 64I/64I CCR2 genotype was more frequent in IBD patients (24.44%) compared to in controls (11.8%) and in CD subjects (29.03%) than in UC patients (14.28%). On the other hand, we found that the A/A CCR5-59029 and heterozygous +/Δ32 CCR5 genotype association frequency was similar in controls (14.7%), in IBD patients (11.11%) and in CD subjects (12.9%). But when comparing UC patients with the control group, this association frequency was found to be lower in UC (7.14%). This difference was not statistically significant. The frequencies of these different genotype combinations are shown in table V.

Our results show a triple association: [A/A CCR5-59029, +/Δ32 CCR5 and +/64I CCR2] in 2 cases: one control and one CD patient.

Table V: Genotype frequencies of different combinations in patients and controls.

Group (n)	**Genotype frequencies* n (%)**		
	MCP-1 G/G – CCR2 +/+	**MCP-1 G/G – CCR2 +/64I**	**MCP-1 G/G – CCR2 64I/64I**
Controls (12)	8 (66.6)	**4 (33.3)**	**-**
Patients (6)	5 (83.3)	**-**	**1 (16.6)**
CD (4)	3 (75)		**1 (25)**
UC(2)	2 (100)	**-**	**-**
	CCR5 A/A – CCR2 +/+	**CCR5 A/A – CCR2 +/64I**	**CCR5 A/A – CCR2 64I/64I**
Controls (34)	19 (56)	11 (32)	**4 (11.8)**
Patients (45)	21(47)	13 (29)	**11 (24.44)**
CD (31)	13 (42)	9 (29)	**9 (29.03)**
UC(14)	8 (57)	4 (29)	**2 (14.28)**
	CCR5 A/A – CCR5 +/+	**CCR5 A/A – CCR5 +/Δ32**	**CCR5 A/A – CCR5 Δ32/Δ32**
Controls (34)	29 85.3)	**5 (14.7)**	**-**
Patients (45)	40 (88.8)	**5 (11.1)**	**-**
CD (31)	27 (87.1)	**4 (12.9)**	**-**
UC(14)	13 (92.8)	**1 (7.1)**	**-**

** The distribution of these genotype frequencies was not statistically significant either between patients and controls or between CD and UC group.*

DISCUSSION

Our Data demonstrate the first association between human chemokine receptor polymorphisms and the activity of inflammatory bowel disease (IBD) in Tunisian population. This pathology is a chronic inflammatory condition of the gastrointestinal tract that manifests as ulcerative colitis (UC) or Crohn's disease (CD). The aetiology of this condition remains unknown [19]. However there are several candidate genes potentially involved in the pathogenesis of IBD [20]. Chemokines are a group of small proinflammatory molecules first described for their role in the recruiting of lymphocytes and monocytes into sites of inflammation. They have now emerged as key regulators in the development, differentiation and anatomic distribution of inflammatory cells [21]. Several functional polymorphisms of these molecules have been described and their association with the susceptibility to UC and CD has been reported[22, 23, 24].

A 32-bp deletion in the CCR5 gene (CCR5Δ32) results in a nonfunctional surface receptor expression unable to bind its chemokine ligands. Consequently, the CCR5-Δ32 allele has been found less frequently in patients with rheumatoïd arthritis[25] and also associated with a reduced risk of developing asthma [26]. Moreover, the majority of exposed but uninfected individuals by HIV are deficient in cell surface CCR5 expression due to homozygous carriage of the Δ32 deletion [27, 28]. Fischereder et al [29] reported that homozygous for CCR5-Δ32 had significantly longer renal transplant survival times than CCR5 heterozygous and CCR5+/+ individuals. Thus, the CCR5-Δ32 allele could be a protective factor for development of certain diseases. Our results agree with this hypothesis. Because we did not observe the CCR5Δ32/Δ32 genotype in our cohort, only the effects of its heterozygous variant was addressed. Indeed, although the difference is not statistically significant, CCR5-Δ32 allele has been found less frequently in Tunisian UC patients and also associated with a less active form of this disease. However, any relationship of such polymorphism with a clinical course of the disease was found in the study. Herfarth et *al.*[24] have also found that carriers of the -Δ32 mutations have less frequent CD finding in the upper part of the gastrointestinal tract and are probably protected against an aggressive form of CD. These authors suggest the hypothesis that polymorphisms in the CCR5 receptor may participate in the course and localization of CD. However, other studies have shown that there was no correlation between the CCR5-Δ32 genotype and the age of IBD-diagnosis, the frequency of surgical intervention, or disease localization [21, 22]. Possible reasons for the conflicting results might include the differences in the ethnic groups and the age at onset of the diseases between these different studies.

A biallelic G/A polymorphism at position-59029 of the CCR5 gene has been also implicated in the HIV-1 transmission and disease progression [30] and its genetic variants have been related to onset of autoimmun diseases such as familial multiple sclerosis [31] , pulmonary sarcoïdosis [32] and type 1 diabetes mellitus with an association between the CCR5-59029G allele and microvascular complications of this disease [33]. These results are in contrast to a previous report which had showed an increase of the A/A genotype in nephropaths compared to type 2 diabetes patients without nephropaty in Japanese population [34]. Our data showed an association in Tunisian patients of CCR5-59029A allele either with the remission form of CD or pancolitis in UC. No explanations have been presented for the conflicting findings. The reason for this divergence might reflect the ethnical gene background. On the other hand, an *in vitro* study has shown that the CCR5-59029A had higher promoter activity than CCR5-59029G and corrolated with the slower rate of HIV-1 infection progression [35]. In this study, the same mechanism probably of the progressive UC evolution in time leading to a wide localization of the lesions has been suggested. Such a functional analysis of CCR5-59029 alleles could confirm this suggestion.

For the polymorphism of the CCR2-V64I, our data reveal that the frequency of muted allele was statistically higher in CD patients in remission disease than those in active form. This result supports the finding of Petrek et al who suggested a possible protective effect of the CCR2-64I allele in Czech patients with pulmonary sarcoïdosis [32] but was in contrast to the studies of Yang et al. Nakajima et al. and Gambelunghe *et al.* [33, 34, 36]. In CCR2-deficient mice an impaired delayed type hypersensitivity granulomas response and decrease production of Th1-type cytokines were observed [37]. Therefore, Futagami S *et al.* [38] have demonstrate that the number of CCR2+/CD68+ cells in postinfectious CD patients was significantly increased compared with those in healthy volunteers and suggest that the migration of inflammatory cells, in particular, duodenal CCR2-positive macrophages, may have an important function in the pathophysiology of CD.

Subsequently, we speculate that in CD individuals carrying a valine to isoleucine substitution (CCR2-64I) in the transmembrane region of this chemokine receptor Th1 reactivity is attenuated with a reduced activity of the disease.

The chemokine MCP-1 plays an important role in the recruitment of mononuclear cells and the maintenance of inflammation in IBD. Furthermore there is evidence for an association of different disease behavior with MCP-1 genotypes 11. Rovin et al. first reported that the polymorphism-2518- A/G in the regulatory region of the MCP-1gene demonstrates ethnic heterogenecity in the general population [39]. In Korean patients with lupus nephretis, Kim et al. reported that -2518A allele of the MCP-1 gene, was associated with the

up-regulation of MCP-1, which might result in a more severe proteinuria [40]. Yang et al. found that the frequencies of the MCP-1-2518A allele and A/A genotype were significantly higher in patients with type 1 diabetes compared with normal controls [33]. These results are in discordance with those reported by Kang et al revealing a significant increase for the risk of late acute rejection in recipients who were homozygous for the MCP-1-2518G polymorphism [41]. Also Krüger et al. reported that the recipients of renal transplants homozygous for the -2518G mutation of the MCP-1 gene are at risk for premature kidney graft failure [42]. Palmieri *et al.* [43] have shown that the -2518 A/G polymorphism seems to be associated with CD but does not influence MCP-1 plasma levels, which in contrast are increased in UC and CD with extensive colonic involvement. Our data indicate no association exists between the -2518 A/G polymorphism and susceptibility to IBD or any clinical course of CD or UC. Nevertheless, it would be important to investigate whether MCP-1 protein expression is correlated with the degree and the maintenance of inflammation in Tunisian IBD population.

Moreover, there was no significant difference in the frequency of the CC-chemokine MCP-1-2518G combined with its chemokine receptor CCR2-64I in controls and in both CD and UC groups studied. However, the frequency of the CCR5Δ32 combined with CCR5-59029 A was found to be lower in UC than in controls. On the other hand, because the genes CCR2 and CCR5 are located next to each other in the chromosome 3p21, combinations of genotypes and haplotypes were analysed. The homozygous -59029 A/A CCR5 genotype associated with the homozygous 64I/64I CCR2 genotype was more frequent in IBD patients compared to in controls and in CD subjects than in UC patients. We may suggest that CCR2 and CCR5 chemokine receptors play a role in the development of CD with a moderate form of the disease via the nonfunctional surface receptor expression unable to recruit a sufficient number of mononuclear cells into the site of inflammation could cause an important structural intestinal damage by the release of proteolytic enzyme and proinflammatory cytokines.

Multivariable logistic regression analyses confirm the univariate results and revealed that the CCR5-59029 G/A and CCR2 V64I genotype were associated to remission form of CD.

In conclusion, the present study supports the involvement of chemokine receptor (CCR2 and CCR5) polymorphisms in various evolving forms of the IBD disease in Tunisian patients. However, given the limites of this transversal study, the results should be treated with caution and deserves to be confirmed by prospective analysis to assess the impact of these polymorphisms on the clinical evolutivity of IBD.

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