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ATG16L1 T300A polymorphism is associated with Crohn's disease in a Northwest Greek cohort, but *ECM1* T130M and G290S polymorphisms are not associated with ulcerative colitis

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Abstract

Background Crohn's disease (CD) and ulcerative colitis (UC) are well-described disease entities with unknown etiopathogenesis. Environmental, genetic, gut microbiota, and host immune response correlations have been implicated. The role of susceptibility gene polymorphisms, such as *ATG16L1* T300A and *ECM1* T130M and G290S, is well-described, although controversial findings have been reported.

Methods Two hundred five patients with inflammatory bowel disease (108 CD and 97 UC), and 223 healthy blood donors (control group) from the Northwest Greece region were genotyped for rs2241880 (T300A), rs3737240 (T130M) and rs13294 (G290S) single nucleotide polymorphisms. Genotyping was performed using the real-time polymerase chain reaction method.

Results The frequency of G allele was significantly higher in CD patients compared to the control group (P=0.029; odds ratio [OR] 1.45, 95% confidence interval [CI] 1.04-2.03). Carriers of two G alleles (T300A), compared to those carrying only one, were 1.3 times more susceptible to CD (P=0.022; OR 2.45, 95%CI 1.14-5.27). In CD patients, the presence of the T300A polymorphism indicates a possible protective effect against developing a penetrating (B3) phenotype, while in UC patients, presence of the T300A polymorphism, indicates a possible protective effect against developing joint-involving extraintestinal manifestations.

Conclusion Our study found a significant association of the T300A polymorphism with CD susceptibility, suggesting that CD occurrence in our population has a strong genetic background, with the T300A G allele having an additive effect.

Keywords Inflammatory bowel disease, Crohn's disease, ulcerative colitis, *ATG16L1*, *ECM1*

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Introduction

Inflammatory bowel disease (IBD) comprises 2 idiopathic relapsing and remitting disorders of the gastrointestinal tract: Crohn's disease (CD) and ulcerative colitis (UC). They are multifactorial, with environmental, genetic, gut microbiota, and host immune response correlations contributing to their etiology [1]. Genetic predisposition to the disease is provided by multiple genes and their distinct allele variants, known as single nucleotide polymorphisms (SNPs). To date, more than 200 susceptible loci have been identified, on a genome-wide scale, representing various pathways in IBD development [2,3]. Furthermore, the role of proteins involved in inflammation, gut defense and epithelial barrier permeability participating in IBD pathogenesis has been elucidated by numerous studies [4,5], and several studies have noted the effect of the genetic background on the phenotype of the disease. For example, in CD patients, carriage of any of the *NOD2* and *JAK2* variants has been found to predict a

more complicated disease course, with ileal involvement and stenosing behavior, while possession of any of *NOD2* variants is protective against colonic disease [6]. Additionally, ileal CD has also been associated with *ATG16L1* variants [7]. On the other hand, in UC patients, severe disease behavior has been associated with polymorphisms of major histocompatibility complex genes [8], and azathioprine use with *ECM1* gene polymorphisms [9].

It has been long known that autophagy-related 16-like 1 gene (*ATG16L1*), and especially its T300A (rs2241880) allele, is associated with susceptibility to CD but not to UC [10-12]. Nevertheless, over the years, various studies on T300A have shown a divergence from this universal susceptibility pattern and variations among different population groups have been widely reported [13-17]. Apart from the correlation between the occurrence of CD and the presence of *ATG16L1* polymorphisms, strong evidence of a predisposition to certain phenotypes of T300A carriers also exists, such as ileal CD in T300A carriers [7].

Likewise, extracellular matrix protein-1 (*ECM1*), a protein involved in epithelial barrier formation, has been associated with UC but not CD [9,18]. Inflammation in UC is limited only to the mucosal surface, and polymorphisms of genes that regulate epithelial barrier permeability play an important role in UC pathogenesis [19]. In particular, polymorphisms of the *ECM1* gene lead to tissue injury, resulting in intestinal ulcers and scarring in UC patients [20]. Two *ECM1* SNPs, rs3737240 (T130M) and rs13294 (G290S), are strongly associated with UC; however, a study from China showed no connection with the disease [21].

Our study area, Northwest (NW) Greece, has remarkable characteristics compared to other Greek regions, as has been previously reported [22]. It used to be a secluded area where population migration was limited, and that might have been crucial in shaping the genetic pool composition. Exceptional in this area is the continuous low incidence of CD compared to UC, despite the phenomenal rise of CD incidence in recent years [23]. Moreover, a relevant study in the past, examining *NOD2*/*CARD15* variants in the aforementioned area [24], showed no coherence with 2 other Greek studies [25,26].

Given the role of *ATG16L1* and *ECM1* polymorphisms in differentiating CD from UC, as well as the discrete associations between SNPs and disease phenotype, and taking into consideration the particularity of our study area, we aimed to evaluate whether our findings correlate with existing data from other regions worldwide and if there is a predictive and/or prognostic association with disease development or a specific clinical phenotype.

Patients and methods

Study population

Our study population (n=428) consisted of 205 unrelated IBD patients (108 CD and 97 UC patients) who attended the Outpatient Clinic and the Gastroenterology Department of the University Hospital of Ioannina, Greece, and 223 unrelated healthy blood donors (control group) from the University Hospital of Ioannina's Blood Bank. All study subjects were from the NW Greece region and are of Caucasian ethnicity. Demographic and clinical data of the study subjects are presented in Table 1. Diagnosis of either CD or UC was based on standard clinical, endoscopic, radiological and histological criteria [27,28]. Both CD phenotype (age at onset, disease location, and behavior) and UC phenotype (extent and severity) were determined according to the Montreal Classification [29]. The presence of extraintestinal manifestations (EIMs) and/or other autoimmune disease was established by relevant specialists. Recruitment of the control group subjects was based on their not having any gut- or liver-related disease. All subjects were informed about the nature of the study and signed the informed consent form.

Genotyping

Genomic DNA was extracted from whole blood samples using a Nucleospin Blood kit (Macherey-Nagel, Germany), according to the manufacturer's protocol. Three SNPs of 2 genes were investigated in this study: namely, rs2241880 (T300A) of *ATG16L1* and rs3737240 (T130M) and rs13294 (G290S) of *ECM1*. Genotyping was carried out using the RotorGene 3000 RealTime-PCR system (Corbett Research, Australia).

The following oligonucleotide primers and SNP-specific probes were used: *ATG16L1*-T300A-Fw: 5'-TGA AGC ATA CTT ACG AAG ACA CAC-3', *ATG16L1*-T300A-Rv: 5'-TGT CTC TTC CTT CCC AGT CC-3', *ATG16L1*-T300A-T: 5'-CCA GAA CCA GGA TGA GTA TCC ACA T-3', *ATG16L1*-T300A-C: 5'-CAG AAC CAG GAT GAG CAT CCA CAT-3', *ECM1*-T130M-Fw: 5'-CCC CAG ATT CTT TCA ATC CTC-3', *ECM1*-T130M-Rv: 5'-AGG ACT CAG GTT CTG GAT GG-3', *ECM1*-T130M-C: 5'-TTT CCC CAT TCC AGG AAC GCC AGC TCC ATT-3', *ECM1*-T130M-T: 5'-TTT CCC CAT TCC AGG AAT GCC AGC TCC ATT-3', *ECM1*-G290S-Fw: 5'-CCC AAC TAT GAC CGG GAC-3', *ECM1*-G290S-Rv: 5'-GCA ACT TAC TGC TTG GTG AG-3', *ECM1*-G290S-G: 5'-CTT GAC CAT TGA CAT CGG TCG AG-3', *ECM1*-G290S-A: 5'-CTT GAC CAT TGA CAT CAG TCG AGT C-3'.

The total reaction volume used for the RT-PCR assay was 20 μL, consisting of 1 μL genomic DNA (65 ng), 10 μL Mastermix (Kapa Probe Fast qPCR kit), 7.8 μL PCR-grade water, 0.4 μL forward primer, 0.4 μL reverse primer and 0.2 μL of each of the 2 SNP-specific probes. The cycling protocol was as follows: enzyme activation at 95°C for 3 min, followed by 40 2-step cycles of denaturation at 95°C for 3 sec and annealing-elongation at 60°C for 20 sec. The protocol was applied to all samples for all 3 SNPs. Following an initial run, 3 samples from each SNP (one wildtype, one mutant and one heterozygote) were verified by dsDNA sequencing and subsequently used as control samples in each and every run.

Oligonucleotide synthesis and dsDNA sequencing services were performed by VBC-Biotech Services GmbH (Vienna, Austria). Allelic discrimination was based on the RotorGene 3000 software.

Table 1 Demographic and clinical characteristics of IBD patients

Characteristics	CD (N=108)	UC (N=97)
Sex (male/female)	66/42	56/41
Age at diagnosis (mean,±SD, range)	32.7±13.4, (13-64)	39.3±14.7, (15-83)
≤16	6 (5.6%)	2 (2.1%)
17-40	76 (70.4%)	50 (51.5%)
>40	26 (24.1%)	45 (46.4%)
Smoking	50 (46.3%)	41 (42.3%)
Disease location		
L1 - Ileal	33 (30.6%)	
L2 - Colonic	32 (29.6%)	
L3 - Ileocolitis	43 (39.8%)	
L4 - Upper gastrointestinal	6 (5.6%)	
Disease behavior		
B1 - Nonstricturing, nonpenetrating	54 (50.0%)	
B2 - Structuring	22 (20.4%)	
B3 - Penetrating	21 (19.4%)	
B2+B3	11 (10.2%)	
p - Perianal disease	26 (24.1%)	
UC extent		
E1 - ulcerative proctitis		10 (10.3%)
E2 - Left sided		61 (62.9%)
E3 - pancolitis		26 (26.8%)
UC severity		
Mild/Moderate		73 (75.3%)
Severe		24 (24.7%)
EIMs	67 (62%)	46 (47.4%)
1 EIM	37 (34.3%)	21 (21.6%)
>1 EIMs	30 (27.8%)	25 (25.8%)
Joint	45 (41.7%)	32 (33.0%)
Osteoporosis	18 (16.7%)	7 (7.2%)
Skin/Oral	26 (24.1%)	18 (18.6%)
Ocular	10 (9.3%)	10 (10.3%)
Other autoimmune disease	6 (5.6%)	1 (1.0%)
Operated	16 (14.8%)	3 (3.1%)
Anti-TNF-α	63 (65.6%)	33 (34.4%)
Cholecystectomy (post-diagnosis)	7 (6.5%)	6 (6.2%)
Appendectomy (pre-diagnosis)	20 (18.5%)	11 (11.3%)
Tonsillectomy (pre-diagnosis)	16 (14.8%)	16 (16.5%)

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; EIMs, extraintestinal manifestations; N, number; SD, standard deviation; TNF, tumor necrosis factor

Statistical analysis

The control group was investigated for conformity with Hardy-Weinberg equilibrium (P>0.05) in all 3 SNPs. Allele and genotype frequencies among groups were calculated using Fisher's exact test. The additive effect of alleles was tested using binary logistic regression analyses. Association assessment of clinical, demographic and genotypic data was implemented using liner regression analysis or Fisher's exact test, where appropriate. The results were expressed as odds ratios (OR) with a confidence interval of 95% (95%CI). A 2-sided P-value of <0.05 was considered as statistically significant. Statistical analyses were performed using the jamovi software: the jamovi project (2019); jamovi (Version 1.0) (https://www.jamovi.org). Additionally, linkage disequilibrium between rs3737240 and rs13294 of *ECM1* was tested using the SNPstats software (http://bioinfo.iconcologia.net).

Results

One hundred eight CD patients, 97 UC patients and 223 healthy individuals (control group) were genotyped in order to examine possible associations of the 3 SNPs with IBD patients in NW Greece. The healthy control group was in Hardy-Weinberg equilibrium with all 3 SNPs. Allele and

Table 2 *ATG16L1* rs2241880 allele and genotype frequencies in CD, UC and control group (Fisher's exact test, odds ratio and confidence intervals were estimated using allele frequencies in 2×2 contingency tables)

	Alleles				Genotypes				
ATG16L1 (rs2241880)	A	G	G allele freq. (%)	P [OR (95%CI)]	AA	AG	GG	GG genotype freq. (%)	P [OR (95%CI)]
CD	77	139	64.4	0.029 [1.45 (1.04-2.03)]	11	55	42	38.9	0.134 [1.48 (0.91-2.40)]
UC	80	114	58.8	0.436 [1.15 (0.82-1.61)]	14	52	31	32.0	0.733 [1.09 (0.65-1.83)]
IBD	157	253	61.7	0.061 [1.30 (0.99-1.71)]	25	107	73	35.6	0.257 [1.29 (0.86-1.93)]
Control group	199	247	55.4		43	113	67	30.0	

CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; OR, odds ratio; CI, confidence interval

genotype frequencies for the T300A *ATG16L1* polymorphism (rs2241880) are presented in Table 2, where AA represents wildtype, AG heterozygotes and GG mutants. The frequency of the G allele of the T300A polymorphism was 64.4%, 58.8% and 55.4% in CD, UC and healthy individuals, respectively. When compared to the control group, the frequency of the G allele in Crohn's disease patients was significantly higher (P=0.029; OR 1.45, 95%CI 1.04-2.03), while it showed no significant association with UC patients.

Investigating the additive effect of the G allele in CD patients, we found that carriers of 2 G alleles (mutant group), compared to those carrying only one G allele (heterozygotes), were 1.3 times more susceptible to CD (Table 3); the difference was statistically significant (GG: P=0.022; OR 2.45, 95%CI 1.14-5.27. AG: P=0.087; OR 1.90, 95%CI 0.91-3.97).

Correspondingly, T130M and G290S *ECM1* polymorphisms (rs3737240, rs13294) are shown in Table 4. For T130M, CC represents wildtype, CT heterozygotes and TT mutants, while for G290S, GG represents wildtype, GA heterozygotes and AA mutants. The frequency of the T allele of T130M was 46.3%, 39.7% and 44.2% for CD, UC and control group, respectively, while the frequency of A allele of G290S mutation was 46.3%, 38.2% and 43.3% respectively. No strong associations between either of the 2 SNPs of the *ECM1* gene and our study group were found. No additive effect for either of the 2 *ECM1* SNPs was found. Furthermore, comparison of the 2 IBD groups (CD and UC) with each other, from a genotypic point of view, showed no significant difference for any of the 3 SNPs (P=0.290; OR 1.27, 95%CI 0.85-1.89 for T300A. P=0.195; OR 1.31, 95%CI 0.88-1.94 for T130M. P=0.109; OR 1.40, 95%CI 0.94-2.07 for G290S).

Moreover, some interesting genotype-phenotype associations among IBD patients carrying T300A SNP were observed. Specifically, in CD patients, the presence of one or two G alleles of the T300A polymorphism (AG+GG genotypes) indicated a possible protective effect against developing a penetrating phenotype (B3 behavior according to Montreal classification [29]) (P=0.015; OR 0.20, 95%CI 0.05-0.74), while in UC patients, the presence of one or two G alleles of the T300A polymorphism (AG+GG genotypes) indicated a possible protective effect against developing joint-involving EIMs (P=0.038; OR 0.31, 95%CI 0.10-0.97; Table 5). However, measure analyses of these associations, performed using the phi-coefficient (Φ) test, showed that these findings were of mild association (Φ=0.251 and Φ=0.211 respectively). Furthermore, in CD patients carrying the T300A SNP (AG+GG genotype) we found an indication of a possible protective effect against the need for cholecystectomy (P=0.022; OR 0.12, 95%CI 0.02-0.60), though with only a mild-to-moderate association (Φ=0.284). However, the number of CD patients who underwent cholecystectomy (post-diagnosis) was small (n=7).

Table 3 Additive effect of G allele, *ATG16L1*; T300A (Binary logistic regression analyses)

Crohn's disease	P-value	Odds ratio	95%CI
AG	0.087	1.90	0.91-3.97
GG	0.022	2.45	1.14-5.27

CI, confidence interval

No association was found between the age at onset, CD location, UC extent and severity, presence of EIMs or other immune disease, need of operation, anti-tumor necrosis factor (TNF)-α treatment, appendectomy or tonsillectomy and any of the three SNPs for either CD or UC patients (data not shown). Also, the 2 polymorphisms of *ECM1* gene were not found to be in linkage disequilibrium.

Discussion

IBD affects millions worldwide and its etiopathology still remains unknown. There is an international scientific consensus that a fine interaction among genetic susceptibility, environmental factors, immune response and gut microbiota may hold the answer. Nevertheless, studies have shown that a global pattern of genetic influence, heritability, environmental triggers and gut fauna does not exist. However, to investigate the matter further, any available data can contribute greatly towards elucidating the true face of IBD; thus, studies from various areas and ethnicities are needed.

This is the first study of *ATG16L1* and *ECM1* gene polymorphisms in our NW Greece cohort, a previously well-described sheltered area [22]. A previous genotypic study from this area focusing on the *NOD2/CARD15* gene showed no association between the studied polymorphisms and CD

Table 4 *ECM1* rs3737240 and rs13294 allele and genotype frequencies in CD, UC and control group (Fisher's exact test, odds ratio and confidence intervals were estimated using allele frequencies in 2×2 contingency tables)

	Alleles				Genotypes				
ECM1 (rs3737240)	C	T	T allele freq. (%)	P [OR (95%CI)]	CC	CT	TT	TT genotype freq. (%)	P [OR (95%CI)]
CD	116	100	46.3	0.617 [1.09 (0.79-1.51)]	26	64	18	16.7	0.550 [0.79 (0.43-1.45)]
UC	117	77	39.7	0.298 [0.83 (0.59-1.17)]	34	49	14	14.4	0.273 [0.67 (0.35-1.28)]
IBD	233	177	43.2	0.78 [0.96 (0.73-1.26)]	60	113	32	15.6	0.257 [0.73 (0.44-1.21)]
Control group	249	197	44.2		71	107	45	20.2	
ECM1 (rs13294)	G	A	A allele freq. (%)	P [OR (95% CI)]	GG	GA	AA	AA genotype freq. (%)	P [OR (95% CI)]
CD	116	100	46.3	0.505 [1.13 (0.81-1.57)]	26	64	18	16.7	0.550 [0.79 (0.43-1.45)]
UC	120	74	38.2	0.257 [0.81 (0.57-1.14)]	36	48	13	13.4	0.159 [0.61 (0.31-1.20)]
IBD	236	174	42.4	0.836 [0.97 (0.74-1.27)]	62	112	31	15.1	0.205 [0.71 (0.43-1.17)]
Control group	253	193	43.3		75	103	45	20.2	

CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; OR, odds ratio; CI, confidence interval

Table 5 *ATG16L1* T300A Genotype and phenotype associations (Fisher's exact test and correlation test)

ATG16L1 T300A (rs2241880)	Crohn's disease			Ulcerative colitis		
	P-value	OR (95%CI)	Φ	P-value	OR (95%CI)	Φ
B3 - Penetrating behavior	0.015	0.20 (0.05-0.74)	0.251	-	-	-
Cholecystectomy	0.022	0.12 (0.02 – 0.60)	0.284	-	-	-
Joint-involving EIMs	-	-	-	0.038	0.31 (0.10-0.97)	0.211

P-value: AG+GG vs. AA

OR, odds ratio; CI, confidence interval; Φ, Phi-coefficient; EIMs, extraintestinal manifestations

susceptibility [24], a divergent result compared to most studies from other areas [30,31]. Furthermore, the role of T300A *ATG16L1* and T130M and G290S *ECM1* gene polymorphisms in the development of CD and UC, respectively, is well-established in the literature [10,20], despite some ethnic variabilities [13,21]. Our study replicated the finding that the T300A *ATG16L1* polymorphism predisposes to CD in our cohort, also identifying an additive effect of the G allele in CD patients, but failed to demonstrate any association between *ECM1* polymorphisms and UC susceptibility.

On the other hand, despite the strong occurrence of the G allele in the CD group compared to the control group, a clear distinction among IBD patients (CD vs. UC analyses) could not be established. Similarly, as mentioned earlier, *ECM1* SNP investigation in our population failed to replicate existing data [9,20] and, again, no distinction among IBD patients was found. Hence, in our study group, it is not possible to differentiate the underlying disease (CD or UC) based on genotypic-phenotypic associations, probably because of ethnic variations, but more patients and more widely associated susceptibility genes are needed to drive to a definite conclusion.

In contrast, we found some interesting protective associations of certain phenotypes and IBD patients. In the CD group, patients who were carriers (AG and GG patients) of G allele of the T300A polymorphism were found to be associated with a possible protective effect against penetrating behavior (B3 phenotype according to the Montreal classification [29]). This finding disagreed with the results of another study, where the majority of the patients were found to exhibit penetrating behavior [7], suggesting that a potential environmental or ethnicity trigger may be present in our cohort and played a role in developing such a phenotype. Again, in the CD group, carriers of the G allele (AG and GG patients) of the T300A polymorphism were found to have a mild association with a protective effect against the need for a cholecystectomy. In the literature, gallbladder disease is well described in IBD patients and is mainly associated with Crohn's disease [32],

though a recent meta-analysis by Zhang *et al* [33] concluded that, despite the apparent association of CD and gallbladder disease, other factors, such as CD location, number of relapses and ileal surgery, were independent variables for developing cholelithiasis; more studies are required to provide a definite answer. Moreover, the G allele (T300A polymorphism) was found to have a protective effect against joint-involving EIMs in UC patients. As described before by Christodoulou *et al* [34], and confirmed by this study, EIMs are not rare in our IBD cohort (62% of CD patients and 47.4% of UC patients) and data from other studies suggest that there is a close genetic correlation between IBD and EIMs [35]. However, such associations between IBD susceptibility genes and EIM occurrence could not be demonstrated in the present study.

When other clinical data (age at onset, CD location, CD behavior, UC extent, UC severity, need of therapeutic operation, anti-TNF-α therapy) were analyzed for any possible linkage with the aforementioned mutations, no significant associations were found, although such associations have been reported in the literature [7,16,17,36,37].

In conclusion, as shown by Tsianos *et al* in 2003 [23], CD is less frequent than UC in our cohort. Thus, the findings of our current study, concerning the significant association of T300A polymorphism with CD susceptibility, point to a strong genetic background that plays a crucial role in CD occurrence in our population, and an additive effect of the T300A G allele, though further investigation including more patients and more susceptibility genes will provide a better understanding.

Summary Box

What is already known:

- More than 200 inflammatory bowel disease susceptibility loci have been identified
- The *ATG16L1* T300A single nucleotide polymorphism (SNP) is associated with susceptibility to Crohn's disease (CD) but not to ulcerative colitis (UC)
- *ECM1* T130M and G290S SNPs are strongly associated with UC but not with CD

What the new findings are:

- The T300A *ATG16L1* polymorphism predisposes to CD in our cohort
- The G allele has an additive effect in CD patients
- The T300A SNP has a protective effect against penetrating behavior and cholecystectomy in CD patients), and against joint-involving extraintestinal manifestations in UC patients
- No association of *ECM1* polymorphisms with UC susceptibility was found in our cohort

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