Polymorphism in the NLRP3 inflammasome-associated *EIF2AK2* gene and inflammatory bowel disease

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Abstract. Inflammatory bowel disease (IBD) is the common name for numerous relapsing inflammatory conditions, and is the collective name for Crohn's disease (CD) and ulcerative colitis (UC). The activation of the inflammasome in the pathogenesis of IBD has recently been identified, however the underlying mechanisms remain unclear. An activator of the inflammasome is double-stranded RNA-dependent protein kinase R, also termed EIF2AK2. A genetic alteration in the *EIF2AK2* gene has previously been shown to be associated with Alzheimer's disease. The present study genotyped samples from a Swedish cohort of patients with IBD and healthy controls for an *EIF2AK2* polymorphism. The rs2254958 polymorphism in the 5'-untranslated region of the *EIF2AK2* gene was genotyped by TaqMan® single nucleotide polymorphism genotyping, followed by allelic discrimination. However, no significant association was determined between the rs2254958 polymorphism and the development of IBD, or clinical outcome. In conclusion, the results of the present study suggest that the rs2254958 polymorphism has a limited effect on the onset or progression of IBD.

Introduction

Inflammatory bowel disease (IBD) is the name for a group of gastrointestinal disorders, of which Crohn's disease (CD) and ulcerative colitis (UC) are the two most common subtypes. IBD is generally hypothesized to be a multifactorial condition caused by a combination of genetic, microbial and environmental factors (1). Among the genetic risk factors involved in IBD, genetic alterations in genes associated with intestinal homeostasis, microbial defense, innate immune system regulation, reactive oxygen species production, autophagy and regulation of adaptive immunity have been identified (2).

The NLRP3 inflammasome has previously been implicated in the pathogenesis of numerous inflammatory diseases, including IBD (3). The intracellular NLRP3 inflammasome consists of the sensor proteins NLRP3, ASC and pro-caspase-1; however, the mechanism for the assembly of the NLRP3 inflammasome remains poorly understood. Various stimuli, such as bacterial muramyl dipeptide, peptidoglycan, bacterial and viral RNA, bacterial toxins, low intracellular K^+ concentrations, ATP and urate crystals are known to induce the formation of the NLRP3 inflammasome (4-10). Upon activation of the NLRP3 inflammasome, caspase-1 is activated, followed by interleukin (IL)-1β, IL-18 and the endogenous danger signal high-mobility group protein B1 (HMGB1) (11-14). Once activated HMGB1 is translocated from the nucleus to the cytoplasm, which facilitates its extracellular secretion (14). Elevated levels of HMGB1 have previously been detected in the stools of patients with IBD (15), thus suggesting a potential role for HMGB1 in the pathogenesis of IBD. Lu *et al* (16) previously demonstrated that one of the molecules responsible for inflammasome activation and HMGB1 release is the double-stranded RNA-dependent protein kinase R (PKR, also known as EIF2AK2) (16). PKR is an intracellular protein encoded by the *EIF2AK2* gene on chromosome 2p22-p21, and has two RNA binding domains and a kinase domain (17). Previous studies have indicated that PKR is involved in various cell regulatory pathways, including antiviral activity, growth suppression, cell cycle regulation, transcriptional regulation and apoptosis (18). Lu *et al* (16) demonstrated that genetic deletion of *EIF2AK2* reduced activation of the NLRP3 inflammasome, whereas overexpression of *EIF2AK2* increased the activity of the NLRP3 inflammasome. Numerous single nucleotide polymorphisms (SNPs) have been shown to be located in the *EIF2AK2* gene. One of these, the rs2254958 SNP is located in the 5'-untranslated region (UTR) and has been associated with Alzheimer's disease, which has previously been identified as an inflammatory disease associated with NLRP3 activation (19,20). The rs2254958 (C/T) polymorphism is located in the evolutionarily conserved SRp55 binding enhancer element of the *EIF2AK2* gene, where interaction with the transcription factor is disrupted by the minor T-allele, but not the major C-allele (19). Bullido *et al* (19) also suggested, via virtual analysis of the 5'-UTR,

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Table I. Clinical characteristics of patients with inflammatory bowel disease.

Characteristic	UC	CD
Number of subjects (%)		
Female	186 (43)	178 (56)
Male	242 (57)	141 (44)
Age at diagnosis, years (mean ± SD)	34.5±14.9	30.6±13.7
First-degree relative with IBD (%)	48 (11)	40 (13)
Smoking habits (%)		
Smoker	55 (13)	103 (34)
Former smoker	148 (36)	52 (17)
Never smoked	215 (51)	152 (49)
Location at diagnosis (%)		
L1 ileal/+upper GI		89 (28)/+4 (1)
L2 colonic/+upper GI		110 (35)/+3 (1)
L3 ileocolonic/+upper GI		101 (32)/+6 (2)
L4 upper GI		2 (1)
Behavior at diagnosis (%)		
B1 non-stricturing, non-penetrating		188 (60)
B2 stricturing		91 (29)
B3 penetrating		35 (11)
Extent at diagnosis (%)		
E1 proctitis	109 (26)	
E2 left sided colitis	139 (33)	
E3 extensive colitis	170 (41)	

UC, ulcerative colitis; CD, Crohn's disease; GI, gastrointestinal; IBD, inflammatory bowel disease; SD, standard deviation.

that the rs2254958 SNP may alter the activity of an exonic splicing enhancer. However, the role of this polymorphism in the development of IBD is currently unknown. The present study aimed to investigate the rs2254958 polymorphism in the *EIF2AK2* gene in a Swedish cohort of patients with IBD. The frequency of the SNP was then compared with the frequency in healthy controls from the same region and examined in association with disease phenotype data.

Materials and methods

Ethics. Ethical approval was obtained from the ethics committee of the Medical Faculty, Uppsala University (Uppsala, Sweden). Informed consent was obtained from all of the patients with IBD, as well as the healthy blood donors. The ethical considerations for the present study followed the principles of the Declaration of Helsinki.

Study subjects. A total of 747 Swedish patients with IBD (CD, n=319; UC, n=428) were recruited at Örebro University Hospital (Örebro, Sweden) between 2007 and 2009. Due to poor genotyping signal, only 730 samples were further analyzed. Diagnosis of IBD was based on standard clinical, endoscopic, radiologic and histologic criteria (21). Medical notes were scrutinized in order to classify disease characteristics, according to the Montreal classification (22). Blood samples were obtained in EDTA tubes and frozen at -20˚C until DNA extraction. Data on the clinical characteristics of the patients are presented in Table I. Healthy blood donors, without any history of gastrointestinal disease (n=1,037), were recruited from Örebro University Hospital as controls. The mean age of the controls was 45 years old and the percentages of females and males were 42 and 58%, respectively.

Genotyping of EIF2AK2 *rs2254958 polymorphism*. The *EIF2AK2* rs2254958 polymorphism was genotyped in a similar manner to that previously described by Fransén *et al* (23). DNA was extracted from whole blood samples using QIAamp DNA Blood Maxi kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The TaqMan® SNP Genotyping assay (C_ _11162026_20; Applied Biosystems/Life Technologies, Foster City, CA, USA) was used to genotype the samples for the rs2254958 polymorphism in the *EIF2AK2* gene. The amplification mixtures contained 20 ng DNA, 1X TaqMan® Genotype PCR Master mix (Applied Biosystems/Life Technologies), 1X TaqMan® SNP Genotyping assay (Applied Biosystems/Life Technologies), including allele-specific probes and primers labeled with the two-allele specific fluorescent reporter dyes VIC® (T allele) and FAM™ (C allele), and Milli-Q water in a 10 μl reaction. The PCR reactions were performed on the 7900HT Fast Real-Time PCR system (Applied Biosystems/Life Technologies), using

Table II. Genotype frequencies of the rs2254958 polymorphism in the *EIF2AK2* gene in patients with inflammatory bowel disease, as compared with healthy controls.

	Genotype	Patients (%)	Controls (%)	OR	CI	P[a]
IBD	TT	127 (17)	200 (19)	1		
	CT	365 (50)	517 (50)	1.1	0.9-1.4	0.42
	CC	238 (33)	320 (31)	1.2	0.9-1.5	0.27
CD	TT	58 (19)	200 (19)	1		
	CT	153 (49)	517 (50)	1.0	0.7-1.4	0.91
	CC	99 (32)	320 (31)	1.1	0.7-1.5	0.73
UC	TT	69 (16)	200 (19)	1		
	CT	212 (51)	517 (50)	1.2	0.9-1.6	0.29
	CC	139 (33)	320 (31)	1.3	0.9-1.8	0.18

[a]Uncorrected P-value. χ^2 test was used to determine P-values. Odds ratio and confidence interval estimated using 2x2 contingency tables. CD, Crohn's disease; UC, ulcerative colitis; C, major allele; T, minor allele; OR, odds ratio; CI, 95% confidence interval.

Table III. Allele frequencies of the rs2254958 polymorphism in the *EIF2AK2* gene in patients with inflammatory bowel disease, as compared with healthy controls.

	Allele	Patients (%)	Controls (%)	OR	CI	P[a]
IBD	T	619 (42)	917 (44)	1		
	C	841 (58)	1157 (56)	1.1	0.9-1.2	0.28
CD	T	269 (43)	917 (44)	1		
	C	351 (57)	1157 (56)	1.0	0.9-1.2	0.72
UC	T	350 (42)	917 (44)	1		
	C	490 (58)	1157 (56)	1.1	0.9-1.3	0.21

χ^2 test used to determine P-values. Odds ratio and confidence interval estimated using 2x2 contingency tables. CD, Crohn's disease; UC, ulcerative colitis; C, major allele; T, minor allele; OR, odds ratio; CI, 95% confidence interval. [a]Uncorrected P-value.

96-well plates. The PCR conditions were set as follows: 10 min at 95˚C, 40 cycles of 15 sec at 95˚C, followed by 60 sec at 60˚C. The amplification was followed by post-read allelic discrimination analysis performed on the same apparatus used for PCR analysis.

Statistical analysis. The rs2254958 polymorphism was tested for Hardy-Weinberg equilibrium. Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA) and Epi Info™ (Centers for Disease Control and Prevention, Atlanta, GA, USA) statistical packages. Associations between categorical variables were assessed by χ^2 test or two-tailed Fisher's exact test where appropriate. The data are presented as uncorrected P-values, odds ratio (OR) and 95% confidence interval (CI). P≤0.05 was considered to indicate a statistically significant difference.

Results

Genotyping of the EIF2AK2 *polymorphism rs2254958 in patients with IBD and healthy blood donors*. The rs2254958 polymorphism in the *EIF2AK2* gene was genotyped by TaqMan® genotyping of blood samples from 747 patients with IBD (319 with CD and 428 with UC) and 1,037 healthy controls. The polymorphism was found in Hardy-Weinberg equilibrium for the IBD and control groups. In the IBD group the genotype frequencies were as follows: 17% TT, 50% CT and 33% CC (Table II). The corresponding percentages for the controls were 19% TT, 50% CT and 31% CC (Table II). The IBD group was then divided into patients with CD and patients with UC; and similar genotype frequencies were evident in these groups, as compared with the whole IBD group (Table II). Furthermore, the allele frequency of the T allele in the whole IBD and control groups was 42 and 44%, respectively (Table III). No significant differences were observed in the genotype or allele frequencies between the controls and the patients with IBD, either when comparing with the whole IBD group, or the separate CD and UC groups (Tables II and III).

The rs2254958 polymorphism was also compared with disease phenotype background data, however no significant association was evident in allele (Tables IV and V) or genotype frequencies (data not shown).

Table IV. Allele frequencies of the *rs2254958* polymorphism in the *EIF2AK2* gene for patients with Crohn's disease, as compared with healthy controls, with regards to sub phenotypes and clinical features.

	Allele frequencies (%)				
	T	C	OR	CI	P
Male					
Controls	522 (44)	674 (56)	1		
Patients	118 (42)	162 (58)	0.9	0.7-1.2	0.64
Female					
Controls	395 (45)	483 (55)	1		
Patients	151 (44)	189 (56)	0.9	0.7-1.2	0.85
Controls total	917 (44)	1157 (56)	1		
Location at diagnosis[a]					
L1 ileal	78 (45)	94 (55)	1.0	0.7-1.4	0.77
L2 colonic	92 (43)	120 (57)	0.9	0.7-1.2	0.81
L3 ileocolonic	80 (40)	118 (60)	0.8	0.6-1.1	0.30
L4 upper GI	3 (75)	1 (25)	-	0.32[b]	
Behavior at diagnosis[a]					
B1 non-stricturing, non-penetrating	149 (41)	211 (59)	0.8	0.7-1.1	0.31
B2 stricturing	84 (47)	96 (53)	1.1	0.8-1.4	0.52
B3 penetrating	31 (44)	39 (56)	1.0	0.6-1.6	0.99

[a]Patients with a combination of two locations were excluded from this overview, [b]Fisher's two tailed exact test used. χ^2 test used to determine P-values, unless otherwise stated. Odds ratio and confidence interval estimated using 2x2 contingency tables. T, minor allele; C, major allele; OR, odds ratio; CI, 95% confidence interval; GI, gastrointestinal.

Table V. Allele frequencies of the *rs2254958* polymorphism in the *EIF2AK2* gene for patients with ulcerative colitis, as compared with healthy controls, with regards to sub phenotypes and clinical features.

	Allele frequencies (%)				
	T	C	OR	CI	P
Male					
Controls	522 (44)	674 (56)	1		
Patients	194 (41)	284 (59)	0.8	0.7-1.0	0.25
Female					
Controls	395 (45)	483 (55)	1		
Patients	156 (43)	206 (57)	0.9	0.7-1.1	0.54
Controls total	917 (44)	1157 (56)	1		
Extent at diagnosis					
E1 proctitis	94 (44)	118 (56)	1.0	0.7-1.3	0.97
E2 left sided colitis	111 (41)	159 (59)	0.8	0.6-1.1	0.33
E3 extensive colitis	137 (41)	201 (59)	0.8	0.6-1.0	0.20

χ^2 test used to determine P-values, unless otherwise stated. Odds ratio and confidence interval estimated using 2x2 contingency tables. T, minor allele; C, major allele; OR, odds ratio; CI, 95% confidence interval.

Discussion

The involvement of the inflammasome in the pathogenesis of IBD, including CD and UC, has been the focus of numerous previous studies (3,24-27). Overexpression of IL-1β and IL-18 in the mucosa of patients with IBD has previously been detected, supporting the role of inflammasome activation in IBD (28). However, conflicting evidence has been presented regarding

the association between IBD and polymorphisms in the genes associated with the NLRP3 inflammasome (24-27,29,30). The present study genotyped DNA samples for a polymorphism in the inflammasome activating and HMGB1 regulating *EIF2AK2* gene in IBD. The C allele and the CC genotype of this polymorphism have previously been associated with Alzheimer's disease (19). However, in the present study, no significant associations were detected in the genotype or allele frequencies of the polymorphism in patients with IBD, as compared with normal controls. Similarly, no associations were observed when the patients with UC and CD were analyzed separately. Furthermore, the present study compared the genotype and allele frequencies with phenotypic features, however no association was identified. The genotype and allele frequencies in the present study were similar to those described in the National Center for Biotechnology Information SNP database for the European population (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2254958). Bullido *et al* (19) previously determined an association between the *EIF2AK2* polymorphism and Alzheimer's disease in a Spanish cohort. Conversely, these results were questioned by a recent study and the findings could not be replicated in a Canadian cohort (31).

The lack of an association between IBD and the rs2254958 polymorphism in the *EIF2AK2* gene in the present study was unexpected. Hypothetically, the lack of association presented in this study cannot exclude any possible association in another population. It may also depend on the functionality of the SNP, or an alternative mechanism for EIF2AK2/PKR activation in IBD. EIF2AK2/PKR was initially suggested to be a key antiviral protein, due to its dsRNA binding functions; however, recent studies have shown that bacterial components, intracellular stress and free fatty acids induce autophosphorylation of PKR (32-34). Therefore, the role of diverse pathogens in PKR sensing for the assembly of the inflammasome in IBD remains to be elucidated.

In conclusion, the present study investigated the role of the rs2254958 *EIF2AK2* polymorphism in patients with IBD and healthy controls; however, no significant association was detected. These results suggest that the rs2254958 polymorphism has a limited role in the development of IBD. Further analysis in additional cohorts and functional analysis of the polymorphism in IBD are therefore required.

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