

## LETTER TO JMG

# Linkage of ulcerative colitis to the pericentromeric region of chromosome 16 in Italian inflammatory bowel disease families is independent of the presence of common *CARD15* mutations

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Crohn's disease (CD) and ulcerative colitis (UC) are the two main clinical subtypes of inflammatory bowel disease (IBD), a common complex disease with a frequency in Western populations of about 1 in 1000.<sup>1</sup> A strong genetic susceptibility to IBD is supported by several epidemiological studies,<sup>2</sup> and the existence of both specific and common susceptibility genes for CD and UC has been postulated.<sup>3</sup>

Several candidate loci for IBD have been identified following whole genome scans in different populations (reviewed in Bonen & Cho<sup>4</sup>). Among these, a locus in the pericentromeric region of chromosome 16 denoted *IBD1*<sup>5</sup> was found consistently in linkage to CD in several independent studies<sup>4</sup> and in a large collaborative study by the IBD International Genetics Consortium.<sup>6</sup> Most of these studies, with few exceptions,<sup>7</sup> reported negative or non-significant linkage of the same locus to UC. Allelic variants of single nucleotide polymorphisms (SNPs) in the *CARD15* gene, located in the *IBD1* region, have since been demonstrated to confer susceptibility to CD, but not to UC in several populations<sup>8-10</sup> (also reviewed in Bonen & Cho<sup>4</sup>).

In a previous study, we reported positive non-parametric linkage (NPL) scores in the *IBD1* region in both our CD and UC families,<sup>11</sup> with the highest scores occurring at markers D16S419 and D16S514, respectively. Marker D16S419 is located approximately 5 cM away from *CARD15*, while D16S514 is located almost 20 cM away on chromosome 16q. To follow up on these results, we typed 10 additional microsatellite markers in the chromosome 16 region of positive linkage in a total of 90 Italian IBD families, and repeated the linkage analysis after excluding 26 families with previously reported *CARD15* SNP susceptibility alleles. The observation of a broad region of positive NPL scores in our UC families suggests that genes conferring susceptibility to this form of IBD may be located in the pericentromeric region of chromosome 16.

## MATERIALS AND METHODS

### Patients and families

Our linkage analysis cohort consists of 90 Italian families with two or more members affected by IBD recruited through a multicentre program of the IG-IBD (Italian Group for Inflammatory Bowel Disease) involving 12 centres throughout Italy. The cohort comprises 41 of 58 families included in our previous analysis of the *IBD1* region<sup>11</sup> for which DNA samples were still available, and 49 new families that had never before been analysed. A total of 108 Italian unrelated healthy controls were also recruited from the same regions of origin of the patients. The local ethics committees of the

### Key points

- Allelic variants of single nucleotide polymorphisms of the *CARD15* gene, located on chromosome 16q, are known to cause susceptibility to Crohn's disease (CD), but not ulcerative colitis (UC). Previous studies suggested that linkage to the *IBD1* region on chromosome 16q was present in both CD and UC families in the Italian population.
- In this study, we tested a group of Italian inflammatory bowel disease families for the three most common *CARD15* single nucleotide polymorphisms known to affect risk of CD (R702W, G908R and L1007fsinsC).
- We show that NPL scores in the *IBD1* region remain positive in those families that tested negative for the presence of the *CARD15* susceptibility alleles, including families of patients with UC.
- Our results suggest that additional genes in the pericentromeric region of chromosome 16 may affect risk of inflammatory bowel disease.

collaborating institutions approved this study, and informed consent was obtained from each participant.

Patients were classified as having CD or UC on the basis of clinical, endoscopic, radiological, and pathological data.<sup>12</sup> In 27 families, all affected members had CD, in 43 families all had UC, and the remaining 20 had individuals affected by both CD and UC (mixed families). The majority of families (72 or 80%) were composed of parents and 2-4 affected siblings, and five families had one affected sibling pair and one additional more distantly related affected member. The other 13 families contained one affected relative pair other than siblings.

### Genotyping

Blood samples were collected into tubes containing sodium citrate and stored at -30°C until extraction. Genomic DNA was then purified from peripheral blood leukocytes according to standard protocols<sup>13</sup> or using a DNA isolation kit (Roche Diagnostics Co., Indianapolis, IN, USA). Eighteen different

**Abbreviations:** CD, Crohn's disease; IBD, inflammatory bowel disease; NPL, non-parametric linkage; SNP, single nucleotide polymorphism; UC, ulcerative colitis

microsatellite markers (D16S409, D16S517, D16S411, D16S3120, D16S416, D16S419, D16S3137, D16S3032, D16S3112, D16S3140, D16S408, D16S3071, D16S3094, D16S3132, D16S3089, D16S514, D16S503, D16S421) from the pericentromeric region of chromosome 16 were used for linkage analysis with PCR reactions and conditions as previously described.<sup>11</sup>

*CARD15* SNPs R702W, G908R and L1007fsinsC were detected using the pyrosequencing approach.<sup>14</sup> Briefly, three fragments of 106, 117 and 104 bp respectively encompassing the three SNPs were PCR amplified using 200 nmol/l of each forward and reverse primer (R702W-F: 5'-TTCCTGGCAGG GCTGTT-3' and R: 5'-biotin-CCAGACACCAGCGGCG-3'; G908R-F: 5'-TAGAGGGGAGGAGGACTGTTAGTT-3 and R: 5'-biotin-CCCCTCGTCACCCACT-3'; L1007fsinsC-F: 5'-biotin-TCTTCTTTCCAGGTTGTCCA-3' and R: 5'-TCCTTACCAGA CTTCCAGGATG-3'). The biotinylated primer in each primer pair allowed subsequent immobilisation to streptavidin coated beads. The amplification was performed in a total volume of 50 µl containing 1× GeneAmp Buffer II, 2.5 mmol/l MgCl<sub>2</sub>, 0.5 mmol/l dNTP, 50 ng genomic DNA and 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and primers (200 nmol/l each). Conditions were 95°C for 10 min followed by 35 cycles at 95°C for 1 min, 62°C for 1 min, 72°C for 1 min and a final step at 72°C for 10 min. PCR products (2 µl) were analysed on a 3% (w/v) agarose gel to verify size and quantity of the amplified fragment. Isolation of single stranded biotinylated templates for pyrosequencing was performed as described previously<sup>15</sup> using solutions provided in the PSQ HS96A Sample Preparation Kit (Pyrosequencing AB, Uppsala, Sweden), streptavidin sepharose HP beads (Amersham Pharmacia Biotech, Uppsala, Sweden) and 96 well microtitre plates. Beads with bound single stranded template were suspended in 12 µl of annealing buffer pH 7.6 (20 mmol/l Tris acetate, 2 mmol/l Mg acetate) and 0.5 µmol/l (6 pmol) of each pyrosequencing primer (R702W-F: 5'-CATCTGAGAAG GCCC-3'; G908R-F: 5'-CTTTTGGCCTTTTCAG -3'; L1007fsins C-F: 5'-TGTCATTCTTTCAAGGG-3') in a PSQ HS 96 plate (Pyrosequencing AB), heated at 94°C for 2 min and then cooled to room temperature to facilitate the annealing of the primer to the template. The plates were placed into the PSQ<sup>TM</sup>HS 96A system following the manufacturer's recommendations. Reactions were performed at 28°C with annealed sequencing primer in addition to manufacturer supplied DNA polymerase, apyrase, luciferase, adenosine triphosphate sulphurylase, adenosine 5'-phosphosulphate and luciferin. Reactions consisted of stepwise elongation of the primer strand by one or more nucleotides upon sequential addition

of the individual dNTPs following the dispensation order entered in the Pyrosequencing SNP Entry software (Pyrosequencing AB). The sequence at each elongation step is inferred by measuring light emission as an indicator of nucleotide incorporation. The resulting sequences were analysed automatically by the SNP Evaluation software v1.1 (Pyrosequencing AB). SNPs were also confirmed by DNA sequence analysis using the ABI BigDye Terminator V3.1 Cycle Sequencing Ready Reaction on a ABI 3100 DNA sequencer (Applied Biosystems) following the manufacturer's recommendations.

### Statistical analysis

*CARD15* SNPs genotype and allele frequencies were calculated from the 90 families' probands and 108 unrelated Italian healthy controls. Genotype and allele frequency distributions in probands and controls were compared by Fisher's exact tests. Relative risks for carriers of one or two *CARD15* SNP variants compared with non-carriers were estimated as odds ratios by means of the Relrisk program [http://linkage.rockefeller.edu/ott/linkutil.htm#relrisk].

Model free multipoint linkage analysis was performed with 18 microsatellite markers using the NPL all statistic from the Genehunter Plus program version 1.2.<sup>16,17</sup> Microsatellite marker allele frequencies were calculated from allele counts in independent individuals in all our families. Map location and genetic distances of microsatellite markers were obtained from the Marshfield Center for Medical Genetics sex average map of chromosome 16 (http://research.marshfieldclinic.org/genetics/). The genetic distance of microsatellite markers from *CARD15* was obtained from the Ensembl Human Contig Map v.12.31.1 (http://www.ensembl.org/), which gave the location of *CARD15* as 25 kb distal to marker D16S3136. In the Marshfield sex average map of chromosome 16, D16S3136 is 0 cM away from marker D16S3120, located at approximately 4 cM from the beginning of the genetic map in our study.

Transmission disequilibrium for microsatellite marker alleles was tested by means of the Fbat computer program.<sup>18</sup>

## RESULTS

### *CARD15* SNP analysis

Genotype and allele frequency distributions at the *CARD15* SNPs R702W, G908R and L1007fsinsC in the IBD families' probands and in 108 unrelated unaffected controls are reported in table 1. Probands from 20 mixed families were assigned to the respective CD or UC category according to their disease diagnosis (8 CD and 12 UC). DNA was missing from one UC proband whose family was included in the

**Table 1** Genotype and allele frequency distributions at three *CARD15* SNPs in IBD probands and controls

SNP	Group	Genotype counts			p‡	Rarer allele frequency	p‡
		1/1	1/2	2/2			
R702W	CD	21	9	0	0.007	0.15	0.01
	UC	46	8	0	0.46	0.07	0.47
	Controls	96	11	1	—	0.06	—
G908R	CD	27	6	0	0.02	0.09	0.02
	UC	53	2	0	1	0.02	0.69
	Controls	103	5	0	—	0.02	—
L1007fsinsC	CD	29	4	2	0.001	0.11	0.001
	UC	52	2	0	1	0.02	1
	Controls	105	3	0	—	0.01	—

1/1, Homozygotes for the common variant; 1/2, heterozygotes; 2/2, homozygotes for the rare variant  
 †p value for difference in genotype frequencies between probands and controls by Fisher's exact test (1/2 and 2/2 grouped together)  
 ‡p value for difference in allele frequencies between probands and controls by Fisher's exact test.

microsatellite linkage analysis. DNA samples from five patients failed to amplify for R702W, and DNA from one patient failed to amplify for G908R.

Genotype frequencies at the three SNPs were in Hardy–Weinberg equilibrium in all three groups except for L1007fsinsC in CD patients ( $\chi^2 = 6.64$ , 1 df,  $p = 0.01$ ), which showed excess of homozygosity for the rarer variant. Allele and genotype frequencies at all three SNPs were significantly different between CD probands and controls, but not between UC probands and controls (table 1).

When genotypes at all three SNPs were combined into a single *CARD15* genotype (table 2), the relative risk of CD for carriers of two variants (either in the homozygous or the compound heterozygous state) compared with non-carriers was highly significant (RR 41.41, 95% CI 4.86 to 352.95,  $p = 0.00001$ ) whereas the relative risk for heterozygotes at only one SNP was not (RR 1.63, 95% CI 0.57 to 4.69,  $p = 0.39$ ). A recessive mode of inheritance for susceptibility to CD linked to *IBD1* has been previously suggested based on a combined linkage and segregation analysis in our patient population.<sup>19</sup>

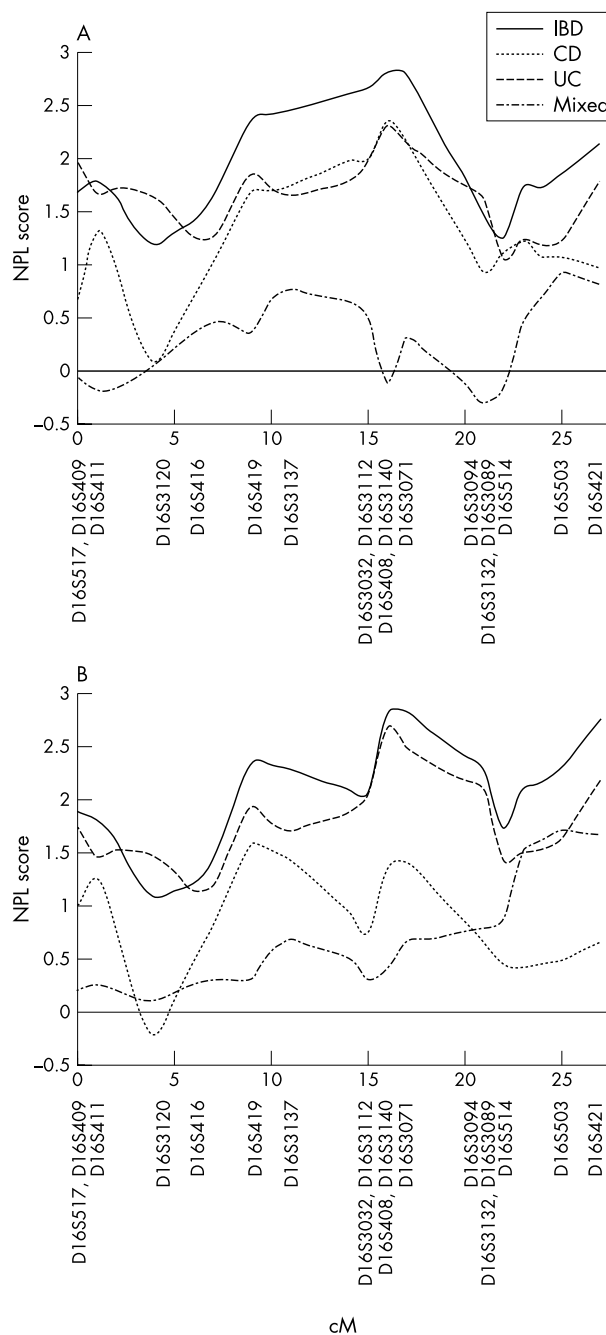
### Linkage analysis

Multipoint model free linkage analysis was first performed using 18 microsatellite markers spanning approximately 28 cM in the pericentromeric region of chromosome 16 (average distance 1.62 cM) on all 90 families grouped according to disease status (all IBD combined, CD, UC, and mixed). Results are shown in fig 1A. The maximum multipoint NPL score occurred at the position of markers D16S408 and D16S3140 (74.44 cM in the Marshfield sex average genetic map) in all 90 families combined ( $p = 0.002$ ), and in the 27 CD ( $p = 0.01$ ) and 43 UC ( $p = 0.01$ ) families. In the 20 mixed families, a non-significant peak  $<1$  was observed near the q-tel end of the interval at marker D16S503 (83.55 cM,  $p = 0.18$ ).

After typing the probands for R702W, G908R and L1007fsinsC, 26 families were removed in which the proband was either heterozygote or homozygote for the rare allele at any one of the three *CARD15* SNPs (13 CD, 6 UC and 7 mixed), and the linkage analysis was repeated (fig 1B).

The highest multipoint NPL score in all 64 families was very similar to the one observed before and still occurred near markers D16S408 and D16S3140 ( $p = 0.002$ ). A second peak of approximately the same magnitude ( $p = 0.003$ ) was observed at the q-tel end of the genetic map close to marker D16S421 (85.94 cM). Two smaller peaks were observed near marker D16S419 (67.40 cM,  $p = 0.01$ ) and further near the centromeric end at markers D16S517 and D16S409 (58.46 cM,  $p = 0.03$ ).

In the CD group, there was no significant evidence of linkage to this region after the *CARD15* positive families were



**Figure 1** Multipoint NPL scores for chromosome 16 microsatellite markers. (A) All families irrespective of *CARD15* SNPs status; (B) only families with probands negative for *CARD15* SNPs analysis.

**Table 2** Combined *CARD15* SNP genotypes in 84 IBD probands typed at all three SNPs and 108 controls

Subjects	Genotype counts				RR <sub>12</sub> (95% CI)	RR <sub>22 or 22'</sub> (95% CI)
	1/1	1/2	2/2	2/2†		
CD	17	6	2	6	1.63 (0.57 to 4.69)	41.41 (4.86 to 352.95)
UC	41	12	0	0	1.36 (0.60 to 3.05)	—
Controls	88	19	1	0	—	—

1/1, homozygotes for the common variant at all three SNPs; 1/2, single heterozygotes; 2/2, homozygotes at one of the three SNPs; 2/2†, heterozygotes at two SNPs.

removed (all  $p$  values  $>0.05$ ). Interestingly, the NPL at the location of marker D16S3120, which is the nearest marker to *CARD15* included in our analysis, was now negative. The maximum NPL was observed at marker D16S419 (67.40 cM,  $p = 0.06$ ), which is about 5 cM away from *CARD15*.

In contrast, linkage to D16S408 was even more significant in the 37 *CARD15* negative UC families ( $p = 0.003$ ). Additional smaller peaks were observed near the ends of the genetic map, and the overall profile was very similar to that described for the whole IBD sample. In the mixed families, linkage to D16S503 near the q-tel end of our interval was more pronounced ( $p = 0.04$ ) when families with *CARD15* SNP positive probands were removed.

### Linkage disequilibrium analysis

Family based tests of association in the three groups of 14 CD, 37 UC, and 13 mixed families that were negative at the *CARD15* SNP analysis did not show any significant association of any of the alleles of 18 microsatellite markers to disease status (all *p* values > 0.05). However, given their small size, it is possible that inability to detect associations in our samples was due to lack of power.

### DISCUSSION

After its initial report by Hugot *et al* in 1996,<sup>3</sup> linkage of CD to the *IBD1* locus in the pericentromeric region of chromosome 16 was consistently replicated in several independent groups of patients, although with some variation in the location of the linkage peak across different studies.<sup>6</sup> This was an unusual finding in the search for susceptibility genes in complex traits by genetic linkage analysis, which is often characterised by lack of replication of initial positive results in subsequent studies. The search for the *IBD1* gene culminated in the discovery that allelic variants of SNPs in the *CARD15* gene conferred an increased risk of developing CD,<sup>8–10</sup> particularly when present on both homologous copies of chromosome 16 in either the homozygous or the compound heterozygous state. Once again, this finding was consistently replicated in several populations,<sup>4</sup> including some in which linkage to the *IBD1* region was less than significant.<sup>20</sup>

Linkage of UC, the other common form of IBD, to the *IBD1* locus was reported usually to be negative or non-significant<sup>6</sup> with few exceptions.<sup>7</sup> We first reported a positive linkage of markers in the *IBD1* region to both CD and UC in our Italian patient population.<sup>11</sup> While we were extending the analysis of the *IBD1* region to a larger sample of families and a more dense map of markers, the identification of the association of CD with *CARD15* SNP alleles<sup>8–10</sup> prompted us to type our families for the same SNPs, and re-evaluate our linkage findings after exclusion of those families in which the probands were carriers of *CARD15* SNP alleles known to confer susceptibility to CD.

Similarly to results in other populations, all three *CARD15* SNPs characterised in this study (R702W, G908R, and L1007fsinsC) showed significantly higher frequencies of the rarer alleles in the CD, but not in the UC probands. Multipoint model free linkage analysis with markers from the *IBD1* region became even more significant in our UC families (with a peak close to marker D16S408, which is about 12 cM away from the location of *CARD15*) after removal of six UC families whose probands were heterozygous carriers of one of the three *CARD15* SNPs. In contrast, the significance of linkage to this region was markedly reduced in our CD group after removal of the families with *CARD15* positive probands, suggesting that the three known SNPs did account for most of the *IBD1* linked susceptibility to CD in our families. A much smaller peak was observed in the mixed families group near the q-tel end of the interval included in our analysis (approximately 20 cM away from *CARD15*). In this case also, significance increased after removal of seven families in which the probands were carriers of one of the three *CARD15* SNPs. These observations point to the existence of a UC susceptibility gene in the *IBD1* region in our families.

The presence of additional IBD loci in the pericentromeric region of chromosome 16 independent of *CARD15* has been suggested by Hampe *et al*<sup>21</sup> on the basis of the observation of positive linkage in their *CARD15* negative IBD families. In their study, linkage was more significant in the CD families than in all IBD families combined, and was marginal in their group of UC families. While linkage was positive across a large pericentromeric region, the observation of positive

association with alleles of a microsatellite marker, D16S3068, located near the centromere on 16p, led these authors to detect significant association to random SNPs in this area. The nearest markers to D16S3068 included in our study are D16S409 and D16S517, located on chromosome 16q approximately 10 cM away from D16S3068 according to the Marshfield chromosome 16 genetic map. Although we observed a small peak near these markers in the IBD and UC groups, our data point to a more distal 16q location for the susceptibility locus in our families. Interestingly, based on the observation of multiple positive linkage peaks in the intervals flanking *CARD15*, Hampe *et al*<sup>21</sup> also suggested that more than one additional IBD gene may be located in this region.

We cannot exclude that rarer *CARD15* SNPs other than those typed in this study are present in our patients and account for some of the positive linkage to this region in our families. In this respect, it is of interest that even in the CD families the maximum NPL score occurred at marker D16S408, which is about 12 cM away from *CARD15*, and that the minimum score occurred at D16S3120, which is the nearest marker to *CARD15* included in our analysis. Nonetheless, 13 CD families out of 27 were found to be positive for the *CARD15* SNPs (with five heterozygous and eight homozygous or compound heterozygous probands). This observation points to the difficulty of correctly mapping susceptibility genes for complex traits by linkage analysis.<sup>20</sup> The relatively small sample size and the presence of both linked and unlinked families may result in a localisation of the linkage peak away from the actual susceptibility gene location. The positive linkage we observed in the *IBD1* region might indicate the presence of different *CARD15* SNPs from those we tested conferring susceptibility to IBD in our population. However, the involvement of rarer *CARD15* SNPs in UC has been excluded in patients from several European populations, including some from Italy.<sup>22</sup> It seems unlikely that *CARD15* plays a different role in our UC patients from that observed in other populations.

In conclusion, these results point to the presence of a UC susceptibility locus in the chromosome 16 pericentromeric region in our families. Whether this is at least in part due to the presence of rarer *CARD15* SNPs or to a completely independent gene, remains to be determined.

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