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ARTICLE

Sex stratification of an inflammatory bowel disease genome search shows male-specific linkage to the HLA region of chromosome 6

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Inflammatory bowel disease (IBD) is a multifactorial disorder, with both genetic and environmental factors contributing to the two clinical phenotypes of Crohn's disease (CD) and ulcerative colitis (UC). The underlying genetic model is thought to involve multiple genes with complex interactions between disease loci, and the *NOD2* gene on chromosome 16 has recently been identified as a CD susceptibility locus. Several genome-wide linkage studies have identified candidate regions, but there has been little replication across studies. Here we investigate the role of sex-specific loci in susceptibility to IBD. Linkage data from our previously reported genome search and follow-up study were stratified by the sex of the affected sib pair. Non-parametric linkage analysis was performed using Genehunter Plus. Simulation studies were used to assess the significance of differences in LOD scores between male and female families for each chromosome. Several regions of sex-specific linkage were identified, including existing and novel candidate loci. The major histocompatibility region on chromosome 6p, referred to as *IBD3*, showed evidence of male-specific linkage with a maximum LOD score of 5.9 in both CD and UC male-affected families. Regions on chromosomes 11, 14 and 18 showed strong evidence of linkage in male-affected families but not in female-affected families. No evidence of sex-specific linkage was found in the *IBD1* or *IBD2* candidate regions of chromosomes 16 and 12. The existence of sex-specific linkage is further evidence of the complex mechanisms involved in IBD and will facilitate future studies to identify susceptibility genes.
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Introduction

The genetic component of inflammatory bowel disease (IBD) includes a complex pattern of inheritance, with multiple susceptibility genes thought to be involved. Recent studies have identified *NOD2* on chromosome 16 as a susceptibility gene for Crohn's disease (CD).[1,2,3] Genome-wide linkage studies to date have reported putative disease susceptibility loci on several chromosomes: candidate regions for inflammatory bowel disease loci have been identified on chromosomes 1, 3, 4, 6, 7, 12, 14 and 16.[4–8] Our previous genome-wide linkage analysis confirmed linkage to chromosomes 16 (*IBD1*) and 12 (*IBD2*), and reported new suggestive evidence for linkage to chromosomes 1, 4, 6, 10, 22 and X.[9] Our follow-

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up study, with dense marker typing and an extended affected sib pair family collection, increased the evidence for linkage to chromosome 6p with a maximum multipoint LOD score of 4.2 at D6S461.[10] This *IBD3* linkage has been replicated in a Canadian population.[11]

Several studies have demonstrated the complex mechanisms involved in IBD, in particular different genetic inheritance models for Crohn's disease (CD) and ulcerative colitis (UC), or phenotypic heterogeneity. For example, a major susceptibility gene in the *IBD1* region with a dominant inheritance for UC and a recessive inheritance for CD has been proposed;[12] it has been suggested that the *IBD2* locus makes a major contribution to UC susceptibility but plays a relatively minor role in susceptibility to CD.[13] Linkage and association have been demonstrated in clinical subgroups defined by age of onset and disease severity.[14,15] Sex-specific differences in IBD are suggested by studies showing linkage to the X chromosome.[9,16] However, there are few established differences in the prevalence of inflammatory bowel disease between males and females.[17] This X chromosome IBD susceptibility locus may therefore be balanced by sex-specific autosomal loci, with the potential for locus heterogeneity and epistatic interactions. No previous IBD linkage studies have investigated this hypothesis, although in other immune-mediated diseases examples of sex-specific autosomal linkages exist. Differential evidence for linkage has been demonstrated in families with affected sib pairs of a particular sex, for example in Type I Diabetes,[18] hypertension[19] and osteoarthritis.[20]

Genome-wide searches for linkage have been performed in many immune-mediated diseases, but results have not been easy to replicate across different studies.[21] Disease genes of small effect are difficult to localise by linkage analysis, and stratification provides a method to increase the statistical power of a linkage study. In this paper, we re-analyse genome search data from inflammatory bowel disease, with stratification of families by the sex of affected individuals. Using the linkage data from our previously reported genome search[9] and chromosome 6p follow-up study,[10] families were stratified by the sex of affected individuals in each family to identify excess allele sharing specific to males or females. Simulation studies were used to assess the significance of chromosomal regions with an observed difference in evidence for linkage between male-only and female-only affected families.

Subjects and methods

As previously reported, 353 white northern European affected sibling pairs from 268 nuclear families were genotyped with 358 microsatellite markers across the genome.[9] A further 75 affected sibling pairs were genotyped with an additional 11 markers in the region of linkage on chromosome 6,[10] which includes the HLA region. The general IBD phenotype was classified as Crohn's disease (CD) or ulcerative colitis (UC); analyses were carried out for each of these phenotypic categories to identify loci specific to either of these sub-phenotypes. Family cohorts were recruited from a number of European centres. Informed, written consent was obtained from all study participants; recruitment protocols were approved by institutional review committees at each participating centre. Methods of family ascertainment, clinical phenotypes and genotyping are described in detail elsewhere.[9,10]

For each phenotype, nuclear families were stratified according to the sex of affected individuals within each family. Analyses were performed using all families (ALL), families with affected females only (F), and affected males only (M). Where a family contained both UC and CD affected siblings, linkage analysis for the CD phenotype classified UC affected individuals as having unknown affection status; similarly for CD cases in UC analysis. By this method, families with more than two affected offspring of mixed sex could be included in the analyses if a sibling pair were concordant for sex and phenotype. A summary of the number of families for each of the phenotypes (IBD, CD, UC), stratified by sex of affected siblings, is given in Table 1.

Multipoint linkage analysis was performed using Genehunter Plus,[22] a modified version of the Genehunter program.[23] This program calculates a non-parametric LOD score using an identity-by-descent linear allele-sharing model. The 'ALL' scoring function was used, allowing allele-sharing to be estimated from all affected individuals simultaneously. For each phenotype, genome-wide LOD scores were calculated for male and female affected families.

A simulation method was used to assess the difference in LOD scores between male and female families by chromosome, providing a test for heterogeneity between sex-specific affected families. The combined cohort of male-only and female-only families was randomly divided into two samples of size identical to male only/female only families for each phenotype. Each sample was analysed and the difference in maximum LOD score between the two groups for each chromosome was calculated. This was repeated 5000 times for a permutation test, and the proportion of simulations for which the observed result exceeded the simulated difference gives the probability of observing such a difference by chance.

Where sex-specific linkage was identified in CD families, the role of locus interaction with *NOD2* was investigated by stratification of families by presence or absence of *NOD2* mutations (3020insC, R702W, L1007P) in CD affecteds.[1–3]

Results

In the genome search family cohort, 60% of affected offspring were female. The distribution of IBD-affected sib-pairs over male-only, female-only and mixed families differs significantly from the expected distribution, given this estimated male–female ratio and assuming random assort-

Table 1 Overview of family cohorts stratified by sex of affected individuals

		CD			*UC*			*IBD*	
No. sibs	*ALL*	*M*	*F*	*ALL*	*M*	*F*	*ALL*	*M*	*F*
Genome search family cohort									
2	114	24	48	78	14	23	230	46	90
3	14	2	4	12	4	2	35	6	8
4	1	0	0	0	0	0	3	0	0
Total	119	26	52	90	18	25	268	52	98
Follow-up study family cohort (chromosome 6)									
2	32	5	12	18	4	5	58	11	19
3	0	0	0	1	1	0	2	1	0
Total	32	5	12	19	5	5	60	12	19

ment of pairs (P=0.02, chi-squared test). An excess of female-only families was observed for both CD and UC phenotypes. This excess was not unexpected, as females, and hence female affected sib-pairs, are easier to recruit as they are generally more willing to participate in epidemiological studies.

Genehunter Plus multipoint LOD scores for CD and UC phenotypes, from all families (ALL), and for the sub-groups of male only (M) and female only (F) affected families, are shown in Figure 1. Regions with a LOD score of at least 2 in either male or female families are shown in Table 2. Regions of linkage with the IBD phenotype are also listed. Eight regions demonstrated 'suggestive' evidence for linkage, as defined by a LOD score of 2.2 in a genome-wide search.[24] Male-specific linkage was found in CD, UC and IBD phenotypes. Only two regions showed female-specific linkage, both for UC, despite the higher numbers of female-only families.

The highest LOD score for IBD was on chromosome 6p21.3 where linkage was identified in male affected families only (see Table 2). A difference between subgroups of comparable size was not observed in any of 5000 simulations. This male-specific linkage to chromosome 6 was also seen in CD affected sib pairs (Table 2), and in UC affected sib pairs (Male LOD=1.53, Female LOD=0, $P>0.05$). The number of families in each subgroup is small for UC and the non-significant difference for UC affected sib pairs may reflect a lack of statistical power. This region corresponds to the previously identified linkage,[9] and includes the HLA region. The dense marker typing on chromosome 6 with additional families increased the evidence for linkage in male only affected families (Figure 2). The maximum multipoint LOD score for IBD occurred at D6S291 (Male LOD=5.91, Female LOD=0.06, $P<0.0002$), with evidence for linkage in both CD and UC phenotypes.

Simulations confirmed seven of the eleven other regions attaining a LOD score over 2 as sex-specific regions, with a significant difference in LOD between male and female families ($P<0.05$). Two regions of linkage on chromosome X correspond to those identified previously from all families in our genome search.[9] The linkage to IBD (Xp22, 34 cM) was identified in male affected families, whereas the second region of linkage (Xq24–26, 139 cM) was in female UC affected families only. Chromosome 1q showed linkage in male families only; this region of linkage is 55 cM proximal to that previously reported without stratification by sex.[9] Male-specific linkage was identified on chromosome 3 (IBD), corresponding to the previously identified candidate locus.[5] The region of linkage in male families shown here on chromosome 14 is approximately 50 cM distal from the putative locus on 14q11.[7,8] Other regions of male-specific linkage not previously identified as candidate regions included chromosome 11q21 and chromosome 18p11, which is more than 70 cM proximal to the regions of linkage previously identified at 18q.[7,8] Distinct male-specific linkage regions were identified on chromosome 11 for CD and UC phenotypes, separated by 64 cM, although the difference in LOD score between male and female families was not significant from simulations. The only autosomal region which showed suggestive linkage in female families was chromosome 20 (UC), although the evidence for a difference between male and female families was marginal (P=0.054). No interesting regions were observed in female CD or IBD families. The established IBD candidate loci on chromosome 16 (*IBD1*) and 12 (*IBD2*) did not exhibit sex-specific linkage.

The male and female specific LOD scores for chromosome 22 were both greater than the overall LOD score for all families (Male LOD=1.38, Female LOD=2.29, ALL LOD=1.28) at 32 cM for IBD. The combined LOD score for male only and female only IBD affected families was 3.66, suggesting that there may be two sex-specific disease loci in this region. A simulation study was used to assess this LOD score for significance. A sample of 149 families (equivalent to 50 male only and 99 female only IBD families) was randomly selected from the ALL family cohort and the maximum LOD score recorded. In 5000 simulations, a maximum LOD score of at least 3.66 was observed only five times (P=0.001).

NOD2 has been confirmed as a CD susceptibility locus. CD families were therefore stratified by presence or absence of NOD2 mutations, and regions showing sex-specific linkage in CD families were re-analysed. The greatest difference in

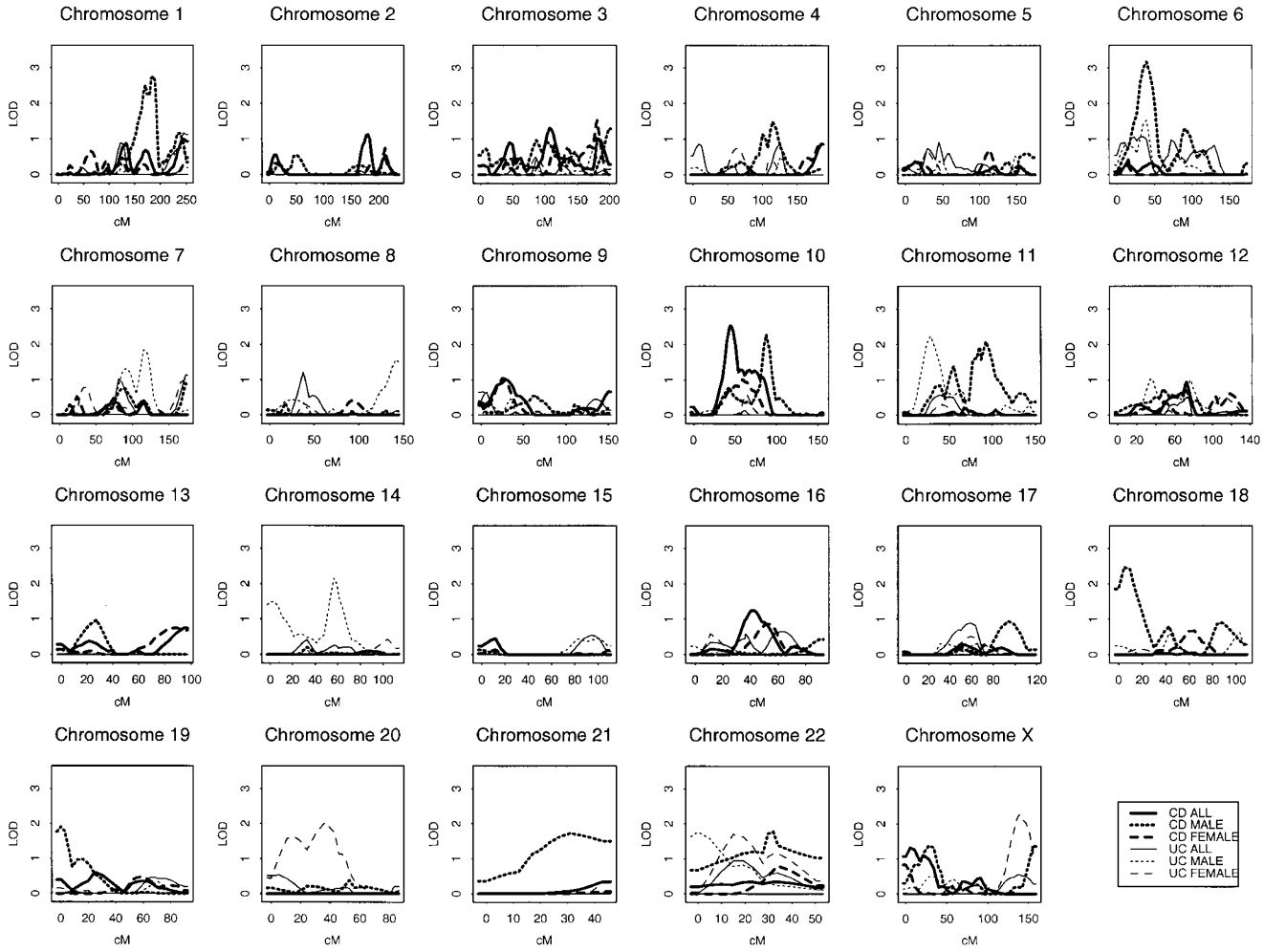

Figure 1 LOD scores for genome search family cohort stratified by sex of affected individuals, for CD and UC phenotypes.

LOD score occurred in male families on chromosome 1, with LOD scores of 2.13 in male NOD2 positive families (n=13), and 0.07 in male NOD2 negative families (n=8), compared with a total male LOD score of 2.76. This effect may, however, be due to the different numbers of families, rather than an epistatic effect between chromosome 1q and *NOD2*. We did not observe any difference in the frequency of *NOD2* mutations between male and female cases with Crohn's disease (unpublished data).

Discussion

In this study, we have identified sex-specific linkage in both previously reported and novel candidate regions, with the highest evidence for linkage in the region of chromosome 6 surrounding the MHC region. Excess allele-sharing at this locus was observed in male-only affected families but not in female-only affected families, implying a disease gene in this region that increases susceptibility in males only. The role of HLA genes in immune-mediated diseases is well established; a number of linkages of IBD to this region have been reported.[10,25,26] HLA alleles are functional candidate loci for IBD susceptibility, playing a central role in the immune response. Several studies have demonstrated an association between HLA class II alleles and susceptibility to both CD and UC.[27,28] The TNFα locus is a functional and positional candidate gene for IBD, but association studies involving TNFα polymorphisms and IBD have given inconsistent results.[10,29] The presence of sex-specific disease genes as well as allelic heterogeneity at a population level may partially explain the lack of replication in these studies. Differences in the frequency of HLA haplotypes between males and females occur in inflammatory bowel disease: DR3-DQ2 frequency was reduced in females with UC[25] and DRB1*15 was increased only in female UC patients.[30] Similar differences in HLA haplotype frequencies by sex occur in other immune-mediated diseases including Type I diabetes,[31] multiple sclerosis[32] and rheumatoid arthritis.[33]

Table 2 Details of linkage regions with maximum LOD score >2

Chr	Region	cM from pter	Phenotype	LOD score ALL	LOD score M	LOD score F	P-value	Markers
1	1q31–32	186	CD	0.16	2.76	0	0.0442	D1S413 D1S249
3	3p12	87	IBD	0.85	2.05	0.05	0.1426	D3S3653
6	6p22	39	CD	0.26	3.18	0	0.0024	D6S276
6	6p22	39	IBD	1.21	4.51	0	<0.0002	D6S276
10	10q22	87	CD	0.81	2.27	0.06	0.1402	D10S201
11	11q21	93	CD	0	2.08	0	0.0060	D11S1358
11	11p15	29	UC	0.38	2.24	0.07	0.0880	D11S902
14	14q23	57	UC	0.27	2.17	0	0.0168	D14S63
18	18p11	5	CD	0	2.46	0	0.0148	D18S59 D18S452
20	20p12–q11	36	UC	0.10	0	2.04	0.0536	D20S186 D20S195
22	22q13	32	IBD	1.28	1.38	2.29	0.0010	D22S283
X	Xq24–26	139	UC	0.55	0.01	2.27	0.3560	DXS1001 DXS1047
X	Xp22	34	IBD	0.77	2.27	0	0.0134	DXS1202

M=male-only affected families; F=female-only affected families; ALL=all families.
P-value is obtained using a permutation test for differences in LOD score between Male and Female families.

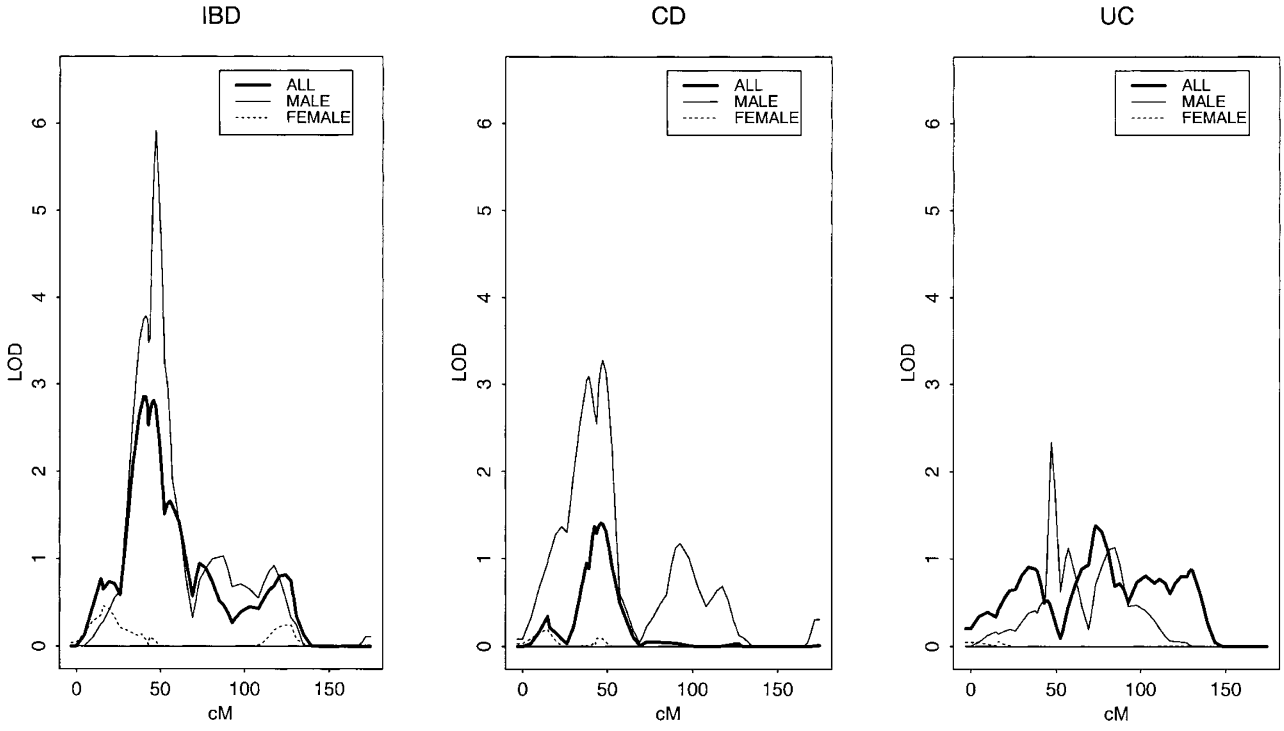

Figure 2 LOD scores on chromosome 6 for follow-up study family cohort stratified by sex of affected individuals, for IBD, CD and UC phenotypes.

Two other regions that showed strong evidence for male-specific linkage were chromosomes 1q31-32 and 18p11 (Table 2 and Figure 1). Neither of these regions was identified by genome scans. The established IBD candidate loci on chromosome 16 (*IBD1*) and chromosome 12 (*IBD2*) did not exhibit sex-specific linkage. Stratifying families by sex of affected individuals can substantially reduce the sample size to detect linkage. Few studies have tested the effect on power of stratifying affected sib pair families, but Leal and Ott[34] showed that if excess identity-by-descent allele-sharing exists

in a subgroup of sibpairs, then power to detect linkage is increased by stratification of the data. However, if no difference in linkage evidence between subgroups exists, stratification by phenotype will reduce power to detect linkage. The majority of the sex-specific linkages observed in this study occur mainly in male-only affected families, in contrast to the excess of female-only affected families for both CD and UC. Regions of male-only linkage would therefore have low power to be detected in the unstratified data, as shown by the low LOD scores for ALL families in Table 1. Our stratification analysis involves multiple testing across phenotypes and families in a genome-wide linkage analysis. No correction for multiple testing has been used, but a conservative Bonferroni correction for simulation tests identifies only chromosomes 6p and 22 as showing significant evidence for sex-specific linkage. Some of the results may therefore represent type I errors, and similar analyses in other IBD genome search linkage studies are necessary to establish which of these sex-specific linkages can be confirmed.

Sex effects in complex disease susceptibility have been reported in other human auto-immune disorders: female specific linkage to chromosomes 2, 6 and 11 was observed in osteoarthritis.[20] Experimental animal models have also identified loci that exhibit sex-specific linkage in several complex diseases, for example in rheumatoid arthritis[35] and Type I diabetes,[36] although the molecular basis for such effects is unknown. It has been proposed that epigenetic factors play an important role in the pathogenesis of IBD,[37] and that sex effects are mediated by androgens and oestrogens. Hormones have a substantial effect on gene expression, and the hormonal differences between males and females could thus lead to differential expression of disease susceptibility genes in males and females.

In conclusion, we have identified several putative regions of sex-specific linkage in IBD, including novel candidate loci. Most importantly, we have shown that evidence for linkage in the HLA region is restricted to families with only male affected individuals. This sex-specific linkage occurs in both CD and UC families. Stratification by sex or other phenotypic factors may reduce etiologic heterogeneity and lead to progress in the search for susceptibility loci in complex diseases.

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