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ARTICLE

Genetic analysis in Italian families with inflammatory bowel disease supports linkage to the *IBD1* locus – A GISC study

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Epidemiological studies suggest that inherited factors influence susceptibility to inflammatory bowel disease (IBD), and some candidate loci have been described. In order to verify whether the same loci are responsible for predisposition to IBD in our population, we carried out a linkage study in a series of 58 Italian families with Crohn's disease (CD) and ulcerative colitis (UC). HLA-DQ alleles, motilin gene, and 34 microsatellites flanking the previously described loci on chromosomes 3, 6, 7, 12 and 16 were analysed by non-parametric linkage analysis in 16 and 23 families with CD and UC, respectively, and in 19 families where CD and UC coexisted. Non parametric analysis using GENEHUNTER yielded maximum NPL scores for marker D16S408 in all IBD families combined (2.71, $P = 0.003$), for marker D16S419 in CD (1.97, $P = 0.026$) and for marker D16S514 in UC families (2.44, $P = 0.007$). These markers map in the previously described *IBD1* region. No significant linkage was found for markers of chromosomes 3, 6, 7 and 12. The present study performed in a Southern European population provides additional support for the conclusion that the *IBD1* locus has a clear role in the genetic susceptibility to IBD.

Keywords: inflammatory bowel disease; ulcerative colitis; Crohn's disease; linkage analysis; genetic predisposition

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Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are common diseases in the Western world with an estimated prevalence rate of about 1:1000 inhabitants.[1] Whilst their etiology remains elusive, epidemiological studies suggest that inherited factors might influence susceptibility to both diseases, especially to CD.[2] Although they are two distinct disorders, there is clinical and therapeutic overlap, which justifies their inclusion under the same heading of inflammatory bowel disease (IBD). Positive family history is the strongest risk factor so far identified for these diseases: given a proband with CD or UC, the relative risk of developing the same disease for first degree relatives is 10 and 8, respectively.[3] Moreover, the coexistence of both diseases in the same family is observed at a frequency substantially greater than that expected for each of them. This finding leads to the hypothesis that multiple genes contribute to both diseases and that phenotypic difference could result from the expression of specific genes.[4]

Hugot *et al* initially studied 25 families with CD and excluded linkage for the entire chromosome 6.[5] In the attempt to disclose possible candidate genes, three genome scans have been undertaken. In two independent series of 41 and 71 European sib pairs with CD, Hugot *et al* identified a putative susceptibility locus (called *IBD1*) near marker D16S409 on chromosome 16 with a maximum lod score of 2.04.[6] Satsangi *et al* performed a two-stage genome scan in two series of 75 and 85 families from the United Kingdom with a total number of 186 affected sib pairs.[7] In this study, in which families with CD (n = 76), UC (n = 60), and mixed disease (n = 31), were included, strong evidence for the presence of susceptibility loci for both UC and CD was found on chromosome 3 (lod score 2.69), 7 (lod score 3.08), and 12 (lod score 5.47). In the same study linkage to D6S276, which lies close to the major histocompatibility complex on chromosome 6, was excluded in CD families, whereas in UC the marker was more frequently shared by affected siblings. More recently a third genome-wide screen performed on 174 North American IBD families found evidence for linkage on chromosomes 1p, 3q and 4q, and confirmed linkage to the pericentromeric region of chromosome 16.[8]

Confirmation of these initial studies in different populations is required in order to extend and test their meaning worldwide. However, replication studies have yielded contradictory results. Whilst most of them have confirmed the data for chromosomes 16 and 12, none has so far been able to confirm linkage for chromosomes 3 and 7.[8–16]

In this study we tested the presence of potential susceptibility loci on chromosomes 3, 6, 7, 12 and 16 in a series of Italian families with IBD.

Materials and Methods

In a multicentre programme of the GISC (Italian Group for the Study of the Colon and the Rectum) involving 12 centres throughout Italy, 85 families with one or more members affected by IBD were recruited. Patients were classified as having CD, UC, or indeterminate colitis on the basis of clinical, endoscopic, radiological, and pathological data.[17] In 22 families all affected members had CD, in 39 UC, and the remaining 24 had CD and UC (mixed families). In each family at least one unaffected relative was also available. All 85 families were included in the transmission disequilibrium analysis (see below). Families with one affected member or with parent/child affected pairs only were excluded as uninformative for the linkage analysis. Therefore, 58 families (16 CD, 23 UC and 19 mixed) with two or more affected members other than parent/child were included in the linkage analysis. The type and number of affected relatives included in these families are shown in Table 1.

From each subject included in the study, peripheral blood was sampled after informed consent. The full purpose of the study was extensively explained to all participating subjects. Blood samples were collected into sodium citrate-containing tubes, frozen as a whole, and stored at –30°C until extraction. Genomic DNA was then purified from peripheral blood leukocytes according to standard protocols.[18] The 35 microsatellite markers, which were used to study five different regions on chromosomes 3, 6, 7, 12 and 16, are shown in Table 2. From chromosome 6, two further markers flanking the HLA region were added: DQ alleles (Amplitype HLA-DQ Perkin-Elmer, Norwalk, CT, USA) and the human motilin gene (*MLN*). For each chromosome, the markers that gave the highest lod score in previous studies were included. Genetic maps and distances assumed in the multipoint analysis were taken from the Généthon database and from

Table 1 Characteristics of the IBD families included in the linkage analysis

Affected relatives	*No of families*		
	CD	*UC*	*Mixed*
2 sibs	10	18	11
2 sibs + 1 first cousin	1	–	–
2 sibs + 1 aunt/uncle	–	1	1
3 sibs	1	–	1
4 sibs	1	–	–
1 aunt/uncle – 1 niece/nephew	2	1	4
2 first cousins	–	2	1
1 grandparent – 1 grandchild	1	1	1
Total	16	23	19

the Ceph/Généthon linkage map (http://www.cephb.f/ceph-.genethon-map.html)[19] or from the Genetic Location Database (http://cedar.genetics.soton.ac.uk). The location of the human motilin gene (*MLN*) on chromosome 6 was assigned close to the HLA–DQα_1 locus as previously described.[20] Allele frequencies used in the genetic analysis were estimated by counting the observed alleles in unaffected, unrelated individuals.

For microsatellite markers, PCR was performed in a 15 µl total reaction volume containing 1.5 µl 10 × Gene Amp PCR Buffer II, 1.5 µl dNTPs (2.5 mM), 1.5 µl $MgCl_2$ (25 mM), 0.12 µl Taq Gold polymerase (5 U/µl), 0.5 µl of each primer (5 to 15 pmol/µl), 50 ng/µl of genomic DNA template. Following 12 min at 95°C to activate the Taq Gold, amplification was achieved by the following protocol: 10 cycles at 94°C for 15 s, 55°C or 56°C for 15 s; 20 cycles at 89°C for 15 s, 55°–56°C for 15 s and 72°C for 15 s. Final extension was at 60°C for 45 s. PCR products were subjected to electrophoresis in 4.75% w/v polyacrylamide gels containing 8 M urea, and run for 6 hours at 2500 V using automated 373 DNA sequencer (Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA). Semi-automated DNA fragment sizing was performed using Genescan software (v. 1.1), and genotyping by means of Genotype software (PE/ABD, Foster city, CA, USA, v. 1.1).

Multipoint non-parametric linkage analysis was carried out with the GENEHUNTER program.[21] This program was chosen because it performs non-parametric linkage analysis with a large number of markers in arbitrary pedigrees of moderate size. Non-parametric linkage analysis is carried out assessing marker allele sharing at each location of the genome among affected individuals and measuring whether they share identical-by-descent alleles more often than expected under random segregation. Under the null hypothesis of no linkage and studying a large number of pedigrees, the non-parametric lod score (NPL) follows asymptotically a standardised normal distribution from which statistical significance is computed. Two nonparametric statistics are implemented: NPL-pairs (which assesses pairwise allele sharing among affected individuals) and NPL-all (which examines all affected individuals in the pedigree simultaneously and computes a score based on identity-by-descent sharing among all affected individuals). In our analysis we used the NPL-all statistics. Transmission disequilibrium test (TDT) was performed using the TDT multiallelic statistic included in the ANALYZE package.[22] This test evaluates whether a marker allele is transmitted from parents to affected children more often than expected under random segregation. Therefore, TDT is a test of association between the disease and the marker alleles.

Table 2 List of polymorphic markers used and their distance

Chromosome	*Marker list and map distance (cM)*
3	D3S3521 (4) D3S1076 (1) D3S3559 (5) D3S1573 (0.5) D3S1568 (5) D3S3717 (5) D3S1592
6	D6S260 (16) D6S464 (1) DQα (0.5) MLN (1) D6S276 (13) D6S426
7	D7S484 (5) D7S2507 (7) D7S2428 (5) D7S519 (5) D7S506 (7) D7S2483 (7) D7S672 (7) D7S669 (7) D7S524 (7) D7S646 (5) D7S527
12	D12S368 (4) D12S1586 (6) D12S355 (1) D12S83 (3) D12S1702
16	D16S517 (1) D16S409 (1) D16S411 (9) D16S419 (8) D16S408 (7) D16S514 (1) D16S503 (3) D16S421

Results

Analysis of 58 families with IBD showed evidence of linkage for chromosome 16 but not for chromosomes 3, 6, 7 and 12.

Chromosome 16

Results of non-parametric multipoint analysis for eight chromosome 16 markers are shown in Table 3 and Figure 1. Identity-by-descent sharing among affected individuals was higher than expected under random segregation in the IBD sample in the whole region investigated. In the IBD families, a peak was obtained with marker D16S408 (NPL score = 2.70, *P* value = 0.0035). Distribution of the NPL score in the CD and in the UC samples (Table 3 and Figure 1) was similar for markers D16S411, D16S419, and D16S408, but differed in the q-telomeric end of the region investigated, in which a NPL score of 2.44 was observed in the UC sample, whilst no significant distortion of allele sharing was observed in the CD sample. Specifically, the analysis of the CD-affected sample identified a 17 cM region between markers D16S411 and D16S408, whereas in the UC families excess identity by

Table 3 Results of multipoint nonparametric linkage analysis using the NPL-all score of GENEHUNTER

	IBD families (58)		*CD families (16)*		*UC families (23)*		*Mixed families (19)*	
	NPL score	*P value*	*NPL score*	*P value*	*NPL score*	*P value*	*NPL score*	*P value*
D16S517	2.04980	0.020489	1.03509	0.150839	2.11397	0.016855	0.32112	0.376186
D16S409	1.97481	0.024479	1.23021	0.110581	1.85748	0.032273	0.29740	0.380952
D16S411	1.91618	0.028025	1.66423	0.049577	1.58546	0.056533	0.10784	0.457304
D16S419	2.20272	0.014037	1.97151	0.026088	1.81884	0.034360	0.07645	0.468463
D16S408	2.70793	0.003456	1.95452	0.026823	2.10631	0.017559	0.64785	0.259548
D16S514	1.87596	0.030701	0.41855	0.335655	2.44923	0.007045	0.20443	0.420375
D16S503	1.79552	0.036691	0.82937	0.203661	1.75080	0.040561	0.45849	0.325335
D16S421	1.50920	0.066090	0.00148	0.492133	2.05271	0.020339	0.37122	0.354732

descent sharing was found over the whole region. No significant linkage was found in the mixed families.

TDT performed using the multiallelic statistic from the ANALYZE package was not significant for eight markers in 85 IBD families and in the subset of 39 UC families with one or more affected individuals. However, an overall χ^2 of 4.64 (1 df, $P = 0.015$) was found for marker D16S408 in 22 CD families, where allele 248 was transmitted 13 times from heterozygous parents to affected individuals as against three non-transmission.

Other Chromosomes

Results of non-parametric linkage analysis in our samples were not significant in the investigated regions on chromosomes 3, 6, 7 and 12 (all P values gt; 0.05; data not shown). Multipoint NPL scores larger than 1 were observed in the UC sample at marker D6S426 (NPL = 1.27) and at locus HLA–DQ (1.11) on chromosome 6; at markers D3S3521 (1.30), D3S1076 (1.20), D3S2559 (1.10), D3S1573 (1.16) and D3S3717 (1.06) on chromosome 3; at markers D7S519 (1.34), D7S2483 (1.43), D7S672 (1.28), D7S524 (1.01) D7S646 (1.42) and D7S527 (1.55) on chromosome 7. In the mixed sample NPL scores larger than 1 were observed at D12S368 and D12S1586 (1.39 and 1.12, respectively) on chromosome 12. All NPL scores were lower than 1 in the CD sample.

Discussion

The present study performed in a sample of Italian IBD families contributes additional evidence supporting the presence of a genetic locus on chromosome 16, which

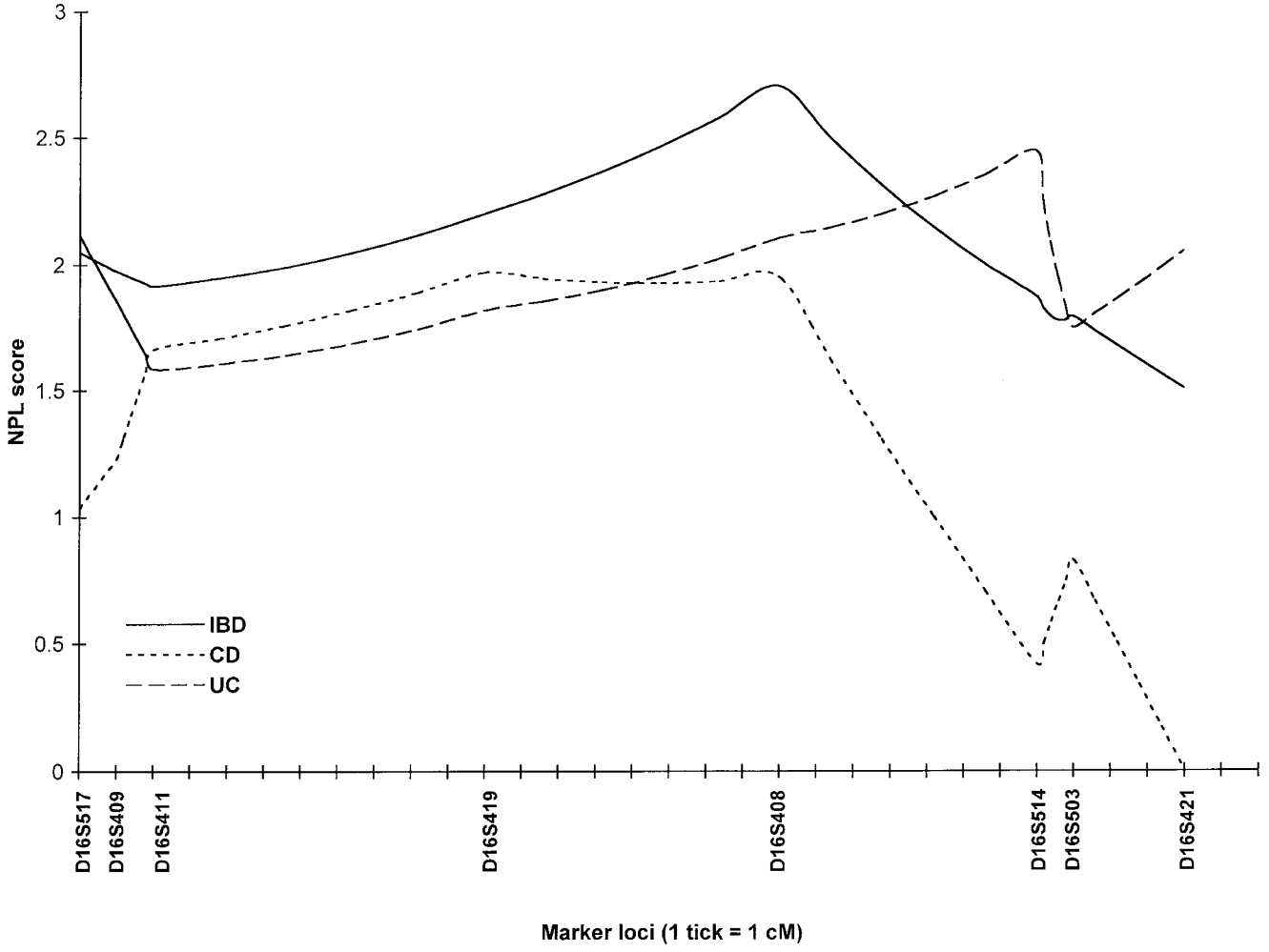

Figure 1 *NPL-all scores obtained with GENEHUNTER for the different subgroups of families with IBD, Crohn's disease and ulcerative colitis with markers of chromosome 16*

appears to encode for predisposing factors to IBD. Although a lod score of at least 3 is first required to establish linkage between a disease locus and a genetic marker, a lower threshold is sufficient to confirm a linkage that has already been found in an independent sample of families.[23] Therefore, our results represent a statistically significant confirmation that a susceptibility locus for CD, named *IBD1*, is located in the pericentromeric region of chromosome 16.[6] This region of the genome contains several candidate genes, including CD11 integrin, CD19, sialophorin and IL-4 receptor, which may be relevant to the pathogenesis of CD. Replication of the initial report has been undertaken in several studies carried out in different populations. Despite some negative reports,[15] most data confirmed that the *IBD1* region contains a gene conferring predisposition to CD, although disagreement exists about the precise location of this locus.[8–11,14,16] Differences between studies may well be ascribed to the variability of the genetic background of the different populations so far evaluated, and to methodological limitations of linkage studies in fine mapping susceptibility loci for complex traits.[24] In our CD families, NPL scores with associated *P* value of less than 0.05 were obtained over the whole region encompassed by D16S411 and D16S408. A possible transmission disequilibrium was observed in our CD families at D16S408, where a multiallelic TDT produced a *P* value of 0.015.

Our study also reports positive linkage results for *IBD1* in 23 UC families. A recent study performed on a large sample of UC families also showed evidence supporting linkage to *IBD1*.[13] Other three studies, which only included a small number of UC families, failed to show any significant result,[9–11] whilst a fourth large sample of European UC families produced mainly inconclusive results.[16] Overall, whether or not *IBD1* is also involved in predisposition to UC is still unclear. In our UC families, a peak NPL score of 2.44 ($P = 0.007$) was detected for D16S514. This locus is about 7 and 15 cm distant from D16S408 and D16S419, respectively, which gave the highest score in our CD families. However, there is considerable overlap in the regions of positive linkage in the CD and UC families, and difference in peak locations might be due to the relatively small number of families. Coexistence of linked and unlinked families in the UC sample may result in overestimation of the recombination fraction and therefore in mapping the disease locus away from its true location. In addition, it has been shown that a susceptibility gene for a complex trait does not necessarily lie in the region of maximum allele sharing, especially when the same gene confers only a modest increase in disease risk.[24] Nonetheless, the possibility of two different loci on chromosome 16 contributing to susceptibility to CD and UC cannot be ruled out.

A putative gene associated with psoriasis has been recently reported on chromosome 16.[25] Since psoriasis is more frequent in patients with Crohn's disease than in controls,[26] it might well be the case that there is an immunomodulatory locus on chromosome 16 influencing the susceptibility to both diseases.

This study, in agreement with other reports,[6,8,14,15] does not confirm previously reported linkage on chromosomes 3, 7 and 12.[7] Multipoint NPL scores larger than 1 were observed over large intervals encompassing the previously reported loci on chromosomes 3 and 7[7] in our UC families only, but all associated *P* values were larger than 0.10. This finding was not completely unexpected, based on the known difficulties in replicating linkage studies in complex traits. Linkage studies entail detection of weak effects that might be even less prominent in different studies, due to genetic heterogeneity of the disease combined with the ethnic variations of allele frequencies. Our non-significant results may just be the consequence of low power derived from our relatively small sample size.

There is considerable evidence supporting an association between some HLA loci and UC or in particular subgroups of UC patients. The HLA DRB1 and DQβ_1 genes have been recently found to influence UC susceptibility and disease behaviour.[27,28] A significant increase of allele sharing by UC siblings for marker D6S276, which lies close to the HLA complex, was previously described.[7] In the present study, we found a NPL score higher than 1.0 in the UC families at locus D6S426 (NPL = 1.27) and marker DQ (NPL = 1.11). Although these results were not statistically significant, the findings are in keeping with previous association studies[29] and further replication in larger series of patients may be warrant.

Finally, this study performed on an IBD population from the Mediterranean area contributes additional support to the overall conclusion that the *IBD1* locus on chromosome 16 has a clear role in genetic susceptibility to IBD. Further studies performed in larger samples will be necessary to establish definitely a role for other loci responsible for predisposition to IBD in our patients.

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