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Replication of Identified Inflammatory Bowel Diseases Genetic Associations: A Case – Control Study in the Tunisian Population

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Abstract

Inflammatory bowel diseases (IBD) — Crohn's disease (CD) and ulcerative colitis (UC) — are chronic gastrointestinal inflammatory disorders with a complex genetic background. A genome wide association scan by the Welcome Trust Case Control Consortium (WTCCC) recently identified several novel susceptibility loci. We performed a replication study in 107 IBD patients (39 CD and 68 UC) and 162 controls. In total, 19 single nucleotide polymorphisms (SNPs) from previously identified susceptibility genes *PTPN11, TNF α, IL23R, PTPN2, PTPN22, IL2 and IL10* were studied. In UC, we confirmed the association with *PTPN2* (rs254215, GG, P=0.013, P_{corr}=0.039; OR= 6.23 (1.18; 32.95)). In CD, we confirmed a marginal association with ((rs11066320, GG, P=0.018, P_{corr}=0.054, OR= 0.4 (0.19; 0.82)). No significant association was found at the allele and genotype levels of SNPs in *TNF α, IL23R, PTPN22, IL2, and IL10.* However, on the haplotype analysis the AG haplotype of *TNFα* was more frequent in CD patients compared to controls (23.1% *vs.* 13.7%; P = 0.039; OR= 1.89 (1.02; 3.5)). The *PTPN11* ATG haplotype was also more frequent in CD patients compared to controls (32.1% vs 21.3%; P = 0.04; OR= 1.75 (1.01; 3.01). These results reveal limited replication in Tunisian population and indicate differences in genetic architecture between populations.

Introduction

Inflammatory bowel diseases (IBD) are disabling inflammatory disorders of the gastrointestinal tract, in which patients frequently suffer from relapsing bouts of intense abdominal pain, typically associated with diarrhoea and rectal bleeding [1]. While Crohn's disease (CD) shows transmural and often discontinuous patches of inflammation, ulcerative colitis (UC) characteristically involves inflammation associated with only the superficial mucosal and sub-mucosal layers of the intestinal wall [1]. Both conditions may have extra-intestinal manifestations, and may be associated with other chronic inflammatory diseases such as arthritis, ankylosing spondylitis or psoriasis [1].

The prevalence of IBD varies depending on ethnicity or race. Ashkenazi Jews have a much higher risk of developing IBD with a 2–4 times higher incidence when compared to non-Jewish Caucasians [2]. However, there is evidence to suggest that the prevalence of IBD increases in populations and regions with industrialization. The highest incidences are reported in northern Europe and North America where they range from 12 to 19/100,000/year and from 5 to 29/100,000/year, respectively and the lower incidences are reported in Asia and Africa, which probably reflects both genetic and environmental factors [1,3].

Currently, environmental risk factors and dysregulated mucosal immune responses are thought to cause chronic relapsing and remitting inflammation in genetically predisposed individuals, although the etiology of IBD remains unclear [4]. Family studies have provided overwhelming evidence that genetic factors play a significant role in determining an individual's susceptibility to inflammatory bowel disease (IBD) [5-8]. The most compelling evidence comes from twin studies conducted in Sweden, Denmark, and the United Kingdom [5-7]. These studies reported Crohn's disease (CD) and ulcerative colitis (UC) concordance rates of 58% and 6-19% for monozygotic twins, and 0.5-7% for dizygotic twin pairs [5-7]. The significantly higher concordance rates observed among monozygotic twin pairs across these studies indicate that there is a strong genetic component to IBD susceptibility.

Genome-wide association studies (GWASs) applying high-density SNP array technology have greatly expanded the number of genetic factors implicated in IBD pathogenesis to include over seventy genes and loci associated with CD and UC, spanning pathways involved in adaptive (*IL23R*, *IL10*, *STAT3, PTPN2, PTPN22, TNFSF15, IL12B, CCR6*) and innate (*CARD15*, *ATG16L1*, *IRGM*) immunity [9-14]. The *TNFα, IL2 and PTPN11*genes may also be considered as a plausible candidate for a genetic association with IBD [14-18].

Although IBD occurs worldwide, its epidemiologic and clinical characteristics vary depending upon the geographic location and the ethnicity of the population. Identifying the susceptibility genes of IBD in populations living in different geographical locations and with different ethnicities may provide significant clues about its etiology and pathophysiology [19].

However, all the published GWAS for IBD studies have been performed in populations of European descent. It will be extremely informative to test whether these new risk alleles associate with disease susceptibility in other ethnic groups.

We undertook a replication study in a cohort of IBD patients from Tunisia. We tested markers involved in adaptive immunity that showed moderate or strong association in previous case-control study.

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Patients and Methods

Patients

Blood samples were obtained from 107 IBD patients (68 with UC, 39 with CD) diagnosed at the Department of Gastroenterlogy of Hedi Chaker University Hospital (Sfax, Tunisia) from March 2004 to May 2008. All patients were from Tunisian descent (North Africa). The diagnosis was based on standard clinical, radiographic, endoscopic, and histopathological criteria [20]. Table 1 summarizes clinical data: Patients with CD were assessed according to the Montreal classification [21] based on the age at diagnosis, localization, and behavior of disease. In patients with UC, anatomic localization was also determined according Montreal classification, using the criteria ulcerative proctitis, left-sided, and extensive. As unaffected controls (HC), 162 healthy Tunisian subjects with no history of digestive system disease, unrelated to each other or to the patients were recruited in this study.

The study was approved by the local ethics committee and all enrolled patients gave their informed consent to participate.

Methods

Genotyping methods: Genomic DNA was extracted from whole blood samples using a standard proteinase K digestion and phenol/chloroform extraction procedure. Genotyping was performed using primer extension chemistry and mass spectrometric analysis (iPlex assay, Sequenom, San Diego, CA) on the Sequenom MassArray at the Instituto Gulbenkian de Ciência, Oeiras, Portugal. We genotyped 3 SNPs in *PTPN11* (rs12423190, rs11066323, rs11066320), 2 SNPs in *TNFα* (rs1800629, rs361525), 1 SNP in *IL23R* (rs11805303), 1 SNP in *PTPN2* (rs2542151), 3 SNPs in *PTPN22* (rs1310182, rs2476601, rs2488457), 4 SNPs in *IL2* (rs2069763, rs1157812, rs2069762, rs6822844), and 5 SNPs in *IL10* (rs3024498, rs3024495, rs1800871, rs1800896, rs1800872). SNP details are given in Table 2. All information about the selected SNPs was extracted from dbSNP public database, built 126 and gene information from the genome browser Ensembl, release 61. For quality control purposes these SNPs were also typed using a panel of HapMap samples.

Assay design was performed according to manufacturer's instructions, whereby the genomic sequence containing the SNP is amplified by multiplex PCR reactions. The amplified product was cleaned using shrimp alkaline phosphatase and used for allele specific primer extension reaction according the MassEXTEND protocol. The reaction mixture was then spotted onto a SpectroCHIP microarray and subjected to the MALDI-TOF mass spectrometry. SpectroTYPER software identifies the SNP-specific peaks and automatically assigns the genotype calls.

	Crohn's disease	Ulcerative colitis	Total (IBD)
Patients (n)	39	68	107
Sex (male/female)	24/15	33/35	57/50
Age (years),mean ± SD	39.4 ± 13.8	40.5 ± 12.4	40.1 ± 13.2
Pathology location Ileum Colon Ileocolon Pancolitis Left-sided Proctitis	 14 11 14	 35 26 7	
Extra intestinal manifestations	18	13	31

Table 1: Clinical description of Crohn's disease and ulcerative colitis patients.

Data analysis: Quality control criteria excluded SNPs with call rate lower than 90% in cases and controls. Genotype frequencies were in Hardy-Weinberg equilibrium for all the typed SNPs (P>0.05) of the Tunisian control population. Case-control association analysis was performed for each SNP. Calculations of allelic and genotypic associations of SNPs with susceptibility to IBD were performed using a home-made program written in R language (www.r-project.org). This is a simple program which computes classical allelic and genotypic chi-square tests in an automated manner to avoid computing tests for each SNP one by one. A result is considered statistically significant when P<0.05. Bonferroni's correction was carried out when single SNP analysis yield significant results. Relative risks were calculated as odds ratio (OR) using 2×2 contingency tables.

Results

Using the iPlex assay, we genotyped 19 SNPs in *PTPN11, TNFα, IL23R, PTPN22, PTPN2, IL2* and *IL10* genes from 107 IBD patients and 162 healthy subjects, all of South Tunisian origin. All SNPs tested except rs11066320 of *PTPN11* were in Hardy-Weinberg equilibrium in the control group.

PTPN11

For the rs11066320 polymorphism of *PTPN11*, a significant increase in the frequency of the A allele was observed in IBD patients (29.7% vs. 20.5% for case and controls, respectively, OR=1.64, 95% CI=1.08-2.49, P=0.02, P_{corr}=0.04) (Table 4). The AG genotype of the SNP rs11066320 was significantly more frequent in IBD patients than controls p=0.019, OR=1.93, but Bonferroni's correction revealed marginal association (P_{corr}=0.057). When stratified by disease type, only CD patients showed a significantly increase frequency of AG genotype (p=0.022) of the SNP rs11066320 compared with controls, but this result was not confirmed after Bonferroni's correction (P_{corr}=0.066) (Table 4). For rs11066323 polymorphism of *PTPN11,* CD patients showed a significantly increase frequency of AA genotype (P=0.027) compared with controls (Table 4). However, statistical significance was lost after Bonferroni's correction (P_{corr}=0.0.81). No significant differences were found in the allelic and genotypic frequencies of the rs12423190 polymorphism among CD and UC patients.

Using the genotyping data, we inferred 4 haplotypes. The haplotype ATG was statistically significantly associated with IBD (*P*=0.026). When stratified by disease type, only CD patients showed a significantly increase frequency of ATG haplotype (p=0.04) as compared with controls (Table 5).

TNF α

For each of the polymorphisms studied, the risk of carrying the variant was compared between CD, UC and control groups, as shown in Table 4. No associations were found at the allele and genotype levels between the polymorphism rs1800629 studied and risk of CD or UC. When combining UC and CD data to increase the statistical power there were still no associations between the polymorphism rs1800629 and risk of IBD. The rs361525 showed no statistically significant effect on patterns of UC disease risk (Table 4). However, CD patients showed a significant difference in the AA genotype frequency (p=0.038; OR=13.36 (1.18; 151.24)) of the SNP rs361525 compared with controls, but this result was not confirmed after Bonferroni's correction (P_{corr}=0.11) (Table 4). Haplotype analysis of two-SNPs was performed

locus	Chr number	Gene	gene name		Position on Chr (bp)	Allele	HW
rs12423190	12	*PTN11*	protein tyrosine phosphatase, non-receptor type 11	intron	112909340	T/C	6,86E-01
rs11066323	12	*PTN11*	protein tyrosine phosphatase, non-receptor type 11	intron	112923361	G/A	9,97E-01
rs11066320	12	*PTN11*	protein tyrosine phosphatase, non-receptor type 11	intron	112906415	G/A	4.56E-03
rs1800629	6	*TNF α*	tumor necrosis factor alpha	5'near gene prom	31543031	G/A	1,61E-01
rs361525	6	*TNF α*	tumor necrosis factor alpha	5'near gene	31543101	G/A	1,94E-01
rs11805303	1	*IL23R*	Interleukine 23 recepteur	intron	67675516	C/T	2,99E-01
rs2542151	18	*PTPN2*	protein tyrosine phosphatase, non-receptor type 2	3'near gene	12779947	G/T	8,38E-01
rs1310182	1	*PTPN22*	Protein tyrosine phosphatase, non-receptor type 22		114373503	A/G	6,18E-01
rs2476601	1	*PTPN22*	Protein tyrosine phosphatase, non-receptor type 22	exon	114377568	A/G	8,72E-01
rs2488457	1	*PTPN22*	Protein tyrosine phosphatase, non-receptor type 22	promoter	114415368	G/C (G/C/A)	6,71E-01
rs2069763	4	*IL2*	Interleukine2	exon	123377482	C/A	1,62E-01
rs11575812	4	*IL2*	Interleukine2	3'near gene	123371049	A/G	7,72E-01
rs2069762	4	*IL2*	Interleukine2		123377980	A/C	2,72E-01
rs6822844	4	*IL2*	Interleukine 2	3'near gene	123509421	G/T	4,98E-01
rs3024498	1	*IL10*	Interleukine 10	3'UTR	206941529	T/C	7,51E-01
rs3024495	1	*IL10*	Interleukine 10	intron	206942413	C/T	9,52E-01
rs1800871	1	*IL10*	Interleukine 10	5'near gene	206946634	A/G	6,86E-02
rs1800896	1	*IL10*	Interleukine 10	5'near gene	206946897	T/C	9,97E-01
rs1800872	1	*IL10*	Interleukine 10	5'near gene	206946407	T/G	2,02E-01

Table 2: Selected polymorphisms in the candidate genes.

Gene/SNP	Genotypes (Reference Allele)	CD (%)	UC (%)	IBD (%)	Controls (%)
PTPN11					
Rs11066320	AA	10.6	10.4	10.5	8.2
	AG	44.7	35.8	39	24.6
	GG	44.7	53.8	50.5	67.2
	A	32.9	28.4	30	20.5
	G	67.1	71.6	70	79.5
Rs12423190	CC	0	0	0	0
	CT	13.2	19.4	17.1	23.4
	TT	86.8	80.6	82.9	76.6
	C	6.6	9.7	8.6	12.8
	T	93.4	90.3	91.4	87.2
Rs11066323	AA	10.8	2.9	5.8	2.6
	AG	24.3	25.4	25	26.9
	GG	64.9	70.2	69.2	70.5
	A	20.3	15.9	18.3	16
	G	79.7	84.1	81.7	84
TNFα					
Rs1800629	AA	5.1	1.5	65.1	3.9
	AG	33.3	31.3	32.1	23.2
	GG	61.6	67.2	2.8	72.9
	A	21.8	17.2	18.8	15.5
	G	78.2	82.8	81.2	84.5
Rs361525	AA	2.7	0	0	0
	AG	8.1	17.9	14.6	18.5
	GG	89.2	82.1	85.4	81.5
	A	6.8	9	8.2	9.3
	G	93.2	91	91.8	90.7
IL23R					
Rs11805303	TT	15.8	17.9	17.2	8.2
	TC	42.1	40.3	40.9	46.8
	CC	42.1	41.8	41.9	45
	T	36.8	38.1	37.6	31.6
	C	36.2	61.9	62.4	68.4
PTPN2					
Rs2542151	GG	2.7	7.4	5.7	1.2
	GT	16.2	13.2	14.3	21.4
	TT	81.1	79.4	80	77.4
	G	10.8	14	12.9	11.9
	T	89.2	86	87.1	88.1
PTPN22					
Rs1310182	AA	18.4	17.9	18.1	13.2
	AG	42.1	41.8	41.9	43.7
	GG	39.5	40.3	40	43.1
	A	39.5	38.8	39	36.3
	G	60.5	61.2	61	63.7
Rs2476601	AA	0	0	0	0
	AG	2.7	4.5	3.9	2.5
	GG	97.3	95.5	96.1	97.5
	A	1.4	2.3	1.9	1.3
	G	98.6	97.7	98.1	98.7
Rs2488457	CC	75	66.7	69.8	67.3
	CG	19.4	30	26	30.1
	GG	5.6	3.3	4.2	2.6
	C	84.7	81.7	82.8	82.4
	G	15.3	18.3	17.2	17.6
IL2					
Rs2069763	AA	0	4.9	3.1	2.5
	AC	22.2	21.3	21.6	18.5
	CC	77.8	73.8	75.3	79
	A	11.1	8.6	13.9	11.8
	C	88.9	84.4	86.1	88.2
Rs11575812	AA	52.8	57.1	56	47.4
	AG	41.7	30.2	34	43.6
	GG	5.5	12.7	10	9
	A	73.6	72.2	73	69.2
	G	26.4	27.8	27	30.8

Rs2069762	AA	32.4	35.8	34.6	32.3
	CA	54.1	41.8	46.2	45.2
	CC	13.5	22.4	19.2	22.5
	A	59.5	56.7	57.7	54.8
	C	40.5	43.3	42.3	45.2
Rs6822844	GG	91.2	82.5	85.6	89.7
	GT	0.8	17.5	14.4	10.3
	TT	0	0	0	0
	G	95.6	91.3	92.8	94.8
	T	0.4	8.7	7.2	5.2
IL10					
Rs1800872	GG	55.6	47	50	43.6
	GT	41.7	45.5	44.1	51.9
	TT	2.7	7.5	5.9	4.5
	G	76.4	69.7	72.1	69.6
	T	23.6	30.3	27.9	30.4
Rs1800871	AA	5.4	8.9	7.7	5.9
	AG	37.8	49.3	45.2	48.3
	GG	56.8	41.8	47.1	45.8
	A	24.3	33.6	30.3	30.1
Rs1800896	G	75.7	66.4	69.7	69.9
	CC	18.4	7.6	11.6	14.9
	CT	36.8	48.5	44.2	47.4
	TT	44.8	43.9	44.2	37.7
	C	36.8	31.8	33.7	38.6
	T	63.2	68.2	66.3	61.4
Rs3024498	CC	2.7	0	0.9	2
	CT	25	29.2	26.2	26.5
	TT	72.3	70.8	72.9	71.5
	C	15.3	14.6	14	15.2
	T	84.7	85.4	86	84.8
Rs3024495	CC	72.3	76.5	75	78.2
	CT	25	22.1	23.1	20.5
	TT	2.7	1.4	1.9	1.3
	C	84.7	87.5	86.5	84.8
	T	15.3	12.5	13.5	15.2

Table 3: Genotype and allele frequencies of *PTPN11, TNFα, IL23R, PTPN2, PTPN22, IL2* and *IL10* in patients with CD, UC, IBD and unaffected controls.

GENE/SNP	Genotype/	CD		UC		IBD	
	Allele	P value[a] (Pcorr)[b]	OR (95 % CI)	P value[a] (Pcorr)[b]	OR (95 % CI)	P value[a] (Pcorr)[b]	OR (95 % CI)
PTPN11							
Rs11066320	AA	0.69		0.6		0.56	
	AG	**0.022 (0.066)**	2.48 (1.17; 5.25)	0.09	1.67(0.89;3.15)	**0.019 (0.057)**	**1.93(1.11; 3.36)**
	GG	**0.018 (0.054)**	**0.4 (0.19; 0.82)**	0.06	0.58 (0.32; 1.06)	**0.011 (0.033)**	**0.51 (0.3; 0.86)**
	A	**0.034 (0.068)**	**1.90(1.08; 3.33)**	0.079	1.50(0.93; 2.42)	**0.02 (0.04)**	**1.64 (1.08; 2.49)**
	G						
Rs12423190	CC	0.48		0.35		0.24	
	CT	0.16		0.54		0.23	
	TT	0.12		0.42		0.15	
	C	0.11		0.35		0.12	
	T						
Rs11066323	AA	**0.027 (0.081)**	**4.61(1.1; 19.37)**	0.84	1.17 (0.21; 6.54)	0.19	2.33 (0.64; 8.46)
	AG	0.69		0.85		0.73	
	GG	0.58		0.92		0.83	
	A	0.19		0.97		0.5	
	G						
TNFα							
Rs1800629	AA	0.75		0.35		0.64	
	AG	0.13		0.2		0.09	
	GG	0.11		0.39		0.15	
	A	0.14		0.67		0.27	
	G						

Rs361525	AA	**0.038 (0.11)**	**13.36 (1.18;151.24)**	-		0.21	
	AG	0.238		0.74		0.39	
	GG	0.44		0.74		0.52	
	A						
	G	0.71		0.75		0.67	
IL23R							
Rs11805303	TT	0.18		**0.029 (0.087)**	**2.50(1.08; 5.81)**	**0.023 (0.069)**	**2.37 (1.11; 5.07)**
	TC	0.72		0.31		0.35	
	CC	0.66		0.73		0.63	
	T						
	C	0.35		0.2		0.16	
PTPN2							
RS2542151	GG	0.53		**0.013 (0.039)**	**6.23(1.18;32.95)**	**0.038 (0.11)**	**4.76 (0.94; 24.04)**
	GT	0.44		0.16		0.15	
	TT	0.58		0.77		0.61	
	G	0.73		0.51		0.76	
	T						

CI: Confidence Interval; OR: Odds Ratio

Significant associations are highlighted in bold. [a] Two-tailed P values were calculated by home-made program written in R language. [b] Corrected P values P_{corr} — after Bonferroni correction— are shown in brackets.

Table 4: Genotypic and allelic association tests of *PTPN11*, *TNFα*, *IL23R* and *PTPN2* SNP polymorphisms with IBD (CD and UC).

using Haploview. The results are summarized in Table 5. Haplotype frequencies were estimated, and association analyses were performed with respect to CD and UC and controls.

The results showed that there was no significant association between these haplotypes for UC patients as compared with control subjects (Table 5). However, we found that the AG haplotype was significantly associated (*P*=0.039) with CD as compared with control subjects.

IL23 R

By screening IBD patients and controls for the *IL23R* polymorphism (rs11805303), significant difference in the TT genotype frequency was detected in IBD patients (p=0.028, OR=2.37 (1.11; 5.07)), but this result was not confirmed after Bonferroni's correction (P_{corr}=0.069). When stratified by disease type, only UC patients showed a significant difference in the TT genotype frequency (p=0.031; OR=2.5(1.08; 5.81)) of the SNP rs11805303 compared with controls. However, statistical significance was lost after Bonferroni's correction (P_{corr}=0.0.87). No association was found between the rs11805303 variant and gender or other phenotypes (Table 4).

PTPN2

For rs2542151 polymorphism of *PTPN2*, a significant genotypic association was observed (GG: P=0.039, OR=4.76 (0.94; 24.04)) in IBD patients when compared with controls, but this result was not confirmed after Bonferroni's correction (P_{corr}=0.11). When stratified by disease type, only UC patients showed a significant difference in the GG genotype frequency (P=0.013; P_{corr}=0.039; OR=6.23 (1.18; 32.95)) of the SNP rs2542151 compared with controls (Table 4).

PTPN22

For *PTPN22*, no significant differences in the frequencies of alleles and genotypes were detected between IBD patients and healthy controls.

The results of haplotype analysis of the three SNPs of *PTPN22*, showed that there was no significant association between these haplotypes for UC and CD patients as compared with control subjects (Table 5).

IL2

For *IL2*, no significant differences in the frequencies of alleles and genotypes were detected between IBD patients and healthy controls. Whereas, for the SNP rs11575812, UC patients showed a lower frequency (30.2%) of the AG genotype compared with controls (43.6%) (Table 3) without reaching significance level (p=0.0675, OR=0.59 (0.32; 1.09)).

The results of haplotype analysis of the four SNPs of *IL2*, showed that there was no significant association between these haplotypes for UC and CD patients as compared with control subjects (Table 5).

IL10

No associations were found at the allele and genotype levels between the five *IL-10* polymorphisms studied and risk of CD or UC. When combining UC and CD data to increase the statistical power there were still no associations between the five *IL-10* polymorphisms and risk of IBD.

We performed the haplotype analysis of the three SNPs in the 5' flanking region of *IL-10* (rs1800871, rs1800872 and rs180096). The results are summarized in Table 5. We showed that there was no significant association between these haplotypes for UC and CD patients as compared with control subjects.

Discussion

In recent years, GWA studies have provided a broad view of the relative contributions of various genomic loci and have substantially improved the understanding of the molecular pathways leading to IBD. GWA have stressed an association between IBD and specific chromosomal loci and thereby have led to the identification of candidate genes which are involved in the development of inflammatory processes [9,14]. As far as we know, no study has been yet performed

haplotype	controls (n=162)	UC patients (n=67)	CD paients (n=39)	IBD paients (n=107)
PTPN11				
GTG	0,498	0,461	0,397	0,437
ATG	0,213	0,284	**0,321***	**0,297****
GTA	0,16	0,159	0,218	0,181
GCG	0,129	0,097	0,064	0,085
TNFα				
GG	0,763	0,746	0,691	0,725
AG	0,137	0,172	**0,231*****	0,193
GA	0,101	0,083	0,078	0,081
PTPN22				
GGC	0,645	0,606	0,618	0,611
AGC	0,175	0,206	0,227	0,214
AGG	0,163	0,156	0,142	0,156
AAG	0,013	0,023	0,013	0,019
IL2				
ACCG	0,442	0,432	0,419	0,427
GCAG	0,254	0,197	0,236	0,21
ACAG	0,131	0,149	0,189	0,155
AAAG	0,119	0,136	0,108	0,134
GCAT	0,052	0,087	0,047	0,074
IL10				
ACC	0,294	0,333	0,38	0,35
GCC	0,39	0,326	0,368	0,341
ATA	0,302	0,294	0,224	0,268
ACA	0,01	0	0,014	0,01
ATC	0	0,039	0,013	0,03

*P=0.04 OR=1.75 (1.01; 3.01); **P=0.026 OR=1.56 (1.05; 2.32); ***P=0.039, OR= 1,89 (1,02; 3,50)

Table 5: *PTPN11, TNFα, PTPN22, IL2* and *IL10* haplotypes frequencies in Crohn's disease (CD), ulcerative colitis (UC), inflammatory bowel disease (IBD) patients and controls.

in North African populations. In this work we evaluated the genetic contribution to IBD susceptibility of polymorphisms in some genes involved in the regulation of immune function.

SHP-2, coded by *PTPN11*, is a key downstream molecule that downregulates T- and B-cell activation through interaction with CTLA4 and PD-1 [22-25]. SHP-2 is recruited to the phosphorylated tyrosine residue in the immunoreceptor tyrosine-based switch motif of the cytoplasmic tail of PD-1 and in the immunoreceptor tyrosine-based inhibitory motif of the cytoplasmic tail of CTLA4 [26,27]. Although the mechanisms of the *PTPN11* polymorphisms identified in this study are unknown, *PTPN11* polymorphisms may affect the expression, affinity to immunoreceptors, and inhibitory activities of SHP-2, leading to the reduction in the SHP-2 down regulatory effect on T- and B-cell activation.

The present study is the first report to demonstrate an association between *PTPN11* polymorphisms and susceptibility to IBD in the Tunisian population. The presence of the allele A and the heterozygous AG genotype for SNP rs11066320 conferred susceptibility to IBD. When stratified by disease type, the AG genotype conferred susceptibility to CD. The AA genotype for rs11066323 conferred susceptibility to CD.

The association of the haplotype ATG with IBD, was statistically significant. These findings imply that *PTPN11* is a genetic determinant for predisposition to the onset of IBD in Tunisian individuals. However, the current population was relatively small, and further study on a larger number of the Tunisian population and on other ethnic populations is necessary to confirm the association between *PTPN11* and IBD.

TNFα is thought to be an important mediator of CD. Increased levels of *TNFα* messenger RNA and protein have been seen in inflamed and normal intestinal mucosa of patients with CD as well as in the serum [28,29]. As reviewed by Najarian and Gottlieb [30], *TNFα* may contribute to CD by causing apoptosis or inhibition of T-helper type 1 downregulatory cells in the lamina propria, thus stimulating mononuclear cells to release interferon gamma, interleukin-2, and *TNFα*. Other authors postulate that *TNFα* is involved in increased intestinal permeability, formation of granulomas, upregulation of endothelial cell adhesion molecules and coagulation pathways [30]. For the SNP rs361525, in the promoter region of *TNFα*, we found that AA genotype was associated with the CD. Furthermore, among the three *TNFα* promoter haplotypes resolved, AG haplotype was over represented in our CD cohort as compared with control subjects (OR=1,89; CI=1,02; 3,50). According to our data, selected studies of SNPs in the TNF promoter region have been found to be associated with IBD [31-33]. A Japanese study reported a significantly increased incidence of polymorphisms in the promoter region of the *TNFα* at positions -1031, -863, and -857 in patients with CD, versus ethnically matched controls [34,35] in a study on Korean patients observed a significantly higher frequency of -238A allele of TNF-α in CD patients with perianal lesions compared with patients without perianal disease. However, other studies that have confirmed evidence for linkage for an IBD susceptibility locus on 6p23 have failed to demonstrate an association to *TNFα* [36]. Santana et al. [37] also showed that TNF-α -308 polymorphism does not appear to be important for the susceptibility in the development of CD but may affect the severity of the disease.

It has been suggested that common *IL23R* gene variations on chromosome1p31 are likely to have a major impact on the differentiation of T helper cells into Th17 cells [38] and may thus contribute to autoimmunity [39]. The identification of susceptibility loci for IBD in *IL23R* and in other genes, whose gene products play a role in the *IL23* / Th17 pathway, strongly suggests that proper *IL23* / Th17 pathway regulation is crucial for intestinal immune homoeostasis and that its dysregulation plays a key role in IBD development [40,41]. Recent studies demonstrated that IL-23 is important for the survival and expansion of Th17 lymphocytes [42]. As a distinct subset of T helper cells, Th17 lymphocytes produce mostly IL17 and to a lesser extent also IL6 and *TNFα* [43]. IL17 acts in vitro and in vivo as a potent inflammatory cytokine. The *IL23*/ IL17cytokine axis is thus a key pathogenic mechanism that mediates the development and progress of inflammation via Th17 cells [42].

We showed a significant increase of the genotypic frequency of SNP rs11805303 of the *IL23R* gene in our UC patients but not in CD patients. This SNP was already reported to be associated with the susceptibility for CD and to be associated with ileal location in New Zealand population [44]. Multiple SNPs showing an association with both CD and UC have been identified in the *IL23R* gene by recent GWA studies. These findings were meanwhile replicated in a number of adult [13,45] and paediatric [46-50] cohorts. However, no associations were seen in a Japanese cohort [51].

PTPN2 (located on chromosome 18) encodes T cell PTP 2, an anti-inflammatory molecule. Dysregulated *PTPN2* is known to be associated with abnormal JAK-STAT signalling, and evidence from mouse knockouts suggests that lack of dephosphorylation within the signalling cascade leads to marked elevation of several proinflammatory cytokines, including IFN-γ, TNF-α and IL12 [52]. The present study demonstrated an association between *PTPN2* polymorphisms and susceptibility to UC in the Tunisian population. *PTPN2* was first published as a susceptibility gene for CD in the GWAS by the WTCCC [53] in which strong associations for CD at four novel loci was reported. *PTPN2* was one of these novel loci and rs2542151 was highly significantly associated with CD. Following the WTCCC results, there was a replication study [54] which showed modest evidence of replication for rs2542151 (P= 0.048) (Pcomb = 3.2×10^{-8}). In addition, a very recent Italian study found no association of *PTPN2* SNP with UC but a weak association of the *PTPN2* SNP rs2542151 with susceptibility to CD (P=0.015; OR=1.34) [55].

PTPN22 (located on chromosome 1) encodes a lymphoid specific phosphatase known as Lyp, a strong negative regulator of T cell activation, either independently or through binding to a variety of adaptor molecules. In our study, there was no evidence for an association with *PTPN22* and we recommend that further replication studies are undertaken to determine if this is a true association. Our results are in agreement with previous studies of Criswell et al. [56], Martin et al. [57] in Spanish subjects, Prescott et al. [58] in British subjects, and van Oene et al. [59] in Canadian patients. However, in the meta-analysis the SNP rs2476601 of *PTPN22* gene was significant with a *P* value of 1.81×10^{-5} (replication $P=1.01 \times 10^{-4}$; combined $P=1.46 \times 10^{-8}$) [9]. In addition, the *PTPN22* 263Q loss-of-function variant showed initial evidence of association with UC in the Spanish cohort (P=0.026, OR=0.61), which was confirmed in the meta-analysis (P=0.013 pooled, OR=0.69) [60]. The lack of replication with *PTPN22* may be falsely negative, possibly for the reason of the inconsistencies across studies regarding case or control exclusion criteria, such that phenotypic and genetic heterogeneity may exist. It is also possible that *PTPN22* is significant only in the context of a particular environmental factor that is not present in the South Tunisian population. Much work, at both the population and molecular levels, remains before dismissing *PTPN22* and its possible association with CD.

Genes encoding for immunoregulatory molecules clearly constitute important candidate susceptibility loci for IBD, and a number of recent studies have highlighted the central roles played by *IL2* and IL-10 in orchestrating the immune response.

IL2, together with IFNγ, is produced by activated Th1 lymphocytes and stimulates the magrophages, natural killer cells and cytotoxic T cells of the cell-mediated immune response. The presence of large numbers of activated T cells in the involved mucosa of IBD patients suggests that *IL2* is likely to be playing at least some role in stimulating inflammation [61,62]. This study revealed no association of the *IL2* polymorphism with IBD in our patient population. Although recent non-synonymous SNP scan [12] did not find evidence for association of *IL2* with UC, previous study in Spanish population did report evidence for associations with both phenotypes, either UC or CD [63]. Although the precise reasons for the lack of replication of the unclear, phenotype and genotype heterogeneity may contribute and some of the earlier studies were based on small sample sizes. *IL10* is produced by Th2 lymphocytes and acts to downregulate macrophages and other antigen-presenting cells (APC). It also inhibits production of cytokines by Th1 lymphocytes in response to these APC, but does not block synthesis of cytokines by Th2 cells [64]. In this study, we did not find evidence for an association with the five polymorphisms studied. Several candidate gene studies that examined the association of the 3 IL-10 promoter polymorphisms with IBD susceptibility have had inconsistent results. In the UC GWAS that identified rs3024505, Franke et al. [65] did not find an association between the 3 promoter polymorphisms and IBD susceptibility. Another very recent study from New Zealand found an association with CD for SNPs rs3024505 and rs1800896, and phenotypic analysis indicated an association of rs3024505 with an early age at first diagnosis, stricturing CD behavior, and requirement for bowel resection [66].

Conclusion

Our replication study clearly reveals an apparent genetic/ allelic heterogeneity at UC/CD loci. It is likely that some SNPs that did not pass correction may be associated with IBD in south Tunisia nonetheless the study did not have sufficient power to detect these associations. In conclusion, the observed disparity in the allele frequency of genetic association study hits in our cohort confirms differences in genetic architecture between populations. However, further studies involving larger number of IBD patients should be performed before arriving at a definitive conclusion regarding the implications of the analyzed genes in IBD susceptibility.

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