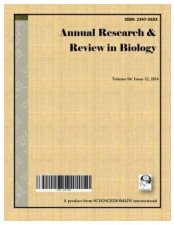

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Association of 10 p Locus with Slovenian Inflammatory Bowel Disease Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Author KR designed the study, wrote the protocol and interpreted the data as well as the writing of the manuscript. Author MG managed the literature searches, gathered the initial data and performed preliminary data analysis. Author UP correspondence for the publication of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate association of 10 p locus in Slovenian inflammatory bowel disease (IBD) patients with different sub-phenotypes.
Methodology: Genotyping of three selected SNPs from 10 p locus was performed in 594 IBD patients, divided into three sub-phenotypes, Crohn's disease (CD), ulcerative colitis (UC) and refractory CD, and in 250 healthy controls with PCR-RFLP technique. Clinical characteristics of patients were compared according to genotype of selected SNPs.
Results: We found statistically significant correlation of all three selected SNPs with different sub-phenotypes of IBD. For SNP rs12777960 on gene *CCNY* we found association with all IBD patients and UC patients separately, where frequency of AA and AC genotype was higher in a group of

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patients compared to controls (*P* = .007). For SNP rs4934697 we found statistically significant association only for refractory CD patients, where frequency of CT and TT genotype was higher in a group of patients compared to controls (*P* = .01). For SNP rs2254252 on gene *SEPHS1* we found association for a group of all IBD patients and CD patients, where frequency of allele T was higher in a group of patients (0.521) compared to healthy controls (0.451, *P* = .009). We identified novel association between age at diagnosis and genotype of SNP rs12777960 in gene *CCNY* where patients with disease susceptible genotype AA were diagnosed younger compared to patients with AC/CC genotype (*P* = .03).

Conclusion: We confirmed candidate SNPs and genes from 10 p region in population of Slovenian IBD patients and found specific correlations of SNPs with different sub-phenotypes of IBD, particularly *SEPHS1* with CD, *CCNY* with UC and region near *CREM* and *CCNY* genes with refractory CD.

Keywords: Inflammatory bowel disease; chromosome 10; association analysis; SNPs.

1. INTRODUCTION

Inflammatory bowel disease (IBD) is chronic inflammatory disorder of gastrointestinal tract where several genes and environmental factors play roles. IBD is divided into two main subtypes, Crohn's disease (CD) and ulcerative colitis (UC). Main difference between both subtypes is that CD can affect any part of the intestine, whereas UC is restricted to colon [1]. The highest reported prevalence value for UC in Europe was 505 per 100,000 persons and for CD 322 per 100,000 persons [2]. Despite the unique features of CD and UC there are also a number of overlapping aspects that suggest a common pathogenesis [3]. Further, genetic epidemiology data provide compelling evidence that the CD and UC are related complex polygenic diseases. Complex disease genetics have been revolutionized in recent years by the advent of genome-wide association studies (GWAs), in which hundreds of thousands of single nucleotide polymorphisms (SNPs) are tested for association with a disease in hundreds or thousands of persons [4]. To date GWAs have identified more than 160 loci in the human genome that contribute to the development of IBD [5]. Approximately one-third of loci described confer susceptibility to both CD and UC and 70% of the IBD loci are shared with other complex diseases or traits [5,6].

Locus on chromosome 10 p has been first identified as CD associated loci in 2008 [7] with SNP rs17582416 located near genes *CREM* and *CCNY*. Further, SNP rs10508459 in gene *SEPHS1* has been also associated with CD [8] and SNP rs3936503 in gene *CCNY* was significantly associated with CD as well as with UC [9]. However, there is lack of evidence about contribution of this locus in different independent populations and since GWA studies mainly focus on risk factors and usually lack analysis of comprehensive genotype/phenotype correlations, was the aim of our study to evaluate association analysis of 10 p locus in Slovenian IBD patients with different sub-phenotypes.

2. MATERIALS AND METHODS

2.1 Subjects

We enrolled 594 Slovenian patients with IBD, including 200 with ulcerative colitis (UC), 340 Crohn's disease (CD) and 54 undetermined IBD. Among the 340 CD patients, a subgroup of 120 refractory CD patients who failed standard therapy with antibiotics, corticoids, and immunosuppressants and developed severe forms of the diseases, such as those with fistulas, were enrolled in the study. The inclusion criteria for those patients was described previously [10]. Demographic data and clinical characteristics of IBD patients according to Montreal classification [11] are shown in Table 1.

As control group 250 healthy unrelated blood donors were analyzed. Sex- and age-matched healthy blood donors were selected as controls in this association study. All of the controls were Slovenian and thus of Caucasian origin representing the general Slovenian population. Experiments were undertaken with the understanding and written consent of each individual and the study was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Study was approved by Slovenian National Committee for Medical Ethics.

2.2 Selection of SNPs

We selected SNPs according to already associated SNPs with IBD from 10 p locus. We selected SNPs rs10508459, rs17582416 and rs3936503. Since restriction fragment length polymorphism (RFLP) tests for those SNPs were not possible, we replaced them with closest SNPs in highest linkage disequilibrium (LD) with selected SNPs, using HapMap and Haploview. SNP rs10508459 was replaced with rs2254252 (D'=1, r2=0,729, LOD=17,53), rs17582416 was replaced with rs4934697 (D'=1, r2=1, LOD=28,31) and SNP rs3936503 was replaced with rs12777960 (D'=1, r2=1, LOD=25,49).

2.3 Genotyping

Genotyping of selected SNPs was performed by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). Primers and restriction enzymes were selected using the freely available program GeneRuner (Hastings Software Inc.). PCR cycling conditions were as follows: Initial denaturation 95℃ for 10 min followed by 35 cycles of 95℃ for 30 s, primer specific temperature for 30 s, and 72℃ for 30 s, in an automated thermal cycler (Biometra, Germany). Composition of a 10 µl PCR mixture was as follow: 1 µl DNA (50 ng); 1 µl forward primer (250 nM), 1 µl reverse primer (250 nM), 0.25 µl DreamTaq DNA Polymerase (1.25U; Thermo Fisher Scientific); 0.2 µl dNTP mix (10 mM each; Thermo Fisher Scientific), 1 µl 10x DreamTaq PCR Buffer (with $MgCl_2$; Thermo Fisher Scientific), PCR grade water up to 10 µl. PCR products were resolved in 2% agarose gel. Primer sequences are presented in Table 2 and PCR conditions are shown in Table 3. PCR products were incubated with restriction enzymes shown in Table 4 for 16 hours (overnight). Digested products were resolved in 2% agarose gel. To determine the sizes of bands after PCR and RFLP, we used GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific). Genotyping was performed for all patients and healthy individuals.

2.4 Statistical Analysis

We used the two-sided Fisher's exact test to compare genotype and allele frequencies between control group and IBD patients as well as between different clinical characteristics of patients. To compare continuous clinical data between different genotypes (dominant and recessive model) and alleles, we used Independent samples t-test in cases of normal distribution of data (Kolmogorov-Smirnov test – $p > 0.05$) or Mann-Whitney U-test in cases, where data significantly deviate from a normal distribution (Kolmogorov-Smirnov test of normality – $p < 0.05$). For statistical analysis, we used the IBM SPSS Statistics 22.0 statistical package.

Table 1. Clinical characteristics of inflammatory bowel disease patients enrolled in this study according to montreal classification [11]

IBD patients		
Age	Mean +/- SD*	41.08 +/- 16.9
Sex	Male/Female (%)	48.6 / 51.4
Age at diagnosis	Mean +/- SD*	27.9 +/- 12.1
	A1 below 17 years (%)	15.7
	A2 between 17 and 40 years (%)	69.4
	A3 above 40 years (%)	14.8
Smoker	Yes/No (%)	25.1/74.9
Previous surgery	Yes/No (%)	41.4/58.6
Crohn's disease patients		
Location	**L1** Ileal (%)	19.5
	L2 Colonic (%)	35.8
	L3 Ileocolonic (%)	44.7
	L4 Isolated upper disease (%)	2.4
Behaviour	**B1** non-stricturing, non-penetrating (%)	24.5
	B1p non-stricturing, non-penetrating, perianal (%)	11.8
	B2 stricturing (%)	18.2
	B2p stricturing, perianal (%)	13.6
	B3 penetrating (%)	24.5
	B3p penetrating, perianal (%)	12.7
Fistula	**Yes/No (%)**	41.8/58.2

Table 2. Primer sequences for selected SNPs

Gene	Location	SNP	Alleles	Forward primer	Reverse primer
CCNY	10 p11.21	rs12777960	A/C	TTCAGACACCTACA GCCCTCT	AAATGCCCTCAA GCAATGAA
/	10 p11.2	rs4934697	C/T	AGACCTTCAGGCCC CAGTT	GCAGTAGGCTGC CAGATCA
SEPHS1	10 p14	rs2254252	C/T	AAGGGGGCAAAAGA GAAATG	CATCTGTCCGGA ACTTGTGG

Table 3. PCR conditions for selected SNPs

Gene	Location	SNP	Primer concentration [nm]	MgCl2 concentration [mM]	Annealing temperature [℃]	Product size after PCR [bp]
CCNY	10 p11.21	rs12777960	250	1.5	60	220
/	10 p11.2	rs4934697	250	1.5	60	246
SEPHS1	10 p14	rs2254252	250	2	60	184

Table 4. Restriction enzymes with optimized amounts, incubation temperature and size of fragments after restriction

Gene	Location	SNP	Restriction enzyme	Amount of restriction enzyme [U]	Incubation temperature [℃]	Size of fragments after restriction [bp]
CCNY	10 p11.21	rs12777960	Mae I	1	37	AA: 200+20 AC: 200+155+45+20 CC: 155+45+20
/	10 p11.2	rs4934697	Mbo II	0,25	37	CC: 246 CT: 246+209+37 TT: 209+37
SEPHS1	10 p14	rs2254252	Rsa I	3	37	CC: 115+47+22 CT: 162+115+47+22 TT: 162+22

3. RESULTS

3.1 Genotyping and Association Study of Selected SNPs

Selected SNPs were genotyped in 250 healthy controls and 594 IBDs patients. All genotype frequencies for control group were in Hardy–Weinberg equilibrium. The association analysis results as well as genotype and allele frequencies were calculated for controls, for IBD patients as a whole group, and separately for each IBD subgroup, including CD, refractory CD and UC patients.

For SNP rs12777960 on gene *CCNY* we found association with all IBD patients and UC patients separately, while for CD patients we did not confirm statistically significant association. When comparing genotype frequencies between all IBD patients and controls, we found higher frequency of AA and AC genotype for SNP rs12777960 in a group of patients compared to controls (P = .007). We also found higher frequency of allele A in a group of IBD patients (0.373) compared to controls (0.308, P = .03). In a group of UC patients we also found higher frequency of AA and AC genotype compared to controls (P = .006). According to the odds ratio for IBD and UC patients bellow 1 C allele is assumed as protective for development of IBD. Association analysis for SNP rs12777960 is shown in Table 5.

For SNP rs4934697 in a region 10 p 11.2 we found statistically significant association only in refractory CD patients, where we found higher frequency of CT and TT genotype in a group of patients compared to controls (P = .01). Association analysis for SNP rs4934697 is shown in Table 6.

For SNP rs2254252 on gene *SEPHS1* we found association in a group of all IBD patients and in CD patients. We found higher frequency of allele T in a group of all IBD patients (0.521) compared

to healthy controls (0.451, *P* = .009). However, in a group of UC patients, statistical significance was at borderline. Association analysis for SNP rs2254252 is shown in Table 7.

The most significant associations are shown in Table 8 with risk allele frequencies and phenotype, for which association has been confirmed.

3.2 Correlation with Clinical Features

Genotype and allele frequencies were compared between clinical characteristics of patients. We found association between age at diagnosis and genotype of SNP rs12777960 on gene *CCNY*. Patients with genotype AA were 23 years old at the diagnosis compared to patients with AC/CC genotype, who were on average 28 years old at the time of diagnosis (*P* = .03). Further, we found higher frequency of disease-susceptible genotype TT of SNP rs4934697 in patients with fistulating disease behavior compared to healthy controls. The frequency of TT genotype of SNP rs4934697 was 18.2% in patients with fistulas compared to controls (9.6%, *P* = .029). We also found correlation between genotypes of selected SNPs and location of the disease and also disease behavior. For SNP rs2254252 we found higher frequency of allele C in patients with ileum location of inflammation (66.7%) compared to patients with colonic or ileocolonic location (45.4%, *P* = .01). Correlation between stricturing disease behavior and SNP rs12777960 was also observed. Frequency of genotype AA was higher in patients with stricturing disease behavior (27.8%) compared to patients without strictures (10.2%, *P* = .045). Statistically significant correlations with clinical characteristics are shown in (see above Table 9).

4. DISCUSSION

In our study, we found strong association of 10 p locus with Slovenian IBD patients with both main subtypes, Crohn's disease (CD) and ulcerative colitis (UC). For SNP rs12777960 we found association with all IBD patients as well as for UC patients separately. Association for CD was at a statistical borderline. Previously, SNP rs3936503 located in gene *CCNY* has been associated with CD as well as UC where frequency of allele A was higher in cases (0.356 for CD and 0.350 for UC) compared to controls (0.306, *P* = .00009 and *P* = .00006, respectively) [9]. Key gene from this region (10 p11.21) remained unclear. In our study we analyzed SNP rs12777960 which is in high linkage disequilibrium (LD) with SNP rs3936503 (D'=1, r^2=1, LOD=25.49). Frequency of allele A has been increased in patients (0.374 for total IBD and 0.378 for UC, compared to controls (0.308, *P* = .0074 and *P* = .0063, respectively). Frequency of allele A has also been increased

Table 5. Association analysis for SNP rs12777960

	CCNY rs12777960				
Genotype or allele	**Crohn's disease (%)**	**Crohn's disease refractory (%)**	**Ulcerative colitis (%)**	**IBD total (%)**	**Controls (%)**
AA	10.71	7.56	8.85	10.50	9.43
AC	50.60	53.78	57.81	53.56	42.77
CC	38.69	38.66	33.33	35.94	47.80
A	0.360	0.345	0.378	0.373	0.308
C	0.640	0.655	0.622	0.627	0.692
	Statistical difference between control group and subtype of disease				**Model**
Genotype					
P value	.752	.669	.855	.769	**AA vs AC+CC**
OR	1.156	0.785	1.072	.888	
95 % CI	.631-2.180	.331-1.861	.518-2.222	.489-1.612	
P value	.052	.144	**.006**	**.007**	**CC vs AC+AA**
OR	1.462	1.453	.546	.613	
95 % CI	.999-2.141	.897-2.355	.354-.841	.429-.875	
Allele					
P value	.115	.410	.056	**.034**	**A vs C**
OR	1.269	1.180	.734	.750	
95 % CI	.954-1.689	.825-1.688	.536-1.006	.574-.979	

Table 6. Association analysis for SNP rs4934697

(10 p 11.2) rs4934697					
Genotype or allele	**Crohn's disease (%)**	**Crohn's disease refractory (%)**	**Ulcerative colitis (%)**	**IBD total (%)**	**Controls (%)**
CC	39.58	32.48	41.41	40.25	46.34
CT	50.45	58.12	50.51	50.53	43.50
TT	9.97	9.40	8.08	9.22	10.16
C	0.648	0.615	0.667	0.655	0.681
T	0.352	0.385	0.333	0.345	0.319
	Statistical difference between control group and subtype of disease				**Model**
Genotype					
P value	.105	**.013**	.336	.122	**CC vs CT+TT**
OR	.753	.557	1.222	1.282	
95 % CI	.539-1.051	.351-0.883	.837-1.783	.948-1.734	
P value	1.000	1.000	.511	.697	**TT vs CT+CC**
OR	1.018	1.090	.777	.898	
95 % CI	.589-1.761	.517-2.298	.403-1.500	.543-1.484	
Allele					
P value	.233	.094	.666	.331	**C vs T**
OR	.595	.750	1.067	1.123	
95 % CI	.670-1.100	.542-1.037	.805-1.415	.896-1.408	

Table 7. Association analysis for SNP rs2254252

SEPHS1 rs2254252					
Genotype or allele	**Crohn's disease (%)**	**Crohn's disease refractory (%)**	**Ulcerative colitis (%)**	**IBD total (%)**	**Controls (%)**
CC	18.44	20.97	21.32	19.20	29.69
CT	57.64	52.42	54.82	57.44	50.39
TT	23.92	26.61	23.86	23.36	19.92
C	0.473	0.472	0.487	0.479	0.549
T	0.527	0.528	0.513	0.521	0.451
	Statistical difference between control group and subtype of disease				**Model**
Genotype					
P value	**.002**	.084	.052	**.001**	**CC vs CT+TT**
OR	.538	.628	1.558	1.776	
95 % CI	.367-.787	.378-1.045	1.010-2.404	1.266-2.492	
P value	.276	.149	.357	.281	**TT vs CT+CC**
OR	.788	.686	1.259	1.225	
95 % CI	.532-1.169	.415-1.134	.804-1.973	.853-1.760	
Allele					
P value	**.010**	.053	.070	**.009**	**C vs T**
OR	.736	0.734	1.280	1.322	
95 % CI	.586-.926	.542-.995	.984-1.665	1.073-1.629	

Table 8. The most significant associations of selected SNPs between patients and controls

Gene	Location	SNP	Risk allele	RAF* controls	RAF* patients	Phenotype	*P* value
CCNY	10 p11.21	rs12777960	A	0.308	0.378	UC	.006
/	10 p11.2	rs4934697	T	0.319	0.385	CD refractory	.01
SEPHS1	10 p14	rs2254252	T	0.451	0.527	CD	.001

** RAF = risk allele frequency*

in CD patients (0.363), however it did not quite reach statistical significance (*P* = .051). Recently in largest IBD GWA this association was further confirmed for SNP rs11010067, which is also in

high LD with both previously mentioned SNPs, namely rs12777960 and rs3936503. This association has been confirmed as IBD general loci implicating CD and UC. As a key gene from this region gene *CREM* has been suggested, however 3 additional genes in locus could also be potential targets, namely *PARD3*, *CUL2* and *CCNY* [5].

Further, we analyzed SNP rs4934697 located in intergenic region near genes *CREM* and *CCNY*. We found association for patients with refractory CD but not with other sub-phenotypes, not even total IBD patients. Frequency of allele T was higher in patients compared to controls. Previously, SNP rs17582416 has been associated with CD in 2008, where frequency of risk allele in control group was 0.345 [7]. The same SNP has also been involved in the orchestration of the secondary immune response in CD versus healthy controls and in UC/IBD unclassified versus healthy controls as well as in CD with colonic involvement [3]. Further first independent replication of association between UC and variant rs17582416 has also been confirmed [12]. In our study, we analyzed SNP rs4934697, which is in high LD with SNP rs17582416 (D'=1, r^2=1, LOD=28.31). We found significant association with refractory CD patients only, where frequency of risk allele T (SNP rs4934697 is C/T) was 0.385 in refractory CD patients and 0.346 in total IBD patients. When we excluded refractory CD patients from a group of all IBD patients, frequency of risk T allele has been even reduced to 0.336, indicating that this SNP could be specific for more severe form of CD.

In our study, we also analyzed SNP rs2254252 located in intron of gene *SEPHS1*. We found statistically significant association with total IBD patients and with CD patients, whether association with UC has been at borderline (*P* = .08). Previously, SNP rs10508459, which is in high LD with SNP rs2254252 (D'=1, r^2=0,729, LOD=17.53), was associated with CD in GWA study, but only in screening panel. In verification panel association was not confirmed [8]. So far, association of SNPs in gene *SEPHS1* has not been extensively studied.

So far, studies have not extensively studied correlation of SNPs from 10 p locus with clinical characteristics of IBD patients. In our study, we found some relevant correlations. One of the most prominent is correlation between age at diagnosis and SNP rs12777960 within gene *CCNY.* Patients with disease susceptible genotype AA were diagnosed younger compared to patients with AC/CC genotype (*P* = .03). Interesting is also correlation between the frequency of disease susceptible TT genotype of SNP rs4934697 (18.2%) in patients with fistulas compared to controls (9.3%, *P* = .029). This correlation is consistent with our finding that SNP rs4934697 is associated with a subgroup of CD patients, who are refractory to standard treatment with corticosteroids and who develop severe complications.

Our study confirmed association of 10 p locus with IBD in Slovenian population. Region covers several genes that could be potential targets, however main gene from this region is still unknown. As key targets, three genes are most often proposed, *CREM*, *CCNY* and *SEPHS*. CREM (cAMP responsive element modulator) is a transcription factor of the leucine-zipper family that also includes CREB, CREB-2, and ATF-1, -2, and -3 [13]. It binds to the cAMP response element. Its action in relation to IBD refers to IL-2, since binding of CREM to the IL-2 promoter in the anergic T cell line A.E7 [14] and SLE T [15, 16] cells limits the production of IL-2 and IL-2 plays a key role in setting the balance between immunity and tolerance, indicating link between development of autoimmune disorders [17]. Another promising target is *CCNY* (cyclin Y). Cyclins belong to a superfamily of eukaryotic proteins that play critical role in activating a group of serine/threonine kinases called cyclin-dependent kinase (CDKs). The function of CCNY

Table 9. The most significant associations of selected SNPs between clinical characteristics of patients

Gen	Location	SNP	Genotype model	Clinical characteristic	*P* value	Protective/susceptible genotype
SEPHS1	10 p14	rs2254252	TT vs CT+CC	Fistula	.018	TT = Protective
			CC vs CT+TT	Location L1	.015	CC = Susceptible
CCNY	10 p11.21	rs12777960	AA vs AC+CC	Behaviour B2	.045	AA = Susceptible
			AA vs AC+CC	Age at diagnosis	.03	AA = Susceptible
/	10 p11.2	rs4934697	TT vs CT+CC	Fistula	.029	TT = Susceptible, compared to control group

in humans is poorly understood, although several *CCNY* isoforms and CCNY-like proteins have been detected and predicted [18]. It is a membrane-binding protein, that can activate the kinase activity of CDK14 and it was found that CCNY/CDK14 signals through the Wnt pathway [19]. The Wnt signaling pathway can promote progenitor cell proliferation, maintain a cycling cell type, and prevent differentiation and is very important in the regulation of cell fate [20]. *SEPHS1* (selenophosphate synthetase 1) is also a potential target. Low serum selenium was found to be associated with CD [21] and it was reported that especially young CD patients have low selenium status [22]. Gentschew L, et al. [21] recently published that SNPs in *SEPHS1* significantly interact with serum selenium levels and CD and proposed involvement of *SEPHS1* with the regulation of oxidative stress and cell growth as well as interaction of *SEPHS1* with p53 to explain association with CD.

5. CONCLUSION

In conclusion, we confirmed candidate SNPs and genes from 10 p region in population of Slovenian IBD patients. We found specific correlation of SNP rs4934697 located in intergenic region near genes *CREM* and *CCNY* with a subgroup of CD patients, who are refractory to standard treatment with corticosteroids and who develop severe complications such as fistulas, but not with other sub-phenotypes, not even total IBD patients. We confirmed association of SNP in gene *CCNY* with UC patients and SNP in gene *SEPHS1* with CD patients. Further, we have identified novel association between age at diagnosis and genotype of SNP rs12777960 in gene *CCNY*. Our study suggests that SNPs from 10 p region should be analyzed for different IBD sub-phenotypes.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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