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The Polymorphism rs3024505 (C/T) Downstream of the *IL10* Gene Is Associated with Crohn's Disease in Serbian Patients with Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD), manifesting as Crohn's disease (CD) and ulcerative colitis (UC), is characterized by recurring episodes of inflammation in gastrointestinal tract, in which aberrant production of regulatory cytokine interleukin-10 (IL-10) presumably plays important role. Single nucleotide polymorphisms (SNPs) that affect IL-10 production, such as rs1800896 (G/A) at position -1082 and rs1800871 (C/T) at position -819 in the promoter region of the *IL10* gene, have been associated with CD and/or UC, but the results were inconsistent. Another SNP that may alter IL-10 production, rs3024505 (C/T) located immediately downstream of the *IL10* gene has been recently identified. T allele of rs3024505 was associated with both UC and CD in Western populations, but the studies from East European countries are lacking. Therefore, our aim was to assess the association of rs3024505, rs1800896 and rs1800871 with Serbian IBD patients. To this end, 107 CD and 99 UC patients and 255 healthy controls were genotyped. As a result, T allele of rs3024505 was associated with CD at allelic, genotypic (GT genotype) and haplotypic (GCCT haplotype) level, suggesting potential role of this variant in susceptibility to CD. In contrast, CD patients carrying C allele of rs3024505 had significantly increased risk of anemia and stricturing/penetrating behavior. No association was observed between rs3024505 and UC or SNPs in *IL10* promoter region and any form of IBD. In conclusion, rs3024505 SNP flanking the *IL10* gene is associated with susceptibility and severity of disease in Serbian CD patients, further validating its role as a potential biomarker in IBD.

Keywords: Crohn's disease; interleukin-10; rs3024505; single nucleotide polymorphisms; ulcerative colitis
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Introduction

Inflammatory bowel disease (IBD), mainly manifesting as Crohn's disease (CD) and ulcerative colitis (UC), represents a heterogeneous group of chronic disorders characterized by recurring episodes of inflammation in gastrointestinal tract. Although the etiology of CD and UC is poorly understood, it is believed that they both result from dysregulated immune response to commensal microorganisms in the gut in genetically susceptible individuals (Abraham and Cho 2009). The balance between the pro-inflammatory and anti-inflammatory cytokines is apparently critical in maintaining homeostasis in the gut immune system and interleukin-10 (IL-10), a cytokine with prominent regulatory function, appears to play crucial role in that process (Kole and Maloy 2014). Mice deficient in IL-10 by gene targeting (*Il10*$^{-/-}$ mice) develop chronic enterocolitis if they are not kept in germ-free environment (Kuhn et al. 1993). Moreover, mutations in the genes for IL-10 or IL-10 receptor are associated with severe colitis with early onset in children, confirming essential role of IL-10 in intestinal homeostasis in humans, too (Engelhardt and Grimbacher 2014). Although many cells are able to produce IL-10, regulatory T cells (Tregs), including Tr1 cells, are predominant source of IL-10 in the intestine, and it is possible that IBD may be caused by defective Treg-mediated suppression of immune responses to commensal gut flora (Kole and Maloy 2014).

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Evidence from the studies on monozygotic twins and their first-degree relatives suggests that as much as 75% of IL-10 production capacity variations appear to be genetically determined (Westendorp et al. 1997). Single nucleotide polymorphisms (SNPs) in cytokine genes or regions in their close proximity are considered to be important in genetic control of the production of cytokines, including IL-10. It is possible that interindividual differences in cytokine levels caused by the polymorphisms in the *IL10* gene may have significant effect on the immune response in intestine and thus affect IBD susceptibility and phenotype. Indeed, the promoter region of the *IL10* gene is polymorphic and some genetic variants were shown to influence IL-10 production. Among them, three biallelic polymorphisms, namely G/A at position -1082 (G-1082A or rs1800896), C/T at position -819 (C-819T or rs1800871) and C/A at position -592 (C-592A or rs1800872) have been most extensively studied (Fig. 1). These polymorphisms are in strong linkage disequilibrium giving rise to only three haplotypes in Caucasians, GCC, ACC and ATA (Turner et al. 1997). Moreover, the complete linkage disequilibrium between C-819T and C-592A polymorphisms has been shown (Turner et al. 1997). Functional significance of these polymorphisms have been demonstrated both *in vitro* (Turner et al. 1997; Tagore et al. 1999) and *in vivo* (Ouma et al. 2008), associating GCC haplotype with high, ACC with intermediate and ATA haplotype with low IL-10 production. A number of studies in different populations and three meta-analyses have addressed the association of one or more of these SNPs in *IL10* promoter region with IBD, but the results were inconsistent (Zhu et al. 2013; Lv et al. 2014; Zou et al. 2014). Thus, the role of these three common *IL10* SNPs in IBD susceptibility, although probable, has not been elucidated yet. On the other hand, a recent genome-wide association study (GWAS) has identified a novel marker of IBD, namely the SNP rs3024505 (C/T), which was strongly associated with UC and modestly with CD (Franke et al. 2008). This polymorphism is located in 3' flanking region of the *IL10* gene, 2,077 nucleotides downstream from the TAG sequence that corresponds to a stop codon in the mRNA (Fig. 1) and it was suggested that it might lie within the 3' untranslated region (3'UTR) of the *IL10* gene in the vicinity of the putative regulatory sequences and affect IL-10 expression (Doecke et al. 2013). Subsequent studies that assessed rs3024505 SNP in IBD patients found associations between this SNP and CD (Wang et al. 2011), UC (Doecke et al. 2013) or both forms of IBD (Andersen et al. 2010), further validating rs3024505 as a potential biomarker of susceptibility to IBD, but more studies in different ethnic populations are needed to confirm this.

It is well known that a substantial geographical and ethnical variations in IBD incidence exist worldwide (M'Koma 2013), and these discrepancies are probably influenced by both environmental and genetic factors. The incidence of IBD is steadily rising in many regions of the world, including countries from Eastern Europe where incidence rates are approaching those seen in Western Europe (Vegh et al. 2014). In Serbia, the incidence and prevalence rates of IBD are not available and are awaited till the end of ongoing multicenter epidemiological study, but our preliminary data suggest that there is a slight predominance of UC (between 51% and 55%) compared to CD (data not published). In addition, recent studies in Croatian population, which is believed to share the same origin and similar demographic characteristics with the Serbian, revealed the incidence rates of 3.5-4.3/100,000 for UC and 1-7/100,000 for CD depending of the region studied, the results being in the intermediate between those obtained for developed and developing countries (Sincic et al. 2006; Pezerovic et al. 2014), so it is plausible to assume that comparable rates would be present in Serbia. So far, significant interethnic differences in allele and genotype frequencies of *IL10* SNPs have been reported in different populations, mostly from Western Europe, Canada, Australia and New Zealand, but data from East European countries, including Serbia, are lacking. Therefore, the aim of this study was to assess the association of G-1082A, C-819T and rs3024505 polymorphisms with CD and UC in a cohort of Serbian IBD patients.

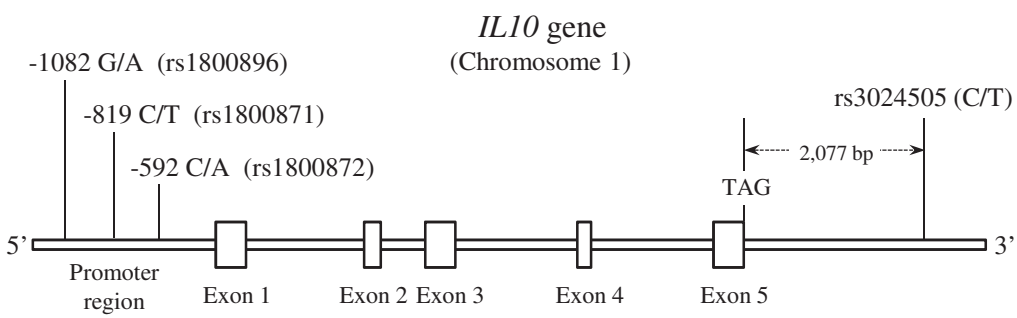

Fig. 1. Schematic representation of the SNPs in the *IL10* gene.
The SNPs are indicated as position with the allele variants (major allele/minor allele) followed by the rs number. Polymorphism rs3024505 (C/T) is located in the 3' flanking region of the *IL10* gene, 2,077 nucleotides downstream of the TAG sequence that corresponds to a stop codon in mRNA (Doecke et al. 2013). Open boxes represent exons.

Patients and Methods

Patients

A total of 206 IBD patients (107 CD and 99 UC) and 255 healthy controls were included in the study. IBD patients were recruited from the Clinic for Gastroenterology and Hepatology, Clinical Center of Serbia in the period from 2012 to 2014. Diagnosis of CD and UC was based on standard clinical, radiological, endoscopic and histological criteria (Van Assche et al. 2010; Dignass et al. 2012) and the phenotype characteristics were defined according to the Montreal classification (Satsangi et al. 2006). The clinical disease activity for CD patients was calculated using Crohn's Disease Activity Index (CDAI) (Harvey and Bradshaw 1980) and for UC patients using Mayo score (Tanaka et al. 2001). Main clinical characteristics of the patients included in the study are presented in Table 1. Matching blood samples were obtained from 255 healthy blood donors using centralized procurement through the National Blood Transfusion Institute. The study was approved by the Ethic

Table 1. Main clinical characteristics of CD and UC patients.

	CD	UC
Number	107	99
Sex – n (%)		
Male	62 (57.9)	54 (54.5)
Female	45 (42.1)	45 (45.5)
Age (years, median, min – max)	40 (18 – 68)	42 (19 – 70)
Age at diagnosis (years, median, min – max)	32.5 (14 – 67)	36.6 (2 – 67)
Disease duration (years, median, min – max)	6.6 (0 – 31)	5.5 (0 – 26)
Localisation – n (%)		
CD Ileal ± UGI	27 + 1 = 28 (26.2)	
Ileocolonic ± UGI	45 + 7 = 52 (48.6)	
Colonic ± UGI	27 + 0 = 27 (25.2)	
UGI (only)	0	
UC Proctitis		2 (2.0)
Left side colitis		35 (35.4)
Extensive colitis		59 (59.6)
IPAA-pauchitis		3 (3.0)
Behaviour – n (%)		
Inflammatory ± perianal	22 + 12 = 34 (31.7)	
Stricturing ± perianal	42 + 15 = 57 (53.3)	
Penetrating ± perianal	11 + 5 = 16 (15.0)	
Perianal (only)	0	
Perianal (any)	32 (29.9)	
Extra-intestinal manifestations – n (%)		
Current	18 (16.8)	27 (27.3)
Previous	16 (15.0)	12 (12.1)
Never	73 (68.2)	60 (60.6)
Activity index (median, min – max)	CDAI 151 (44 – 400)	Mayo 6 (0 – 12)
Early disease (up to 18 months) – n (%)	40 (37.4)	45 (45.5)
Surgery – n (%)	53 (49.5)	3 (3)
Age at Surgery (years, median, min – max)	34.7 (19 – 66)	48.7 (30 – 65)
Disease duration to surgery (years, median)	2.9	2.3
Corticosteroid-dependent – n (%)	54 (50.5)	32 (32.3)
Corticosteroid-refractory – n (%)	14 (13.1)	4 (4.0)
Anti-TNF therapy		
Yes	42 (39.3)	7 (7.1)
No	65 (60.7)	92 (92.9)
Smoking – n (%)		
Current	25 (23.4)	10 (10.1)
Ex smoker	11 (10.3)	19 (19.2)
Never	71 (66.3)	70 (70.7)

UGI, upper gastrointestinal; IPAA, ileo-pauch anal anastomosis; CDAI, Crohn's disease activity index; TNF, tumor necrosis factor.

Committees of Clinical Center of Serbia and the School of Medicine of University of Belgrade, and informed consent was obtained from all subjects.

Genotyping

Genomic DNA was isolated from peripheral blood cells using the GeneJET whole blood genomic DNA purification mini kit (Fermentas Thermo Fisher Scientific Inc., Germany). Genotyping was performed using real-time PCR with commercial TaqMan probes (C_1747360_10 for rs1800896, C_1747362_10 for rs1800871 and C_15983681_20 for rs3024505; all from Applied Biosystems Inc - ABI, Foster City, USA) with Maxima Probe qPCR Master Mix (Fermentas Thermo Fisher Scientific Inc.) following procedure recommended by the manufacturer of probes. Data for controls samples for rs1800896 and rs1800871 were partly taken from another study in Serbian population (Perovic et al. 2016). Alleles and genotypes for rs1800872 SNP were inferred from rs1800871 results with allele A instead of allele T, since these two polymorphisms are shown to be in complete linkage disequilibrium (Turner et al. 1997).

Statistical analysis

Comparisons of allele, genotype and haplotype frequencies between cases and controls were performed using the Pearson Chi-square test or Fisher's exact test where appropriate. Two-tailed p values, odds ratios (OR) and 95% confidence intervals (CI) were calculated. P values less than 0.05 were considered significant. The estimation of haplotype and diplotype frequencies was done by the Expectation-Maximization algorithm using Arlequin 3.5.1.3 (Excoffier and Lischer 2010). Statistical analyses were performed using the statistical package for social sciences, version 20 (SPSS, Chicago, USA).

Results

Genotypes of all analyzed *IL10* SNPs were in Hardy-Weinberg equilibrium in all the cases and in the control group. The results of genotyping for rs1800896 (G-1082A), rs1800871 (C-819T) and rs3024505 SNPs in our study population are presented in Table 2. We did not perform analysis for rs1800872 (C-592A) polymorphism, since it has been shown that C-819T and C-592A polymorphisms were in complete linkage disequilibrium in Caucasians (Turner et al. 1997). The frequencies of alleles and genotypes of polymorphisms within the promoter region of the *IL10* gene (G-1082A and C-819T) were not significantly different in patients (CD, UC and all IBD group) compared to controls. In contrast, polymorphism rs3024505 downstream of the *IL10* gene was associated with CD patients and IBD group as a whole, but not with UC patients. Namely, minor T allele was more frequent in CD patients than in healthy controls (22.4% vs. 13.7%; p = 0.0038, OR = 1.818, 95% CI [1.208-2.734]) suggesting predisposing role of T allele of rs3024505 in CD. Likewise, carriers of T allele were also associated with CD patients (p = 0.0069, OR = 1.928, 95% CI [1.193-3.117]). In addition, both TT and CT genotypes were more frequent in CD group compared to con-

Table 2. Allele and genotype frequencies of *IL10* SNPs in controls and all IBD, CD and UC groups.

***IL10* SNP**		**Controls**[1] (n = 255) n (%)	**All IBD** (n = 206) n (%)	p value[2]	**CD** (n = 107) n (%)	p value[2]	**UC** (n = 99) n (%)	p value[2]
G-1082A (rs1800896)								
Allele	G	207 (40.6)	178 (43.2)	0.424	94 (43.9)	0.406	84 (42.4)	0.655
	A	303 (59.4)	234 (56.8)		120 (56.1)		114 (57.6)	
Genotype	GG	44 (17.2)	35 (17.0)		21 (19.6)		14 (14.1)	
	GA	119 (46.7)	108 (52.4)	0.407	52 (48.6)	0.705	56 (56.6)	0.247
	AA	92 (36.1)	63 (30.6)		34 (31.8)		29 (29.3)	
C-819T (rs1800871)[3]								
Allele	C	380 (74.5)	298 (72.3)	0.454	159 (74.3)	1.000	139 (70.2)	0.245
	T	130 (25.5)	114 (27.7)		55 (25.7)		59 (29.8)	
Genotype	CC	142 (55.7)	109 (52.9)		61 (57.0)		48 (48.5)	
	CT	96 (37.6)	80 (38.8)	0.745	37 (34.6)	0.763	43 (43.4)	0.472
	TT	17 (6.7)	17 (8.3)		9 (8.4)		8 (8.1)	
rs3024505								
Allele	C	440 (86.3)	328 (79.6)	**0.007**	166 (77.6)	**0.0038**	162 (81.8)	0.136
	T	70 (13.7)	84 (20.4)		48 (22.4)		36 (18.2)	
Genotype	CC	191 (74.9)	131 (63.6)		65 (60.7)		66 (66.7)	
	CT	58 (22.7)	66 (32.0)	**0.028**	36 (33.6)	**0.018**	30 (30.3)	0.297
	TT	6 (2.4)	9 (4.4)		6 (5.6)		3 (3.0)	

[1]Data for controls samples for rs1800896 and rs1800871 were partly taken from another study (Perovic et al. 2016).

[2]p values were calculated using Chi-square test.

[3]C-819T and C-592A *IL-10* SNPs are in complete linkage disequilibrium (Turner et al. 1997).

trols, but the difference reached statistical significance only in heterozygotes, probably due to low number of TT homozygotes in our cohort (5.6 % vs. 2.4%; p = 0.126, OR = 2.465 [0.776-7.825] for TT allele and 33.6% vs. 22.7%; p = 0.031, OR = 1.722 [1.048-2.829] for CT genotype). In line with these findings, CC genotype was more common in healthy subjects (74.9% vs. 60.7%; p = 0.0069, OR = 0.519, 95% CI [0.321-0.838]). Similar results were obtained when IBD group as a whole was compared to controls (Table 2).

In order to further evaluate the role of *IL10* SNPs, and T allele of rs3024505 in particular, in IBD susceptibility, we also determined distribution of haplotypes and diplotypes (haplotype combinations) in our cohort. Three SNPs in the promoter region of *IL10* are shown to be in strong linkage disequilibrium and in most studies were analyzed together as the three-locus haplotype (G-1082A, C-819T and C-592A). Therefore, in order to avoid any possible confusion that might arise, we decided to include C-592A SNP in our haplotype analysis. Genotypes for C-592A polymorphism were inferred from C-819T results with allele A instead of allele T, as these two polymorphisms were shown to be in complete linkage disequilibrium (Turner et al. 1997). This allowed us to classify our patients into four-locus haplotypes (G-1082A, C-819T, C-592A and rs3024505). As a result, a total of 6 haplotypes were identified, but two rare haplotypes (GTAT and ATAT), with the frequencies less than 1%, were excluded from further statistical analysis (Table 3). The frequencies of GCCT haplotype and GCCT carriers were higher in CD group than in control group (p = 0.003, OR = 1.848 [1.227-2.783] for GCCT haplotype and p = 0.0054, OR = 1.969 [1.217-3.186] for GCCT carriers). Similarly to rs3024505 genotype distribution, GCCT haplotype was also associated with IBD group as a whole. In contrast, no significant difference in haplotype distribution was detected in UC patients in comparison to the control group (Table 3). In addition, we detected 12 different diplotypes in our population, but none of them was associated with CD, UC or all IBD group (data not shown).

Finally, we also assessed whether distributions of alleles, genotypes, haplotypes and diplotypes correlate with some clinical characteristics of CD and UC in our cohort. To this end, we stratified our patients according to gender, age at diagnosis, localization, behavior, disease activity, presence of extra-intestinal manifestations (EIM), surgery due to disease, therapy response, presence of anemia and smoking habit. However, since G-1082A and C-819T SNPs were not significantly associated with any form of IBD in this study, with relatively high p values (all above 0.2), only rs3024505 polymorphism was included in the genotype-phenotype analysis. As a result, the associations were observed in relation to anemia and disease behavior in CD. Namely, carriers of C allele of rs3024505 were more common in the group of patients with anemia (p = 0.036, OR = 6.696 [1.152-38.924]). As for disease behavior, C allele carrier status was associated with stricturing/penetrating disease phenotype (p = 0.012, OR = 12.414 [1.389-110.911]). No other characteristic of CD correlated with allele and genotypes of rs3024505. Likewise, no association was found between rs3024505 SNP and UC phenotype in our study.

Discussion

In the present case-control study we determined allele and genotype patterns of three SNPs in *IL10* locus (G-1082A, C-819T and rs3024505) in IBD patients and healthy controls from Serbia. The polymorphism rs3024505 flanking the *IL10* gene was significantly associated with CD patients, indentifying T allele as a potential susceptibility marker in CD. Moreover, a haplotype containing T allele (GCCT) was also more frequent in CD patients compared to controls. In contrast, polymorphisms in promoter region of the *IL10* gene (G-1082A and C-819T) were not associated with any form of IBD.

Due to its prominent immunosuppressive and anti-inflammatory function, IL-10 is regarded as a key player in the regulation of immune response and control of inflammation (Ouyang et al. 2011). Therefore, defects in IL-10 expression and function or IL-10 signaling pathway are believed to have important role in autoimmune and chronic inflammatory diseases, including IBD (Ouyang et al. 2011; Kole and Maloy 2014). It is possible that alterations in

Table 3. Haplotype frequencies of *IL10* SNPs in controls and all IBD, CD and UC groups.

***IL10* Haplotypes**[1]	**Controls** (n = 255)	**All IBD** (n = 206)		**CD** (n = 107)		**UC** (n = 99)	
	n (%)	n (%)	p value[2]	n (%)	p value[2]	n (%)	p value[2]
G C C C	138 (27.1)	94 (22.8)	0.140	46 (21.5)	0.117	48 (24.2)	0.446
G C C T	69 (13.5)	83 (20.2)	**0.007**	48 (22.4)	**0.003**	35 (17.7)	0.162
A C C C	173 (33.9)	121 (29.4)	0.141	65 (30.4)	0.354	56 (28.3)	0.150
A T A C	129 (25.3)	113 (27.4)	0.462	55 (25.7)	0.920	58 (29.3)	0.279
G T A T	0 (0.0)	1 (0.2)	/	0 (0.0)	/	1 (0.5)	/
A T A T	1 (0.2)	0 (0.0)	/	0 (0.0)	/	0 (0.0)	/
Total	510 (100)	412 (100)		214 (100)		198 (100)	

[1]The order of polymorphisms is -1082, -819, -592 and rs3024505.

[2]p values were calculated using Chi-square test.

capacity to produce IL-10 levels caused by functional polymorphisms in promoter region of the *IL10* gene, such as G-1082A, C-819T and C-592A, may determine susceptibility to develop IBD and/or phenotype of disease in the individuals carrying particular combination of alleles. ATA haplotype has already been associated with lower IL-10 production *in vitro*, both in healthy subjects (Turner et al. 1997; Koss et al. 2000) and IBD patients (Tagore et al. 1999), or *in vivo* in Kenyan children infected with *Plasmodium falciparum* (Ouma et al. 2008). In addition, ATA haplotype was also associated with increased basal serum IL-10 levels in healthy controls, but not CD patients (Wang et al. 2011). The G allele at position -1082 was shown to have the most important role in the regulation of high constitutive IL-10 mRNA levels in healthy population (Suarez et al. 2003). On the other hand, the allele A of C-592A SNP may lead to the formation of a binding site for the ETS family of transcription factors (which is absent in carriers of -592C allele) resulting in diminished transcription and IL-10 production observed for -592A variant (Shin et al. 2000). The association studies for the three SNPs in IBD gave conflicting results. Initially, G-1082A was associated with UC in a small cohort of British IBD patients (Tagore et al. 1999). In subsequent studies, *IL10* promoter polymorphisms were sporadically associated with IBD. For example, G-1082A SNPs was associated with UC and/or CD in Spanish, Australian, New Zealand, Italian, and Mexican studies (Fernandez et al. 2005; Fowler et al. 2005; Castro-Santos et al. 2006; Hong et al. 2008; Tedde et al. 2008; Garza-Gonzalez et al. 2010; Wang et al. 2011), but the findings were not consistent, showing associations for both G and A allele (or genotypes containing those alleles). The polymorphism C-592A was also associated with CD in Canadian pediatric patients (Amre et al. 2009). However, most studies failed to find any association between *IL10* promoter SNPs and susceptibility to develop IBD (Klein et al. 2000; Koss et al. 2000; Kim et al. 2003; Balding et al. 2004; Cantor et al. 2005; Klausz et al. 2005; Celik et al. 2006; Sanchez et al. 2009; Andersen et al. 2010; Ahirwar et al. 2012; Doecke et al. 2013; Bank et al. 2014; Lopez-Hernandez et al. 2015). Moreover, three meta-analysis, performed so far, were not consistent either, with each one showing associations of some of the three SNPs with one form of IBD that were not replicated by others (Zhu et al. 2013; Lv et al. 2014; Zou et al. 2014). Interestingly, GWAS analyses did not find any evidence of association for those SNPs in *IL10* promoter region with UC or CD (Barrett et al. 2008; Franke et al. 2008). The absence of association of G-1082A and C-819T with any form of IBD in our study is in concordance with these findings. The reasons for the discrepancy in the results from different studies are unclear, but they are most likely to be explained by ethnic diversity. In line with this notion, allele frequencies of C-819T and G-1082A, in particular, in our control group differed significantly from a number of other studies, including several studies from Europe done in Caucasians (data not shown), suggesting some ethnic specificity of Serbian population with regard to promoter region of the *IL10* gene. Other reasons for reported inconsistency between studies may include differences in study design and selection of control population and small sample size leading to a lack of association due to low power. In the present study, we have not performed the analysis for C-592A polymorphism. Yet, given that C-819T and C-592A SNPs are in strong linkage disequilibrium with concordance of almost 100% in most studies and since the p values for C-819T polymorphisms in our study were in the range between 0.245 and 1, it is likely that C-592A would not be associated with CD or UC in our cohort, either. Thus, it seems plausible that G-1082A, C-819T and C-592A SNPs in promoter region of *IL10* are not associated with CD or UC and could not be considered as potential biomarkers of susceptibility to develop IBD in Serbian population. However, it is also possible that some of the associations observed in other populations exist in Serbian population too, but we failed to detect it due to relatively limited number of patients in our study.

Contrary to *IL10* promoter SNPs, rs3024505 polymorphism downstream of the *IL10* gene was associated with CD and IBD group as a whole in our study, at both the allelic and the genotypic level. Allele T, genotype CT and carriers of T allele were all more frequent in CD and all IBD group compared to controls, while similar trend was also observed for TT genotype, although it did not reach statistical significance. Four-locus haplotype (-1082, -819, -592 and rs3024505) analysis, demonstrating the association of CD with allele T-containing haplotype GCCT, but not GCCC, further corroborated findings from the SNP analysis, thus validating potential role of T allele as a risk marker of CD. The polymorphism 3024505 was identified for the first time as susceptibility marker of UC in the multicentre GWAS performed in five European populations from Germany, UK, Belgium, Netherlands and Greece (Franke et al. 2008). In the same study, a modest, but significant association with CD was also observed in German IBD patients. The subsequent GWA studies clearly associated rs3024505 with both forms of IBD, including early-onset UC and CD (Imielinski et al. 2009; Franke et al. 2010; Anderson et al. 2011). The replication case-control studies that followed, found the association of T allele and TT (or CT) genotype of rs3024505 SNP with UC and/or CD in Danish, Dutch, New Zealand, Australian and North Indian IBD patients (Andersen et al. 2010; Festen et al. 2010; Juyal et al. 2011; Doecke et al. 2013; Bank et al. 2014), with the exception for two studies that found no association with CD in Danish and Canadian pediatric cohorts (Amre et al. 2009; Bank et al. 2014). The only meta-analysis done so far, that included data from just 2 publications with 6 populations of UC patients, also associated rs3024505 with UC (Doecke et al. 2013). Thus, rs3024505 seems to be the obvious candidate for susceptibility biomarker in IBD patients from Western Europe, but

it is not clear whether such an association exists in the populations from other European regions whose populations may have different origin, including those from Eastern Europe. The results of this study imply that this is really the case, at least for CD. Indeed, in our study fairly large difference in minor allele frequency (MAF) was observed (22.4% in CD patients compared to 13.7% in controls) with one of the strongest odds ratios recorded so far in the case-control studies (OR = 1.818, 95% CI [1.208-2.734]). As for UC, the lack of association with our UC patients might suggest that T allele of rs3024505 does not represent a risk marker in Serbian population. However, both T allele and T carriers were more frequent in UC group than in controls and the differences, although not statistically significant, were close to a threshold level ($p = 0.136$ and $p = 0.119$, respectively). Therefore, it is possible that, because of relatively small sample size, we had low power to detect these associations with a level of statistical significance, so it is on the future studies with more subjects involved to delineate this.

The biological significance of rs3024505 polymorphism in IBD remains unclear. This polymorphism is located in the 3' flanking region of the *IL10* gene, in the putative 3' UTR (Doecke et al. 2013). Whether it is directly involved in the regulation of IL-10 production is not clear, but it is known that the *IL10* gene expression is regulated at the post-transcriptional level (Le et al. 1997). In the close proximity of rs3024505, a highly conserved stretch of DNA is located, which contain putative motif for binding of AP-1 (Activator protein 1) (Franke et al. 2008), transcription factor that regulates gene expression in response to different stimuli, such as cytokines, growth factors or infections (Hess et al. 2004). In addition, recent *in silico* transcription factor binding site analyses identified two new binding sites that surround rs3204505 SNP, one for Sp1 (Specificity protein-1) and another for USF (Upstream stimulatory factor) (Doecke et al. 2013). Thus, this region may contain regulatory sequences and affect IL-10 gene expression. Previous studies were not able to observe any effect of rs3024505 on serum IL-10 levels in IBD patients and healthy controls (Wang et al. 2011). Nevertheless, it is possible that T variant of rs3024505 may have some effect on expression levels of IL-10 and hence decrease its production and anti-inflammatory activity and consequently confer susceptibility and/or influence the pathophysiology of IBD. Alternatively, rs3024505 SNP may serve merely as marker in linkage disequilibrium with another yet undetected causal variant(s). Therefore, further sequencing and functional experiments are needed to decipher functional capacity of this region with regard to IL-10 production and IBD susceptibility.

Furthermore, we also performed genotype-phenotype analysis in the present study. To this end, we classified our patient population into clinical subgroups based on the age of onset, localization, behavior and activity of disease, therapy response, presence of EIM and anemia, need for surgery and smoking habit. The only associations observed were between C allele of rs3024505 SNP in the *IL10* gene and the presence of anemia and stricturing/penetrating phenotype in CD patients. The clinical impacts of these findings are presently unclear. Thus far, the polymorphisms in *IL10* locus (including rs3024505) have not been associated with anemia in IBD patients. The role of IL-10 in anemia in chronic inflammation has not been elucidated yet, but it has been shown that the administration of IL-10 can induce anemia in CD patients, probably through the induction of imbalances in iron homeostasis, leading to hyperferritinemia and limited iron availability to erythroid progenitor cells (Tilg et al. 2002a). Therefore, in view of the abovementioned potential functional relevance of rs3024505, it is plausible to assume that C allele might increase *IL10* expression causing a sustained production of IL-10 that may eventually lead to anemia in chronic inflammation present in CD patients. On the other hand, the association between C allele of rs3024505 SNP in the *IL10* gene and an aggressive CD phenotype may be surprising, taking into account that this variant did not correlate with disease susceptibility in this study, as well as in other studies (Andersen et al. 2010; Wang et al. 2011). Our results are in contrast with the findings from New Zealand study, where individuals carrying the T allele of rs3024505 had a significantly increased risk of stricturing CD behavior (Wang et al. 2011). The observed discrepancy might be due to inter-ethnic differences between the populations, but the reason could also be the composition of the cohorts in the two studies. In our study, all IBD patients were recruited from the tertiary referral center and, consequently, patients with severe form of disease were overrepresented. Indeed, as many as 53.3% of our CD patients had stricturing form of disease compared to only 30.4% in New Zealand study. Nevertheless, we are not able to explain the association of putative protective allele (C allele of rs3024505) with more severe form of disease in our patients. It has long been recognized that, besides its anti-inflammatory functions, IL-10 may also have pro-inflammatory role in immune response (Groux and Cottrez 2003). For example, IL-10 stimulates production of pro-inflammatory mediators, such as interferon-γ, when it is administered to patients with CD (Tilg et al. 2002b). We can only speculate whether such immunostimulatory function of IL-10 is responsible for the observed association in our study, but it is on the future studies to clarify this. In addition, our findings also need to be interpreted keeping in mind the potential for false positives given that the associations we observed might not withstand correction for multiple testing. In the light of the obtained p values and the number of statistical tests performed, we cannot exclude that some of our positive findings may be due to chance. Therefore, a replication of associations in independent ethnically similar cohort using the same assessment methods as in this study is needed to validate our findings.

In summary, this is the first report on the role of *IL10* gene polymorphisms in IBD susceptibility and severity

from an East European country. The rs3024505 polymorphism flanking the *IL10* gene was associated with the risk of CD in Serbian IBD patients. The associations of rs3024505 with anemia and stricturing/penetrating phenotype were also observed in our CD patients. On the other hand, none of the polymorphisms in promoter region of the *IL10* gene were associated with the risk of CD or UC. Thus, the results from our study add further evidence that support important role of rs3024505 polymorphism in susceptibility and severity of CD, which may prove valuable for the future meta-analyses in the search for reliable biomarkers in IBD and eventually help to clarify the role of IL-10 in the pathogenesis of this inflammatory disorder.

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Conflict of Interest

The authors declare no conflict of interest.

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