

# Association of Crohn's disease and ulcerative colitis with haplotypes of the *MLH1* gene in Italian inflammatory bowel disease patients

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Crohn's disease (CD) and ulcerative colitis (UC), the two clinical entities comprising idiopathic inflammatory bowel disease (IBD), are complex disorders with a proven genetic predisposition.<sup>1</sup> Several systematic genome wide searches for susceptibility genes in patients with IBD have reported linkage to specific regions of the genome.<sup>2–8</sup> In particular, many replication studies, including a large international cooperative study<sup>9</sup> and our own,<sup>10</sup> have confirmed linkage to a locus on chromosome 16, named *IBD1*.<sup>2</sup> Association of susceptibility to CD with allelic variants of *NOD2*, located in 16q12, has been recently reported.<sup>11–13</sup>

One of the worst complications of IBD, especially in UC, is the increased risk of colorectal cancer.<sup>14</sup> Pokorny *et al*<sup>15</sup> studied three polymorphisms located inside or near the *MLH1* gene, one of the DNA mismatch repair genes implicated in hereditary non-polyposis colon cancer (HNPCC),<sup>16</sup> in a cohort of IBD patients. They found that specific *MLH1* haplotypes were associated with the presence and family history of the disease in both CD and UC. Interestingly, markers from chromosome 3p21 located less than 10 cM away from the *MLH1* gene showed evidence of linkage to IBD in a genome wide search.<sup>3</sup>

Following the initial report of Pokorny *et al*,<sup>15</sup> we performed a case-control study with the aim of investigating the association of polymorphisms of the *MLH1* gene with CD and UC in our population of Italian IBD patients.

## MATERIAL AND METHODS

Our sample consisted of a total of 52 CD, 60 UC unrelated patients, and 51 unrelated unaffected controls. All patients were Italians and originated from southern Italy. Diagnosis of IBD was based on standard criteria.<sup>17,18</sup> Controls were unrelated blood donors originating from the same southern Italian region. All subjects gave informed consent to participate in the present study, which was approved by the IRB of the Ospedale CSS-IRCCS in San Giovanni Rotondo.

Three polymorphic markers located within or near the *MLH1* gene were selected for the analysis (table 1). These were the 5' flanking microsatellite D3S1768, the microsatellite D3S1611 located in intron 12, and the A/G single nucleotide polymorphism (SNP) located in intron 14 (IVS14) at nucleotide position 1668–19, also analysed by Pokorny *et al*.<sup>15</sup>

Genomic DNA was isolated from peripheral lymphocytes following standard procedures.<sup>19</sup> Fluorescent dye labelled forward primers and unlabelled reverse primers for microsatellite analysis were custom synthesised (PE, Applied Biosystems Division), and PCR conditions were as previously described.<sup>10</sup>

Single strand conformation polymorphism (SSCP) was used to search for the IVS14 SNP.<sup>20</sup> The oligonucleotide sequences and PCR annealing temperature were according to Han *et al*.<sup>20</sup> DNA fragments displaying abnormal SSCP patterns were sequenced. PCR products were purified with CENTRISEQ

**Table 1** Results of marker-disease association analysis

Marker	Map position in cM*	Map position in Mb†	CD LRT p value‡	UC LRT p value‡
D3S1768	61.52	39.70	0.00035	NS
D3S1611	61.52	40.49	0.00104	0.00004
IVS14	NA	40.77	NS	NS

\*Map position in cM according to Marshfield Clinic's Center for Medical Genetics sex average map (<http://research.marshfieldclinic.org/genetics/>).

†Map position in Mb according to LDB ([http://cedar.genetics.soton.ac.uk/public\\_html/ldb.html](http://cedar.genetics.soton.ac.uk/public_html/ldb.html)).

‡p values of likelihood ratio test of association from DISEQ program.<sup>23</sup>

NS=not significant (p>0.05); NA=not available.

spin column, following the manufacturer's instructions (PRINCETON Separations). Automatic sequencing was performed by cycle sequencing with the Big Dye Terminator kit (Perkin-Elmer, Applied Biosystems Division), and the resulting *MLH1* sequences were compared with the published ones.<sup>21,22</sup>

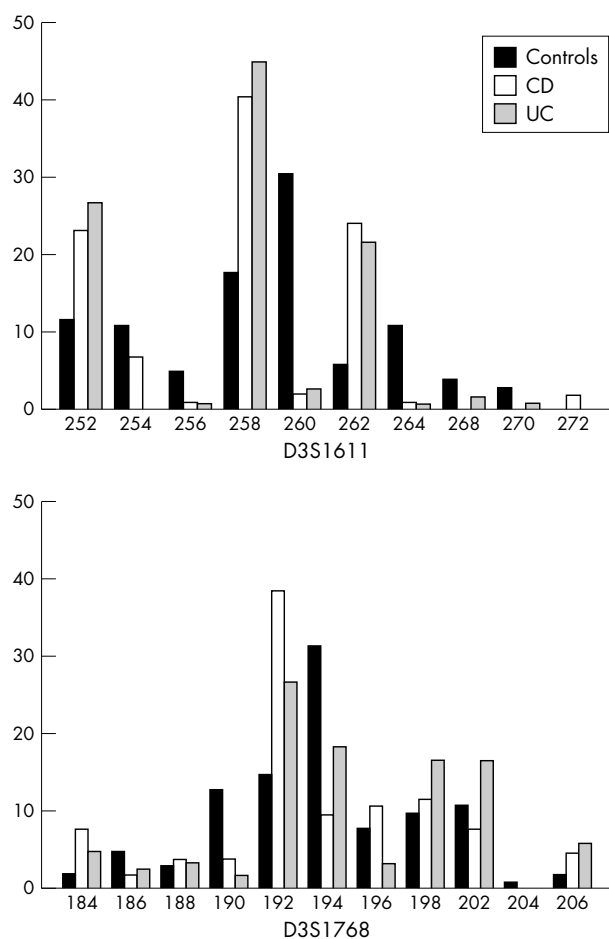
Association of single marker alleles with IBD status was evaluated using the DISEQ program by means of the likelihood based test proposed by Terwilliger,<sup>23</sup> which has been shown to be a powerful test in the linkage disequilibrium analysis of multiallelic markers. Linkage disequilibrium analysis between markers and haplotype analysis were carried out using the EHPLUS and PMPLUS programs.<sup>24</sup>

## RESULTS

Marker allele frequencies in cases and controls for the two microsatellite markers are shown in fig 1. The frequency of allele 192 of D3S1768 was increased in both CD (0.39) and UC (0.27) compared to controls (0.15). Three alleles of D3S1611 were observed more frequently in both CD and UC patients than in controls: 252 (CD=0.23, UC=0.27, controls=0.13), 258 (CD=0.40, UC=0.45, controls=0.18), and 262 (CD=0.24, UC=0.22, controls=0.06). No difference was observed in the allele frequency distribution of the IVS14 SNP (allele A: CD=0.55, UC=0.52, controls=0.54; allele G: CD=0.45, UC=0.48, controls=0.46).

Results of association tests between IBD status and the three markers using the DISEQ program<sup>23</sup> are shown in table 1. Association was significant in CD patients for markers

**Abbreviations:** CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; HNPCC, hereditary non-polyposis colon cancer; SNP, single nucleotide polymorphism



**Figure 1** Allele frequency distributions of microsatellite markers D3S1611 and D3S1768 in CD, UC, and controls.

D3S1768 and D3S1611, while it was significant only for D3S1611 in UC patients and not significant for the IVS14 SNP in either group of patients.

Linkage disequilibrium analysis between markers carried out using the EHPLUS program<sup>24</sup> showed that significant linkage disequilibrium exists between D3S1611 and the IVS14 SNP in all three groups (all  $p$  values  $<0.0001$ ), whereas linkage disequilibrium is not significant between the two microsatellite markers and between D3S1768 and the IVS14 SNP (all  $p$  values  $>0.05$ ). In particular, alleles 252 and 262 of D3S1611 are associated with IVS14 SNP allele A, and allele 258 is associated with allele G in all three groups.

To compare our results to those of Pokorny *et al.*,<sup>15</sup> we tested for association of haplotypes defined by D3S1611 and the IVS14 SNP with CD and UC, by means of the heterogeneity test implemented in PMPLUS.<sup>24</sup> Significant association exists in both groups of patients ( $p < 0.0001$ ). In particular, haplotype 252/A has an estimated frequency of 0.23 in CD, 0.22 in UC, and 0.13 in controls; haplotype 258/G of 0.37 in CD, 0.39 in UC, and 0.16 in controls; haplotype 262/A of 0.23 in CD, 0.21 in UC, and 0.06 in controls. The most frequent (0.23) haplotype among controls, 260/G, has an estimated frequency of 0.02 in both CD and UC.

## DISCUSSION

Much evidence supports the hypothesis that an IBD susceptibility locus may be located on chromosome 3p, including results from a genome wide search,<sup>3</sup> a fine mapping study,<sup>25</sup> and a recent study in a Finnish group of patients.<sup>26</sup> In our previous linkage study in IBD families,<sup>10</sup> we found non-

parametric linkage scores larger than 1 in our subset of UC families for five chromosome 3p markers.

Pokorny *et al.*<sup>15</sup> first reported a significant association of haplotypes of the *MLH1* gene, located on chromosome 3p, with IBD. In our patient population, a significant association was observed of both CD and UC with alleles of D3S1611, a microsatellite located in *MLH1* intron 12. In addition, significant association was found between CD and alleles of one flanking microsatellite marker, D3S1768. These observations suggest that one or more allelic variants of *MLH1*, or of another gene located nearby, may contribute to susceptibility to IBD in our population.

In the study of Pokorny *et al.*,<sup>15</sup> although the single marker associations were not significant, significant association was found in CD and UC with three haplotypes defined by different combinations of alleles at markers D3S1611 and IVS14 1668-19 A/G. We also found that three haplotypes of these two markers (252/A, 258/G, and 262/A) are present at increased frequency in our IBD patients compared to a group of unaffected controls. Since Pokorny *et al.*<sup>15</sup> did not report the exact size of the microsatellite alleles, or the nucleotide base at the IVS14 SNP, it is difficult to compare our results to theirs in terms of which alleles are associated with each clinical group in the two populations. However, it is interesting that they also found that more than one *MLH1* haplotype is associated with IBD in their patients, suggesting the presence of multiple susceptibility alleles.

Another candidate gene located in the same chromosomal area has recently been investigated, the human *GNAI2* gene coding for G protein, Galphai2.<sup>27</sup> No detectable mutation in codon 179 of this gene has been found in familial IBD and colon cancer patients studied.<sup>27</sup> Paavola *et al.*<sup>26</sup> also studied whether risk of IBD is associated with a 32 bp deletion variant of the chemokine receptor *CCR5* gene on chromosome 3p21, but did not find any significant association.

Further studies are needed to investigate the presence of specific mutations of the *MLH1* gene in patients with IBD, and specifically in the presence of dysplasia or associated colorectal cancer. Finally, even if *MLH1* proves not to be directly implicated in the pathogenesis of IBD, our results and those of Pokorny *et al.*<sup>15</sup> may indicate linkage disequilibrium with a gene located close to it, and thus additional mapping studies in this area are warranted.

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