Polymorphism of the *IRGM* Gene Might Predispose to Fistulizing Behavior in Crohn's Disease

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OBJECTIVES: **Recently, genome-wide association analyses have identified single nucleotide polymorphisms in the *IRGM gene* (rs1000113 and rs4958847) as strong candidate susceptibility factors for Crohn's disease (CD). The aim of our study was to test whether these variants are associated with inflammatory bowel disease (IBD) in adult- and childhood-onset Italian patients.**

METHODS: **Allele and genotype frequencies of rs1000113 and rs4958847 were determined in 823 CD (265 younger than 19 years at diagnosis), 353 ulcerative colitis (UC) (130 younger than 19 years at diagnosis), and 578 controls. Genotype distributions were examined both within IBD clinical sub-phenotypes and *CARD15* genotypes.**

RESULTS: **rs1000113 and rs4958847 were both associated with adult-onset ($P=2\times10^{-4}$; $P=2.5\times10^{-3}$, respectively) and childhood-onset ($P=4\times10^{-4}$; $P=8\times10^{-3}$, respectively) CD cohorts. Similarly, the genotype frequencies remained significantly different for both variants (adult rs1000113, $P=1\times10^{-4}$; rs4958847, $P=1\times10^{-3}$; pediatric rs1000113, $P=2.3\times10^{-4}$; rs4958847, $P=9.6\times10^{-3}$). At logistic regression, the rs4958847 polymorphism was associated with fistulizing behavior ($P=0.037$, OR = 1.54, CI = 1.02–2.31) and perianal fistulas ($P=0.045$, OR = 1.55, CI = 1.01–2.38). Conversely, no association with UC and sub-phenotypes was shown.**

CONCLUSIONS: **We replicated the previously reported associations between CD and rs1000113 and rs4958847, confirming that *IRGM* is a susceptibility locus only for CD, either adult- or early-onset in the Italian population; furthermore, we have also shown its influence on specific clinical features (fistulizing disease).**

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INTRODUCTION

Crohn's disease (CD) seems to result from an impairment of the gut epithelial barrier and a dysregulated immune response toward intestinal bacteria, with the latter leading to the establishment of chronic inflammation in a genetically susceptible host (1). The characterization of the genetic background in CD is emerging rapidly, especially by means of genome-wide association (GWA) studies. Using this approach, several novel CD-associated risk loci, including *IL23R* (2), *ATG16L1* (3) and *IRGM* (4,5), have been identified. In the Wellcome Trust Case–Control Consortium (WTCCC) GWA scan, a highly significant association between variants flanking *IRGM* (immunity-related GTPase protein type M) and susceptibility to Crohn's disease has been shown, with the strongest signal being at rs1000113 ($P=5.1\times10^{-8}$) (4). Replication for association was obtained in an independent case–control sample (rs4958847: $P=3.1\times10^{-4}$) and in the combined panels of 2,930 cases and 4,962 controls ($P=3.8\times10^{-9}$) (5). The *IRGM* gene located on chromosome 5q33.1 has a central function in the autophagy pathway. Autophagy is essentially the mechanism for the removal of damaged cells and organelles, but its key function in defense against intracellular microorganisms has also been recently recognized (6). Defective elimination of intracellular bacteria secondary to autophagy derangement provides a

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plausible link with the pathogenesis of Crohn's disease (CD). In contrast, the *IRGM* gene seems not to be involved in the predisposition to UC (7,8).

Given the potential clinical and biological relevance of *IRGM* as a genetic factor influencing susceptibility to CD, it is important to confirm and extend this association in an independent CD cohort. Our aim was to assess the contribution of *IRGM* variants, rs1000113 and rs4958847, in determining disease susceptibility and phenotype in adult- and childhood-onset CD in the Italian population. In addition, we investigated the possible association with UC, and interaction with CARD15 gene variants.

METHODS

Study volunteers

We genotyped the rs1000113 and rs4958847 polymorphisms in 823 (468 male) CD patients, including 265 patients with an initial diagnosis of the disease before their 19th birthday, 353 (172 male) UC (including 130 patients younger than 19 years at diagnosis), and 578 healthy controls, comprising blood donors and locally recruited healthy controls. All volunteers were Italian and Caucasian, recruited from January 2004 to December 2005.

The rs1000113 polymorphism was chosen as it gave the strongest signal in the WTCCC study ($P = 5.1\times10^{-8}$), the first article reporting the *IRGM* gene (4). Moreover, because the two single nucleotide polymorphisms reported in Parkes *et al.*'s study (5) are in close linkage disequilibrium (LD), we selected the rs4958847 from among the two variants, as it is not in complete LD with rs1000113 ($D' = 0.72$; $r^2 = 0.17$). The sample size of the study population gave a power of >98% at the 5% significance level to replicate an odds ratio of 1.36 or higher, assuming a minor allele frequency (MAF) of at least 11% in the control population, as reported by earlier studies (4,5).

Diagnosis of IBD was based on accepted criteria (9,10) and followed the Montreal classification system. Detailed phenotypic data were available for members of this cohort and concerned the family history of IBD, presence of one or more extraintestinal manifestations, and previous bowel resection. Written informed consent from all patients and ethical approval for the study was obtained. This work was supported by a grant from the Italian Minister of the Health.

Genotyping

DNA was extracted from peripheral venous blood according to standard protocols (11) and genotyped at the Molecular Laboratory of the Unit of Gastroenterology of the San Giovanni Rotondo Hospital, Italy. Study participants were analyzed for rs1000113 (C/T) and rs4958847 (A/G) *IRGM* polymorphisms (SNPs) on chromosome 5q33.1 (position 150220269 and 150219780, respectively) and genotyped using the Custom Taqman® SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Briefly, PCR reaction (15 ml) was carried out in 1× TaqMan Universal PCR Master Mix, 1× Genotyping Assay Mix, and 50 ng of genomic DNA. After the temperature reached 50 °C for 2 min and initial denaturation at 94 °C for 10 min, the reaction was subjected to amplification for 40 cycles at 94 °C for 15 s, and 60 °C for 1 min. A wild-type heterozygote and homozygote mutant, validated by sequencing using the ABI310 DNA sequencer (Applied Biosystems), according to the manufacturer's recommendation, and an internal control DNA were included as controls in each plate. The genotyping call rate for both polymorphisms was >98%. The three common *CARD15* (rs2066844, rs2066845 and rs2066847) variants were genotyped as described earlier (12).

Statistical analysis

Data were evaluated using the SPSS software package version 11.5 (Chicago, IL, USA). Each marker was tested for the Hardy–Weinberg equilibrium in both the IBD and control cohorts using the Arlequin software version 2.0 (http://lgb.unige.ch/arlequin). Marker linkage disequilibrium analysis and haplotype frequency were performed with the Haploview Software version 3.2 (http://www.broad.mit.edu/personal/jvbarret/haploview). For comparison between the categorical variables, χ^2 test or Fisher's exact test was used where appropriate. Odds ratios were calculated for the minor allele at each SNP. Interactions between different polymorphisms were tested using logistic regression with the SPSS software. Genotype–phenotype associations were analyzed by means of univariate and multivariate logistic regressions with the SPSS software; this approach allowed taking into account a dose–response effect (heterozygote or homozygote), the possible interactions between genes, and the effect of potential confounding variables (i.e., duration of follow-up, disease localization, etc.). Armitage's trend test was assessed using Finetti software (http://ihg.gsf.de/linkage/download/finetti.zip). Power calculation was carried out using PS software (http://biostat.mc.vanderbilt.edu).

RESULTS

Demographic and clinical characteristics of patients, including their *CARD15* status, are listed in **Table 1**. The two *IRGM* polymorphisms conformed to the Hardy–Weinberg predictions both in controls and patients, and resulted in strong linkage disequilibrium ($D' = 0.97$). Evidence of association of both SNPs was found by comparing genotype and allele frequencies between CD patients, but not UC patients, and controls (**Table 2**).

Allele frequencies of the rs1000113 variant differed significantly between CD (16%) and controls (11%) ($P = 3\times10^{-5}$, OR = 1.62, CI = 1.29–2.03). Similarly, the frequency of the rs4958847 SNP amounted to 22% in patients and 17% in controls ($P = 8\times10^{-4}$, OR = 1.39, CI = 1.15–1.69). When comparing the allelic frequencies of rs1000113 and rs4958847 in adult-onset ($P = 2\times10^{-4}$; $P = 2.5\times10^{-3}$ respectively) and childhood-onset ($P = 4\times10^{-4}$; $P = 8\times10^{-3}$ respectively) CD cohorts

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

	CD (n=823)	UC (n=353)
Age at diagnosis mean ± s.d. (range)	30 ± 15 (1–79)	25 ± 16 (1–83)
Duration of follow-up mean ± s.d. (range)	8 ± 7 (1–37)	5 ± 5 (1–33)
Age at diagnosis (years)		
≤16 (A1)	245 (30)	
17–40 (A2)	435 (53)	
>40 (A3)	143 (17)	
Gender (male/female) (% male)	468/355 (57)	172/181 (49)
Localization CD, n *(%)*		
Ileum (L1)	288 (35)	
Colon (L2)	204 (25)	
Ileocolon (L3)	324 (39)	
Upper GI tract (L4)	56 (7)	
Only upper GI	7	
Localization UC, n *(%)*		
Rectum		26 (8)
Left-side colitis		152 (48)
Pancolitis		142 (44)
Disease type, n *(%)*	Montreal	
Inflammatory	405 (49)	
Stricturing	215 (26)	
Fistulizing	203 (25)	
Perianal, y/n, (%)	140/683 (17)	5/314 (2)
Familial, y/n (%)	69/754 (8)	37/316 (10)
CARD15 (pos/neg)	309/514 (38)	55/298 (16)
1 risk allele	234 (76)	50 (91)
2 risk alleles	75 (24)	5 (9)
Resective surgery, y/n (%)	250/573 (30)	18/302 (6)
Smoking, n *(%)*		
Yes	210 (32)	52 (16)
Ex	94 (15)	60 (19)
No	344 (53)	204 (65)
ANCA, pos/neg (%)	36/264 (12)	83/114 (42)
ASCA, pos/neg (%)	173/82 (68)	17/110 (13)

ANCA, antineutrophil cytoplasmic antibody; ASCA, anti-*Saccharomyces cerevisiae* antibody; CD, Crohn's disease; GI, gastrointestinal; UC, ulcerative colitis.
Note that some data are missing.

with controls, the association was still present (**Table 2**). Similarly, the genotype frequencies remained significantly different for both variants after stratifying the cohort in adult- (rs1000113, $P=1\times10^{-4}$; rs4958847, $P=1\times10^{-3}$) and pediatric-onset CD (rs1000113, $P=1\times10^{-4}$; rs4958847, $P=9.6\times10^{-3}$). Armitage's trend test for rs1000113 and rs4958847 in CD patients was 0.00003 and 0.00078, respectively. The GT haplotype frequency was significantly increased in CD patients (16%) compared with controls (10.7%) ($P<0.01$, data not shown), but not in UC patients.

A combined genotype–phenotype analysis showed a significant association of the *IRGM* rs4958847 polymorphism with fistulizing behavior ($P=0.0245$, OR = 1.48, CI = 1.05–2.09) and perianal fistulas ($P=0.0103$, OR = 1.61, CI = 1.12–2.33) when carriers of the GG/AG genotype were compared with those carrying the AA wild-type genotype (**Table 3**). However, after stratifying the CD population with respect to age at diagnosis, the association persisted only in the adult-onset subset of patients (fistulizing: $P=0.013$; perianal: $P=0.05$), probably because of the small size of the pediatric sample. A similar trend was found for the rs1000113 polymorphism, which did not reach statistical significance. At logistic regression analysis, using a custom/stepwise model (forward entry), after correction for all other covariates (age at diagnosis, disease localization, duration of follow-up, smoking status, etc.), the correlation with internal ($P=0.037$, OR = 1.54, CI = 1.02–2.31) and perianal ($P=0.045$, OR = 1.55, CI = 1.01–2.38) fistulizing disease was confirmed, without a dose–effect relationship. Conversely, no significant statistical association with UC subphenotypes was found (data not shown). To test for statistical interaction, the individual contributions of the three common CARD15 polymorphisms (at least 1 variant), IRGM rs1000113 (TT/CT vs CC) and rs4958847 (GG/AG vs AA) SNPs were analyzed by logistic regression analysis in 823 CD and 578 controls. No evidence for statistical interaction ($P>0.05$) was observed (data not shown), thus implying that each gene contributes independently to disease risk.

DISCUSSION

The autophagy-inducing *IRGM* gene has recently been identified as a potential susceptibility factor for Crohn's disease. In this study, we report an independent replication in the Italian population of the findings of WTCCC (4) and Parkes *et al.* (5) do so in the case of British CD cohorts. We confirmed the identification of the rs1000113 and rs4958847 variants as independent major CD susceptibility loci both in early-onset ($P=4\times10^{-4}$; $P=8\times10^{-3}$, respectively) and adult-onset ($P=2\times10^{-4}$; $P=2.5\times10^{-3}$) patients, compared with healthy controls. It is relevant that the strength of the association in children was almost identical to that detected in adults, with a comparable OR for both *IRGM* variants (rs1000113: $OR_{ped}=1.69$; $OR_{adult}=1.59$; rs4958847: $OR_{ped}=1.41$; $OR_{adult}=1.39$). In contrast, similar to other recent reports (7,8), no association with UC was found.

Furthermore, in our cohort of CD patients, *IRGM* variations seem to influence both disease susceptibility and behavior. In fact, in contrast with the British study (5), a genotypephenotype association of the IRGM rs4958847 polymorphism with fistulizing behavior ($P=0.0245$) and perianal

Table 2. Genotype and allele frequencies of *IRGM* polymorphisms in CD and UC patients

	Genotypes (*n*, %)									Alleles (*n*, %)						
rs1000113	**TT**		**CT**		**CC**		**Total**	***P* value (TT CT vs. CC)**	**OR 95% CI**	**T**		**C**		**Total**	***P* value (T vs. C)**	**OR 95% CI**
CD	19	2%	232	28%	572	70%	823	1×10^{-5}	1.75 (1.36–2.25)	270	16%	1376	84%	1646	3×10^{-5}	1.62 (1.29–2.03)
Adult	13	2%	154	28%	391	70%	558	1×10^{-4}	1.70 (1.30–2.23)	180	16%	936	84%	1116	2×10^{-4}	1.59 (1.24–2.03)
Pediatric	6	2%	78	29%	181	68%	265	2.3×10^{-4}	1.85 (1.33–2.57)	90	17%	440	83%	530	4×10^{-4}	1.69 (1.26–2.26)
UC	5	1%	80	23%	266	76%	351		NS	90	13%	612	87%	702		NS
Adult	2	1%	47	21%	172	78%	221		NS	51	12%	391	88%	442		NS
Pediatric	3	2%	33	25%	94	72%	130		NS	39	15%	221	85%	260		NS
Controls	9	2%	107	19%	462	80%	578		—	125	11%	1031	89%	1156		—
rs4958847	**GG**		**AG**		**AA**			**(GG AG vs. AA)**		**G**		**A**			**(G vs. A)**	
CD	36	4%	286	35%	484	60%	806	5×10^{-4}	1.50 (1.19–1.88)	358	22%	1254	78%	1612	8×10^{-4}	1.39 (1.15–1.69)
Adult	23	4%	194	36%	326	60%	543	1×10^{-3}	1.50 (1.17–1.92)	240	22%	846	78%	1086	2.5×10^{-3}	1.39 (1.12–1.71)
Pediatric	13	5%	92	35%	158	60%	263	9.6×10^{-3}	1.49 (1.10–2.03)	118	22%	408	78%	526	8×10^{-3}	1.41 (1.09–1.83)
UC	11	3%	102	29%	235	68%	348		NS	124	18%	572	82%	696		NS
Adult	6	3%	66	30%	149	67%	221		NS	78	18%	364	82%	442		NS
Pediatric	5	4%	36	28%	86	68%	127		NS	46	18%	208	82%	254		NS
Controls	18	3%	155	28%	389	69%	562		—	191	17%	933	83%	1124		—

CD, Crohn's disease; UC, ulcerative colitis; NS, not significant.

Table 3. Genotype frequencies of *IRGM* rs4958847 and rs1000113 in CD patients stratified by subphenotypes

	rs4958847						rs1000113					
	AA (*n*=484)		AG (*n*=286)		GG (*n*=36)		CC (*n*=572)		CT (*n*=232)		TT (*n*=9)	
Age at diagnosis (years)												
≤16 (A1)	147	60%	85	35%	11	5%	169	69%	71	29%	5	2%
17–40 (A2)	250	59%	154	37%	17	4%	300	69%	126	29%	9	2%
>40 (A3)	87	61%	47	33%	8	6%	103	72%	35	25%	5	3%
Gender												
Male	277	61%	159	35%	21	4%	327	70%	131	28%	10	2%
Female	207	59%	127	36%	15	5%	245	69%	101	28%	9	3%
Localization												
Ileum (L1)	166	58%	107	38%	12	4%	195	68%	86	30%	7	2%
Colon (L2)	121	61%	69	35%	8	4%	143	70%	56	28%	5	2%
Ileocolon (L3)	191	60%	109	35%	16	5%	228	70%	89	28%	7	2%
Upper GI (L4)	35	66%	16	30%	2	4%	43	77%	11	20%	2	3%
Disease type												
Inflammatory (B1)	246	63%	133	34%	14	4%	285	70%	110	27%	10	3%
Stricturing (B2)	132	62%	70	33%	11	5%	152	71%	57	26%	6	3%
Penetrating (B3)	106*	53%	83	42%	11	5%	135	67%	65	32%	3	1%
Perianal disease												
Yes	70**	51%	63	45%	6	4%	91	65%	47	34%	2	1%
No	414	62%	223	33%	30	5%	481	70%	185	27%	17	3%
Familial												
Yes	41	61%	25	37%	1	2%	49	71%	19	28%	1	1%
No	443	60%	261	35%	35	5%	523	70%	213	28%	18	2%
Resective surgery												
Yes	142	58%	99	40%	6	2%	173	69%	73	29%	4	2%
No	342	61%	187	34%	30	5%	399	70%	159	28%	15	2%
Smoking history												
Yes	122	60%	75	37%	7	3%	146	70%	60	29%	4	2%
Ex	47	51%	41	45%	4	4%	58	62%	35	37%	1	1%
No	204	61%	1 17	35%	15	4%	241	70%	95	28%	8	2%
ANCA												
Positive	24	67%	9	25%	3	8%	25	69%	11	31%	0	0%
Negative	150	59%	92	36%	12	5%	182	69%	77	29%	5	2%
ASCA												
Positive	101	61%	59	35%	6	4%	125	72%	45	26%	3	2%
Negative	44	56%	32	41%	2	3%	51	62%	30	37%	1	1%

ANCA, antineutrophil cytoplasmic antibody; ASCA, anti-*Saccharomyces cerevisiae* antibody; CD, Crohn's disease; GI, gastrointestinal; UC, ulcerative colitis.
**P*=0.0245, OR 1.48, IC 95% (1.05–2.09)—B3 vs. B1 (GG AG vs. AA) (univariate analysis); *P* =0.037, OR 1.54, 95% CI (1.02–2.31) (logistic regression). ***P*=0.0103, OR 1.61, 95% CI (1.12–2.33)—(GG AG vs. AA) (univariate analysis); *P*=0.045, OR 1.55, 95% CI (1.01–2.38) (logistic regression).

fistulas ($P=0.0103$) was shown at logistic regression after correction for confounders. However, after stratifying the CD population with respect to age at diagnosis, the association persisted only in the adult subset of patients (fistulizing: $P=0.013$; perianal fistulas: $P=0.05$), although the possibility that our study may have been underpowered in the pediatric cohort cannot be ruled out. The reason for discrepancy with British cohorts of the geno/pheno association might rely on patients' selection and phenotype definition; the fistulizing behavior was more frequent in our series, whereas perianal localization (not only fistulas) was reported more frequently in British patients.

Finally, we failed to show a significant interaction between CARD15 and IRGM variants in both the pediatric and the adult cohorts, but we cannot exclude the possibility that an analysis of larger sample sizes might unveil such an association and gene–gene interactions. Indeed, our study reaffirmed that the *IRGM* gene is a risk factor for CD, even in the absence of CARD15 alterations.

The exact function of *IRGM* in CD etiology and pathogenesis is unknown. *IRGM* has a pivotal function in the autophagy pathway. *IRGM1*-deficient mice have a reduced defense against intracellular pathogens such as *Toxoplasma gondii* and Listeria monocytogenes (13); similarly, the human ortholog IRGM acts as its functional equivalent in the autophagy pathway and participates in conferring resistance against mycobacterial infections (14). Unlike the IRG family in mice, the human IRGM is not responsive to IFN-γ and is constitutively expressed (15). Moreover, autophagy has an important function in the clearance of apoptotic bodies (16), and the failure of this mechanism might contribute to persistent inflammation in CD. However, sequencing of this gene by Parkes *et al.* (5) did not identify any causal amino acid changes. It therefore remains unclear whether the *IRGM* gene itself is associated with disease susceptibility and whether efforts should aim at identifying the causative variant(s) in this region or in strong LD with associated SNPs.

An intriguing issue is genetic predisposition and age at diagnosis. One compelling hypothesis suggests that pediatric-onset IBD is more likely to be influenced by genetics compared with late-onset disease, as there might be less time for environmental modifiers to influence disease susceptibility or behavior (17,18). However, none of the gene variants disclosed so far, including *IRGM*, explain early-onset disease and the specific phenotypic differences displayed in children with IBD (9,19). This finding indicates that the possible interaction between the environment and the variants so far identified might not be timedependent. The use of GWA studies in an exclusive early-onset IBD population will probably enable further identification of genes of relevance in childhood-onset disease.

We have replicated in an Italian population of IBD patients *IRGM* variations influencing susceptibility to Crohn's disease, but not UC, in both early- and adult-onset. Furthermore, we have also shown influence on specific clinical features (fistulizing disease) but not on age at diagnosis. These data complement the results of the GWA studies on IBD and might have clinical implications (i.e., different response to antibiotic therapy).

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CONFLICT OF INTEREST

Guarantor of the article: V. Annese, MD.

Specific author contributions: Anna Latiano: conception and design, supervising the project and writing of the manuscript; Orazio Palmieri: supervising genotyping, data handling and data management; Salvatore Cucchiara, Massimo Castro, Renata D'Incà, Graziella Guariso, Bruno Dallapiccola, Angelo Andriulli: collecting patient DNA and clinical information of the IBD cohort; Maria Rosa Valvano: statistical analysis; Tiziana Latiano: IRGM and CARD15 genotyping; Vito Annese: supervising manuscript preparation and patient recruitment.

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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ An association of the *IRGM* (immunity-related GTPase protein type M) variants (rs1000113 and rs4958847) with Crohn's disease (CD) has been shown and replicated in British cohorts.
- ✓ *IRGM* has a pivotal function in the autophagy pathway.
- ✓ The *IRGM* polymorphism does not seem to be associated with ulcerative colitis (UC).

WHAT IS NEW HERE

- ✓ We replicated the association between the variants in the *IRGM* gene in both early- and adult-onset CD in the Italian population.
- ✓ We confirmed that *IRGM* is not associated with UC.
- ✓ A genotype-phenotype association of the IRGM rs4958847 polymorphism with fistulizing behavior and perianal fistulae has been shown in CD.
- ✓ Associations between *IRGM* and CD are likely independent of the CARD15 gene.

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