

CLINICAL RESEARCH

Clinical significance of NOD2/CARD15 and Toll-like receptor 4 gene single nucleotide polymorphisms in inflammatory bowel disease

Luciana Rigoli, Claudio Romano, Rosario Alberto Caruso, Maria A Lo Presti, Chiara Di Bella, Vincenzo Procopio, Giuseppina Lo Giudice, Maria Amorini, Giuseppe Costantino, Maria D Sergi, Caterina Cuppari, Giovanna Elisa Calabrò, Romina Gallizzi, Carmelo Damiano Salpietro, Walter Fries

Luciana Rigoli, Claudio Romano, Chiara Di Bella, Vincenzo Procopio, Giuseppina Lo Giudice, Maria Amorini, Caterina Cuppari, Giovanna Elisa Calabrò, Romina Gallizzi, Carmelo Damiano Salpietro, Dipartimento di Scienze Pediatriche Mediche e Chirurgiche, Università di Messina, Messina 98125, Italy

Rosario Alberto Caruso, Dipartimento di Patologia Umana, Università di Messina, Messina 98125, Italy

Maria A Lo Presti, Giuseppe Costantino, Maria D Sergi, Walter Fries, Dipartimento di Medicina Interna e Terapia Medica, Università di Messina, Messina 98125, Italy

Author contributions: Rigoli L, Romano C, Caruso RA and Fries W contributed equally to this work; Rigoli L, Romano C and Fries W designed research; Lo Presti MA, Di Bella C, Procopio V, Lo Giudice G, Amorini M, Costantino G, Sergi MD, Cuppari C, Calabrò GE, Gallizzi R performed research; Rigoli L and Salpietro CD contributed new reagents/analytic tools; Rigoli L, Romano C, Caruso RA and Fries W analyzed data; and Rigoli L wrote the paper.

Correspondence to: Luciana Rigoli, Dipartimento di Scienze Pediatriche Mediche e Chirurgiche, UO di Genetica ed Immunologia Pediatrica, pad. NI, Policlinico Universitario, Messina 98125, Italy. luciana.rigoli@unime.it

Telephone: +39-90-2212120 Fax: +39-90-2213788

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Abstract

AIM: To evaluate the role of genetic factors in the pathogenesis of Crohn's disease (CD) and ulcerative colitis (UC), we investigated the single nucleotide polymorphisms (SNPs) of NOD2/CARD15 (R702W, G908R and L1007finsC), and Toll-like receptor 4 (TLR4) genes (D299G and T399I) in a selected inflammatory bowel disease (IBD) population coming from Southern Italy.

METHODS: Allele and genotype frequencies of NOD2/CARD15 (R702W, G908R and L1007finsC) and TLR4 (D299G and T399I) SNPs were examined in 133 CD patients, in 45 UC patients, and in 103 healthy controls. A genotype-phenotype correlation was performed.

RESULTS: NOD2/CARD15 R702W mutation was significantly more frequent in CD (9.8%) than in controls (2.4%, $P = 0.001$) and in UC (2.3%, $P = 0.03$). No sig-

nificant difference was found between UC patients and control group ($P > 0.05$). In CD and UC patients, no significant association with G908R variant was found. L1007finsC SNP showed an association with CD (9.8%) compared with controls (2.9%, $P = 0.002$) and UC patients (2.3%, $P = 0.01$). Moreover, in CD patients, G908R and L1007finsC mutations were significantly associated with different phenotypes compared to CD wild-type patients. No association of IBD with the TLR4 SNPs was found in either cohort (allele frequencies: D299G-controls 3.9%, CD 3.7%, UC 3.4%, $P > 0.05$; T399I-controls 2.9%, CD 3.0%, UC 3.4%, $P > 0.05$).

CONCLUSION: These findings confirm that, in our IBD patients selected from Southern Italy, the NOD2/CARD15, but not TLR4 SNPs, are associated with increased risk of CD.

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Key words: Crohn's disease; Ulcerative colitis; NOD2/CARD15 gene; Toll-like receptor 4 gene; Single nucleotide polymorphisms

Peer reviewers: Nanne KH de Boer, MD, PhD, Department of Gastroenterology and Hepatology, VU University Medical Center, PO Box 7057, Amsterdam 1007 MB, The Netherlands; Elias A Kouroumalis, Professor, Department of Gastroenterology, University of Crete, Medical School, Department of Gastroenterology, University Hospital, PO Box 1352, Heraklion, Crete 71110, Greece

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are

idiopathic chronic inflammatory bowel disease (IBD). The molecular basis of their pathogenesis is not completely clear, but contributing factors may include persistent bacterial infection, a defective mucosal barrier, and an imbalance in the regulation of the intestinal immune response^[1].

Animal models of IBD support the concept that genetic factors, environmental triggers, and immune dysregulation may have a potential role in developing uncontrolled intestinal inflammation that determines the typical endoscopic manifestations and mucosal lesions compatible with CD or UC^[1-4].

Over the last decade, multiple genome-wide linkage searches have delineated numerous genomic regions containing putative IBD risk factors. In studies performed on unselected populations, an average of 8%-10% of CD patients and 6%-8% of UC patients have at least one relative affected by some type of IBD. However, these values vary from study to study and percentages of CD familial aggregation of less than 4% and more than 20% have been reported^[2].

Moreover, studies on twins demonstrate a greater genetic influence for CD compared with UC; combined study concordance rates for monozygotic twins are 36% for CD and 16% for UC^[5].

Recently, an association between CD and mutations in the NOD2/CARD15 gene located on chromosome 16q12 (IBD1) has been reported. NOD2/CARD15 acts as an intracellular receptor in monocytes for bacterial components, triggering activation of NF κ B and thus leading to subsequent activation of the inflammatory response. Within the NOD2/CARD15 gene, three mutations have been identified as being associated with CD: two missense mutations (Arg702Trp in exon 4 and Gly908Arg in exon 8) and an insertion mutation of a C in exon 11 (1007finsC), the latter resulting in a truncated NOD2/CARD15 protein. These NOD2/CARD15 variants alter the structure of either the leucine-rich repeat (LRR) domain of the protein or the adjacent region. The activating function of nuclear factor NF κ B is regulated by the carboxy-terminal LRR domain, which has an inhibitory role and also acts as an intracellular receptor for components of microbial pathogens. These observations suggest that the NOD2/CARD15 gene can confer susceptibility to CD by altering the recognition of these components and/or by over-activating NF κ B in monocytes^[6-9]. The question arises as to how NOD2/CARD15 mutations and impaired NF κ B activation can confer susceptibility to CD. It has been suggested that the answer most likely lies within the leucine-rich repeats (LRR) of the NOD2/CARD15 gene and the family of Toll-like receptors (TLRs). These receptors, a family composed of at least 10 mammalian homologs of *Drosophila* Toll, serve as pattern recognition receptors for various microbial products and can mediate production of proinflammatory cytokines^[10]. Toll-like receptor 4 (TLR4) functions as the main receptor for lipopolysaccharide (LPS) of Gram-negative bacteria^[11]. After the recognition of pathogen-associated molecular patterns, the TLRs activate signal transduction pathways

of the innate immune response genes including inflammatory cytokines and the NF κ B signalling pathway^[12]. TLR4 is expressed in macrophages, dendritic cells, endothelial cells, and, less abundantly, in intestinal epithelial cells, which are partly tolerant to LPS, thereby preventing an exaggerated immune response caused by the large number of bacteria in the intestinal lumen^[13].

Mutations of the TLR4 gene are known to abolish responses to endotoxin in mice, as shown for the mice strains C3H/HeJ and C57BL/10SeCr^[14]. Therefore, the ability to recognize bacterial wall products and to activate proinflammatory mechanisms by TLRs may be of great importance for immune reactions in the intestinal mucosa. The recently characterised D299G and T399I single nucleotide polymorphisms (SNPs) of TLR4 gene are probably associated with impaired LPS signalling and increased susceptibility to Gram negative infections^[15].

In this study, we investigated the frequencies of the three NOD2/CARD15 gene mutations (Arg702Trp, Gly908Arg and 1007finsC) and of the TLR4 gene D299G and T399I SNPs in a group of 178 Italian adult patients affected by IBD: 133 patients with CD and 45 with UC. The allele frequencies of the NOD2/CARD15 and TLR4 genes were evaluated, and a detailed genotype-phenotype correlation was performed.

MATERIALS AND METHODS

Study population

The study population was comprised of 133 patients with CD (70 males, 63 females; mean age, 43.5 \pm 10.7 years), 45 with UC (27 males, 18 females; mean age, 43.2 \pm 11.0 years) and 103 healthy, unrelated controls (68 males, 35 females; mean age, 46.6 \pm 9.8 years). Patients were consecutively recruited from Department of Paediatrics and Department of Medicine, University Hospital of Messina, Italy. All patients were from Eastern Sicily and Calabria (Southern Italy). Informed consent was obtained from each participant.

Diagnosis of CD and UC was established according to accepted clinical, endoscopic radiological, and histological criteria^[16]. A detailed clinical questionnaire concerning different features of the disease was employed. The Vienna classification was used for CD phenotypes^[17], while localization was defined based on the largest extent of the disease, according to X-ray, endoscopy, or surgical reports.

The following data of patients with CD and UC were collected: age, age at diagnosis, gender, familial or spontaneous disease (familial disease was considered if one first or second-degree relative had IBD), disease localization, disease behaviour, extraintestinal manifestations (arthritis, affections of eyes or skin, primary sclerosing cholangitis), type and site of surgery. Disease localization was defined as the maximum extent of digestive tract involvement at the latest follow-up.

Patients were eligible if IBD was confirmed, and they had undergone full colonoscopy with biopsy and/or surgical resection.

Table 1 Primers sequences and restriction enzymes used for genotyping TLR4 and NOD2/CARD15

Gene locus	SNPs		Sequence	Restriction enzyme
NOD2/CARD15	R702W	For	5' TTCAGATCACAGCAGCCTTC 3'	<i>MspI</i>
		Rev	5' CCCACACTGCAAAATGTCAAC 3'	
	G908R	For	5' AGCCACTGAAAACCTCTTGG 3'	<i>HhaI</i>
		Rev	5' TCTTCACCTGATCTCCCAA 3'	
	L1007finsC	For	5' CCTGCAGTCTCTTAACTGG 3'	<i>NlaIV</i>
		Rev	5' CTTACCAGACTTCCAGGATG 3'	
TLR4	D299G	For	5' TTAGAAATGAAGAAAACCTGGAAAAG 3'	<i>BsaBI</i>
		Rev	5' TTGTCAAACAATTAATAAGTGATTAATA 3'	
	T399I	For	5' GGTGTCTGTTCTCAAAGTGATTTGGGAGAA 3'	<i>HinfI</i>
		Rev	5' CCTGAAGACTGGAGAGTGAGTTAAATGCT 3'	

A group of 103 healthy, unrelated subjects coming from the Sicily and Calabria regions (mainly students, blood donors and hospital employees) were selected as controls.

DNA extraction

Genomic DNA was isolated from 1 mL of peripheral blood anticoagulated with EDTA as previously described^[18]. DNA samples of the patients and control subjects were analyzed for the variants of NOD2/CARD15 and TLR4 genes by melting curve analysis.

Genotyping of the NOD2/CARD15 mutations

To detect the R702W, G908R, and L1007finsC mutations, we performed a polymerase chain reaction (PCR) using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life Sciences Division, UK), 400 $\mu\text{mol/L}$ dNTPs, and 0.1 $\mu\text{mol/L}$ of each primer in a total volume of 25 μL . After an initial denaturation for 5 min at 95°C, PCR was performed by 35 cycles of denaturing at 95°C for 30 s, annealing at 65°C for 40 s, primer extension at 72°C for 30 s. The final extension was performed at 72°C for 7 min. PCR reactions were carried out using a GeneAmp PCR system 2700 (Applied Biosystem, CA, USA).

Genotyping of each SNP was performed by enzymatic digestion at 37°C, overnight. After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA).

The specific primers PCR and the restriction enzymes (New England Biolabs, Ipswich, MA) for each SNP are given in Table 1.

Wild-type/mutant genotype was confirmed by automatic sequencing using the ABI-PRISM Big Dye™ Terminator v. 3.0 Cycle sequencing Ready Reaction Kit (Applied Biosystems, CA, USA). The sequencing products were purified using DyeEx Spin Kits (Qiagen) and visualized on an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems, CA, USA).

Genotyping of the TLR4 polymorphisms

The two D299G and T399I SNPs of the TLR4 gene were determined by PCR-RFLP.

We performed PCR using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life Sciences Division, UK), 400 $\mu\text{mol/L}$ dNTPs, and 0.1 $\mu\text{mol/L}$ of each primer in

a total volume of 25 μL .

For D299G SNP, cycle conditions were an initial denaturation for 5 min at 95°C, followed by 32 cycles of denaturing at 95°C for 30 s, annealing at 51°C for 30 s, primer extension at 72°C for 30 s, followed by a final extension at 72°C for 7 min. For T399I SNP, cycle conditions were an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturing at 95°C for 45 s, annealing at 55°C for 30 s, primer extension at 72°C for 45 s, followed by a final extension at 72°C for 7 min.

The specific primers PCR and the restriction enzymes for each SNP are given in Table 1.

The amplified samples of TLR4 gene D299G and T399I SNPs were digested at 37°C, overnight, with the *BsaBI* and *HinfI* restriction enzymes (New England Biolabs, Ipswich, MA, USA), respectively.

After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA).

As previously described here, the results of enzymatic digestion were confirmed by DNA sequence analysis of representative samples of each SNP.

Statistical analysis

Data are given as mean \pm SD. Allele and genotypes frequencies in patients and in controls were compared by χ^2 test or Fisher exact test, when an expected value was < 0.5 ; *P* values were considered significant at a level of < 0.05 . Odds ratio (OR) and *P* values were calculated using a standard package (StataCorp. Stata Statistical Software: Release 8.0 College Station, TX: Stata Corporation 2001).

Allele frequencies were tested for the Hardy-Weinberg equilibrium. Cases and controls were compared using Pearson's χ^2 test.

RESULTS

Allele frequencies in IBD patients NOD2/CARD15 gene SNPs

In CD patients, the frequency of R702W mutation was significantly higher (9.8%) than in controls (2.4%, *P* = 0.001; OR, 4.09; 95% CI, 1.5-11.9) and in UC (2.3%, *P* = 0.03; OR, 4.49; 95% CI, 1.02-19.8; Table 2). No significant difference of the G908R mutation allele frequency was found

Table 2 NOD2/CARD15 and TLR4 SNPs allele frequencies of CD patients *vs* control group and UC patients

Polymorphisms of NOD2/CARD15 and TLR4 genes	CD (n = 133)	Allele frequency (%)	Controls (n = 103)	Allele frequency (%)	¹ P	OR (95% CI)	UC (n = 45)	Allele frequency (%)	² P	OR (95% CI)
<i>R702W</i>										
Wild-type	107 (80.4%)	9.8	98 (95.1%)	2.4	0.001	4.09 (1.5-11.9)	43 (95.5%)	2.3	0.03	4.49 (1.02-19.8)
Heterozygous	26 (19.6%)		5 (4.9%)				2 (4.5%)			
<i>G908R</i>										
Wild-type	120 (90.2%)	4.5	94 (91.2%)	4.3	NS	1.01 (0.3-3.5)	41 (91.1%)	4.4	NS	0.90 (0.28-2.92)
Heterozygous	13 (9.8%)		9 (8.8%)				4 (8.9%)			
<i>L1007finsC</i>										
Wild-type	107 (80.4%)	9.8	97 (94.1%)	2.9	0.002	3.92 (1.55-9.95)	43 (95.5%)	2.3	0.01	5.22 (1.19-22.98)
Heterozygous	26 (19.6%)		6 (5.9%)				2 (4.5%)			
<i>D299G</i>										
Wild-type	123 (92.5%)	3.7	95 (92.2%)	3.9%	NS	0.96 (0.37-2.54)	42 (93.3%)	3.4	NS	0.87 (0.23-3.35)
Heterozygous	10 (7.5%)		8 (7.8%)				3 (6.7%)			
<i>T399I</i>										
Wild-type	125 (94%)	3.0	97 (94.1%)	2.9%	NS	1.15 (0.28-4.64)	42 (93.3%)	3.4	NS	1.11 (0.28-4.40)
Heterozygous	8 (6.0%)		6 (5.9%)				3 (6.7%)			

No patients homozygous for NOD2/CARD15 gene R702W, G908R and L1007finsC SNPs were found in this study population; No patients homozygous for TLR4 gene D299G and T399I SNPs were found in this study population. ¹CD patients *vs* control group; ²CD patients *vs* UC patients. NS: No significance.

between CD (4.5%) and the control group (4.3%; $P > 0.05$; OR, 1.01; 95% CI, 0.3-3.5), and between CD and UC patients (4.4%, 0.05; OR, 0.90; 95% CI, 0.28-2.92; Table 2).

The frequency of the frameshift mutation L1007finsC was significantly higher in CD patients (9.8%) compared with controls (2.9%, $P = 0.002$; OR, 3.92; 95% CI, 1.55-9.95) or patients with UC (2.3%, $P = 0.01$; OR, 5.22; 95% CI, 1.19-22.98; Table 2).

In UC patients, the allele frequencies of the R702W, G908R, and 1007finsC mutations were not significantly different from the control group (R702W: $P > 0.05$; OR, 0.91; 95% CI, 0.17-4.88 and G908R: $P > 0.05$; OR, 1.01; 95% CI, 0.3-3.5 and L1007finsC: $P > 0.05$; OR, 0.75; 95% CI, 0.15-3.88).

No homozygous carriers of the three NOD2/CARD15 mutations were found in the study and control populations.

The NOD2/CARD15 allele frequencies were in Hardy-Weinberg equilibrium in all patients and in control subjects.

TLR4 gene SNPs

The results of the genotype analyses in 133 patients with CD, in 45 patients with UC and in 103 control individuals, with regard to the TLR4 D299G and T399I SNPs are shown in Table 2.

In CD patients, the frequency of the D299G SNP (3.7%) was not significantly different from the controls (3.9%, $P > 0.05$; OR, 0.96; 95% CI, 0.37-2.54) or from UC patients (3.4%, $P > 0.05$; OR, 0.87; 95% CI, 0.23-3.35; Table 2). The T399I SNP allele frequency was not significantly different between CD patients (3.0%) and control group (2.9%, $P > 0.05$; OR, 1.15; 95% CI, 0.28-4.64); or between CD (3.0%) and UC patients (3.4%, $P > 0.05$; OR 1.11, 95% CI 0.28-4.40; Table 2). No significant difference was found between UC patients and control group as regards the D299G SNP ($P > 0.05$;

OR, 0.84; 95% CI, 0.21-3.36) or the T399I SNP ($P > 0.05$; OR, 1.15; 95% CI, 0.28-4.64).

No homozygous carriers of the two SNPs were found in the study and control populations.

The TLR4 allele frequencies were in Hardy-Weinberg equilibrium in all patients and in the control group.

Genotype-phenotype correlations

When the contribution of each SNP of the NOD2/CARD15 gene was investigated, the major support to the genotype-phenotype correlation could be ascribed to the G908R and the L1007finsC alleles (Table 3). In particular, in CD patients, the occurrence of one risk allele of G908R was associated with stenosing phenotype ($P = 0.03$) and resective surgery ($P = 0.003$).

An increased frequency of ileal localization (80.7%, $P = 0.001$) and resective surgery (53.9%, $P = 0.01$) was found in CD L1007finsC heterozygotes compared with CD patients with wild-type NOD2/CARD15 gene (ileum 36.8% and resective surgery 26.4%, respectively).

Moreover, the clinical features of all CD patients were analysed with respect to the presence of one or two risk alleles of each SNP (heterozygous or compound heterozygous) of any NOD2/CARD15 variants (Table 3).

By univariate analysis, the presence of one risk allele was significantly associated with ileal localization ($P = 0.04$) and resective surgery ($P = 0.03$). These significant associations increased in the compound heterozygotes ($P = 0.03$ and $P < 0.0001$, respectively). Moreover, the presence of two risk alleles was significantly associated with stenosing disease ($P = 0.02$, Table 3).

In CD patients, TLR4 D299G and T399I SNPs were not found to be associated with age at diagnosis, sex, localization, disease type, resective surgery and extraintestinal manifestations.

Similarly, in UC patients, these TLR4 gene SNPs were not associated with any studied clinicopathological parameter.

Table 3 Genotype-phenotype correlations in CD patients

Total CD patients (n = 133)	CARD15 no risk alleles (n = 68, 51.1%)	R702W 1 risk allele (n = 26, 19.6%)	G908R 1 risk allele (n = 13, 9.7%)	L1007finsC 1 risk allele (n = 26, 19.6%)	CARD15 at least 1 risk allele (n = 65, 48.8%)	CARD15 compound heterozygous (n = 48, 36.1%)
Age (mean ± SD)	41.5 ± 11.2	41.2 ± 11.9	43.02 ± 10.9	42.0 ± 12.8	42.3 ± 12.1	42.7 ± 11.9
Sex (m/f, 70/63)	32/36	13/13	8/5	10/16	30/35	25/23
Localization (%)						
Ileum (n = 61)	25 (36.8)	11 (42.3)	4 (30.8)	21 (80.7)	38 (58.5)	30 (62.5)
Ileo-colon (n = 39)	22 (32.3)	10 (38.5)	4 (30.8)	3 (11.5)	15 (23.0)	10 (20.8)
Colon (n = 30)	18 (26.5)	5 (19.5)	5 (38.4)	2 (7.8)	12 (18.5)	8 (16.7)
Upper GI (n = 3)	3 (4.4%)				0	0
P		> 0.05 ¹	> 0.05 ¹	0.001 ¹	0.04 ²	0.03 ³
Disease type (%)						
Inflammatory (n = 37)	14 (20.6)	7 (27.0)	6 (46.0)	10 (38.5)	23 (35.4)	8 (16.6)
Stenosing (n = 56)	32 (47.0)	11 (42.3)	7 (54.0)	6 (23.0)	24 (37.0)	34 (70.8)
Fistulizing (n = 40)	22 (32.4)	8 (30.7)	0	10 (38.5)	18 (27.6)	6 (12.6)
P		> 0.05 ¹	0.03 ¹	> 0.05 ¹	> 0.05 ²	0.02 ³
Resective Surgery (%)	18 (26.4)	9 (34.6)	9 (69.2)	14 (53.9)	32 (49.2)	31 (64.6)
P		> 0.05 ¹	0.003 ¹	0.01 ¹	0.01 ²	0.000 ³
Extraintestinal manifestations (n = 18, %)	10 (55.5)	3 (11.5)	1 (7.7)	4 (15.3)	8 (13.3)	3 (6.2)

No patients homozygous for R702W, G908R and L1007finsC SNPs were found in this study population. ¹CD patients no risk allele vs CD patients with risk allele; ²CARD15 at least 1 risk allele vs CD patients no risk allele; ³CARD15 compound heterozygous vs CD patients no risk allele.

DISCUSSION

In our study, we investigated the prevalence of NOD2/CARD15 and TLR4 genetic variants in CD and UC patients. Moreover, we compared the results with clinical phenotype characteristics of IBD of our patients to identify a possible genotype-phenotype association.

There are several controversial data about the role of the SNPs of the NOD2/CARD15 (R702W, G908R and L1007finsC), and TLR4 genes (D299G and T399I) in the pathogenesis of CD. Indeed, there are significant phenotypic differences that exist among populations.

The NOD2/CARD15 mutations are absent in Asian CD populations and controls^[19-21]. In this case, the findings indicate that the NOD2/CARD15 is not a major contributor to CD susceptibility in the Japanese population. Similar data have been found in Turkish patients with IBD^[22].

The highest recorded frequencies are reported in a small study of 55 paediatric patients in Europe with two thirds of the patients having at least one NOD2/CARD15 mutation^[23]. Within Europe, there is evidence of a north-south gradient with lower allele frequencies in the Celtic and Scandinavian countries compared to Southern Europe^[23].

To our knowledge, this is the first study in a large series of sporadic IBD patients coming from Eastern Sicily and Calabria. Indeed, previous reports regarded a Sicilian, small town population in which a familial study was performed^[24]. Other studies have examined a group of sporadic Sicilian IBD patients, but the number of cases was smaller than in our study^[25].

In our study, the reported rates of 48.8% of patients carrying at least one NOD2/CARD15 mutation in CD and 19.4% in controls are consistent with previously reported rates of 30%-50% in CD and 7%-20% in controls from other European regions^[26-31]. Moreover, 36.1% had two mutations (compound heterozygotes). Recently,

Renda *et al* examined a group of 182 CD patients coming from Western Sicily and they found that 56 patients (30%) had at least one mutation of the NOD2/CARD15 gene^[32]. This percentage was lower in respect to our data (48.8%). This difference may be ascribed to a different ethnic background. Indeed, the patients of our study coming both Eastern Sicily and Calabria. Today, populations genetically similar to that of the Northern Italy (as well as of the Northern Africa) are present in the Eastern Sicily. This heterogeneous population, during the middle age, might explain the genetic differences in the patient CD samples of the Eastern in respect to Western Sicily^[33].

The allele frequencies of the R702W (9.8%) and L1007finsC (9.8%) mutations were significantly higher in CD patients compared to UC patients and controls, whereas the frequency of the G908R (4.5%) mutation was similar in CD and UC patients, and in the control group. Collectively, our study confirmed previous studies, which reported increased mutation carrier frequencies of one of the three variant alleles in CD patients compared to UC patients or healthy controls^[2,28,32,34-36].

We also found that different risk alleles might be associated with different clinical features: in particular, the G908R allele seems to be associated with stenosing phenotype and need for surgery. The L1007finsC seems to correlate with ileal localization and resective surgery. These data suggest a more aggressive course of the disease in carriers of risk alleles. The strongest observed effect for ileal location is consistent with the proposed involvement of ileal Paneth cells in the pathophysiology of NOD2/CARD15-mediated disease susceptibility^[30,36,37]. NOD2/CARD15 mutations may, thus, abrogate normal Paneth cell behaviour, explaining preferential involvement of the terminal ileum^[26].

Moreover, in our study, the risk of developing CD with a more aggressive course was increased in compound heterozygotes. In other populations, stronger associations

have been reported for homozygotes and compound heterozygotes than for simple heterozygotes. One copy of the risk alleles confers a 2-4-fold risk for developing CD, whereas double-dose carriage increases the risk by 20-40-fold^[23]. Our study is in agreement with such a gene-dosage effect, although at lower levels.

In our IBD patients, we also examined the allele frequencies of the TLR4 D299G and T399I SNPs and the possible genotype-phenotype correlation.

With regard to the role of the TLR4 gene in the pathogenesis of IBD, several studies have been conducted leading to divergent results^[23]. The allele frequency of the D299G mutation ranges between 8%-13% in CD, 0%-10% in UC and 3%-15% in healthy controls^[38]. This TLR4 SNP was associated with CD and UC in a Belgian study^[39]. This association was replicated in Dutch, German, Australian and Greek populations with CD, and an association with colonic disease has been described^[8,34,35,40]. In one German cohort, an association was demonstrated between UC and the TLR4 T399I SNP^[41]. However, there is substantial heterogeneity between populations, and no association was noted in Scottish CD patients^[30].

In our study, we found no difference in the prevalence of these mutations in our CD and UC patients, and controls. Recently, other studies have failed to find the association of the D299G and T399I SNPs of TLR4 gene^[24,42-45]. In a retrospective German and Hungarian cohort study, patients with CD and UC were genotyped for the presence of the CD14 c.1-260C>T promoter variant and the TLR4 D299G variant. In this study, in German and Hungarian populations, IBD appears to be associated with the CD14 c.1-260C>T promoter variant, but not with the TLR4 D299G variant^[45]. Recent data suggest that neither of these 2 variants is causal, but they may be in linkage disequilibrium with, as yet unidentified, causal variants^[46-48].

We examined also whether the TLR4 D299G and T399I SNPs could be related to particular CD or UC phenotypes. Detailed analysis did not show any association of the examined TLR4 gene variants with either CD or UC patient subgroups. In other studies, in CD patients, an association has been reported between D299G SNP and ileal localization and structuring phenotype^[49]. Our data are similar to those previously reported^[28]. These contrasting results can be ascribed to the different ethnic background of the various IBD populations studied.

Although several studies have been performed, further research is warranted to clarify the role of the genetic variants of NOD2/CARD15 and TLR4, and to investigate whether these genetic risk factors might be confirmed and considered clinically relevant. Indeed, an eventual goal in the genomic study of IBD is to identify these biologically relevant genotype-phenotype associations and to apply them to clinical practice.

bowel disease (IBD) with genetic risk factors. There is evidence that NOD2/CARD15 and Toll-like receptor 4 (TLR4) genes may be involved in their pathogenesis.

Research frontiers

In our study, we found that some single nucleotide polymorphisms (SNPs) of the NOD2/CARD15 gene, but not of the TLR4 gene, were significantly more frequent in CD patients. Therefore, it is possible that the NOD2/CARD15 gene plays an important role in the pathogenesis of IBD.

Innovations and breakthroughs

We evaluated the allele and genotype frequencies of the more frequent SNPs of NOD2/CARD15 and TLR4 genes in a selected IBD population coming from Eastern Sicily and Calabria (Southern Italy), a geographical area for which very few data exist.

Applications

Genotyping of patients with CD could be an important diagnostic tool in clinical practice for identifying high-risk patients with specific diagnostics and therapeutic needs.

Peer review

This study underlines the association of the NOD2/CARD15 genotype with the behaviour and location of CD also in patients coming from Eastern Sicily and Calabria (Italy). Moreover, the CD patients carrying at least one major variant of NOD2/CARD15 gene had an aggressive clinical course. Test strategies with NOD2/CARD15 variations to predict the clinical course of CD could lead to the development of new therapeutic paradigms.

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COMMENTS

Background

Crohn's disease (CD) and ulcerative colitis (UC) are idiopathic inflammatory

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