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Genetic analysis in Finnish families with inflammatory bowel disease supports linkage to chromosome 3p21

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In inflammatory bowel diseases (IBD), certain chromosomal candidate loci have been repeatedly identified by independent studies in different populations. To investigate the contribution of the loci on chromosomes 1, 3, 7, 12, 14, and 16 to the susceptibility of IBD in Finnish population, where the predominant feature is the excess of ulcerative colitis (UC) families compared to Crohn's disease (CD) families, we carried out linkage analyses using 93 Finnish, multiply-affected IBD families. We observed nominal evidence for linkage to chromosome 3p21, consistent with earlier reports. The lod scores peaked at D3S2432, with a maximum two-point lod score of 1.68 ($P=0.0027$). In addition, we studied whether risk of IBD is associated with functional variants of two positional candidate genes; the chemokine receptor *CCR5* gene on chromosome 3p21 and the interleukin-4 receptor α -subunit gene (*IL4RA*) on chromosome 16. We did not find any significant correlation between a 32-bp deletion variant of *CCR5* or a single nucleotide change A1902G (Gln576Arg) of *IL4RA*, and IBD phenotypes, with the exception that in the UC group homozygosity for the G1902 allele of *IL4RA* was less frequent (0.019 vs 0.049, $P=0.038$). In conclusion, our study, carried out in a genetically homogenous population, suggests that chromosome 3 may contain a susceptibility gene for IBD. *European Journal of Human Genetics* (2001) 9, 328–334.

Keywords: chromosome mapping; genetic predisposition to disease; inflammatory bowel diseases; linkage; lod score; microsatellite repeats

Introduction

Studies of the genetic susceptibility to inflammatory bowel disease (IBD) have resulted in the discovery of several chromosomal areas of interest during recent years. The most encouraging results concern Crohn's disease (CD) which is linked in most studied populations to chromosome 16 pericentromeric region (IBD1 locus),^{1–10} and in

two independent studies to chromosome 14.^{11,12} Evidence for a relatively strong genetic basis of CD has also earlier been obtained by epidemiological data. For instance, the reported concordance rate of the disease between monozygotic twins is greater for CD (20–44%) than ulcerative colitis (UC, 6–16%).¹³ Linkage to another promising IBD locus (IBD2) on chromosome 12 has been supported by many independent studies, especially in UC families.^{2,7,10,11,14} The locus on chromosome 6p referred to as IBD3 locus is not as definitively replicated as the IBD1 and IBD2 loci.^{15–17} Linkage to chromosome 1 in American Chaldean population is the only linkage report, so far, from an isolated population,¹⁸ that strengthens and restricts the assignment of the susceptibility locus previously reported in outbred population.⁶

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The Finnish population represents a genetic isolate and may provide advantages in genetic studies on complex diseases. Based on epidemiological data from the south-central part of the country, the prevalence of UC has been estimated as 121/100 000 and that of CD as 44/100 000 among the Finns.¹⁹ As the first part of a systematic search for IBD susceptibility genes in the Finnish population, we conducted a linkage study on the most attractive candidate loci on chromosomes 1, 3, 7, 12, 14 and 16 in 93 IBD families. In addition, functional alterations in two positional candidate genes,^{20–22} one encoding the chemokine receptor CCR5 on chromosome 3p21 and the other encoding the interleukin-4 receptor alpha subunit (IL4RA) on chromosome 16, were analysed in a separate case-control study of unrelated individuals.

Materials and methods

Family ascertainment and sample collection

We recruited IBD patients from all over Finland using strict diagnostic criteria based on typical clinical characteristics as well as diagnostic findings on both visual inspection by successful ileocolonoscopy and histopathological examination of biopsy specimens. The clinical diagnosis of each patient was confirmed by at least two members of the research group. Individual families consisting of at least two siblings with a diagnosis of either CD, UC or indeterminate colitis, were identified and collected. Families with a history of IBD in the relatives of both parents of the sib-pairs were excluded. Eventually 93 IBD families, containing a total of 124 sib-pairs, were accepted into the linkage study (Table 1). Both parents were available for analysis in 38% of the 93 families. The definition for a mixed family denotes sibships with both UC and CD; only in five families one of the siblings had indeterminate colitis. In the association study, we genotyped 155 familial (with a known 1st degree affected relative) IBD patients, 280 sporadic IBD patients, and 183 healthy controls for the common CCR5 and IL4RA polymorphisms. The controls were healthy voluntary blood

donors (91 men, 92 women) aged 40 to 50 years (mean 45 years) collected from the Finnish Red Cross Blood Service, and had their residencies in the capital region of Finland where approximately 70% of the patients were living. In addition, we genotyped the whole family cohort (Table 1) for the IL4RA polymorphism. Informed written consent was obtained from all study participants, and the study protocol was accepted by the Ethical Review Committee of Helsinki University Central Hospital.

Genetic analysis

All the blood samples for DNA isolation were collected in accordance with the Helsinki declaration. Microsatellite markers spanning the IBD candidate regions originated from the marker collections of Génethon and the Cooperative Human Linkage Center (CHLC) (<http://lpg.nci.nih.gov/CHLC/>). Genotyping was performed using 96-well format both in PCR with fluorescently labelled primers and in electrophoresis of the pooled amplified products. Data collection was based on the use of an ABI377 automated DNA sequencer (Perkin Elmer, Applied Biosystems), and data analyses were performed with the Genescan 2.0 and Genotyper software programs. The genotype data were processed using LINKBASE, the Windows-based database system designed to connect the genotype data produced by automatic sequencers to linkage analysis programs (<http://www.ktl.fi/molbio/software/linkbase>).

Analyses of candidate gene polymorphisms

Genotyping of the CCR5 insertion/deletion polymorphism was performed by amplifying DNA by PCR using primers 5'-TGT T T G C G T C T C T C C C A G - 3' and 5'-C A C A G C C C T G T G C C T C T T - 3' resulting in a 232 bp product for the wild type allele and a 200 bp product for the mutant allele ($\Delta 32$) upon agarose gel electrophoresis. The A1902G polymorphism of the IL4RA gene was genotyped according to the protocol described by Noguchi *et al.*²³

Statistical analysis

In order to take advantage of the extended nature of the family cohort, two-point pairwise linkage analyses using affecteds-only dominant and recessive pseudomarker analysis were performed,²⁴ using the MLINK program of the LINKAGE package,²⁵ FASTLINK version 2.2.²⁶ This method is analogous to comparison of the proportion of alleles affected relatives share identical by descent in families of general structure.²⁴ A two-point analysis was preferred to multipoint analysis because the latter is more sensitive to misspecification of intermarker distances and allele frequencies.²⁴ *P* values <0.05 are presented in the tables, to allow the comparison of lod scores and non-parametric lod (NPL) scores that were used in most of the previous IBD reports. We also present for each marker the NPL scores calculated by using the Genehunter 2.0 beta software.²⁷ Multipoint NPL analyses for each chromosome were also performed but they

Table 1 Structure of the family cohort

Sibship size	UC	Mixed	CD	All
2	35	13	15	63
3	5	3		8
4	1	1		2
2+3 ^a		1		1
2+ARP ^b	6	5	1	12
3+ARP	3			3
ARPs only	3	1		4
Total families	53	24	16	93
Total sib-pairs	71	37	16	124

^aTwo and three sibs in successive generations.

^bARP denotes for affected pair; uncle or aunt/niece or nephew pair, grandparent/grandchild pair, half-sibling pair, or cousin pair.

did not significantly add information content on the families that we studied. The identity-by-descent status in the affected sib-pairs was analysed by the SIBPAIR program. Linkage disequilibrium was tested with a haplotype relative risk test (HRR-test) and genetic heterogeneity using HOMOG, as incorporated in the ANALYZE package.²⁸ For each marker, the allele frequencies were estimated from the total study sample.

Power analyses were performed to estimate the probability of detecting linkage under various gene effect sizes, eg, proportion of linked families (α), using the SIMSIBS program which was also used for the simulation analyses in the articles by Terwilliger *et al.* and Göring *et al.*^{24,29} The extended nature of our family cohort was not taken into account, but the estimates were based on the total amount of sib-pairs of our family cohort. On the other hand, these power calculations assume that genotypes were known for all individuals. We assumed the average marker heterozygosity to be 80% and recombination fraction of 0.05 between marker and disease loci. Based on the calculations, under this sample size (only sibships included) our family cohort could permit detection of major loci ($\alpha > 0.40$) contributing to susceptibility to IBD (data not shown). The statistical analysis for association in the candidate gene study was performed by using Pearson chi-square statistics with the BMDP software (BMDP Statistical Software Inc., Los Angeles, CA, USA).

Results

Linkage analyses of candidate chromosomal loci

The family cohort consisted of 53 UC families, 16 CD families, and 24 mixed families, containing 229 affected individuals (114 male and 115 female) and 145 non-affected individuals. The majority of the patients (68.4%) had diagnosis of UC ($n=156$). Five patients had the diagnosis of indeterminate colitis and 68 patients the diagnosis of CD. The age of the patients ranged from 12 to 86 years (mean and median, 47 years). The mean age of disease onset (age at diagnosis) was slightly lower in CD families (mean 28.4 years, median 27.5 years) compared to UC families (mean 33 years, median 30 years) or mixed families (mean 33 years, median 32.5 years).

Nominal evidence for linkage was observed on chromosome 3p21. The lod scores peaked at D3S2432 where the maximum two-point lod score obtained by the recessive pseudomarker analysis was 1.68 ($P=0.0027$, $\theta=0.26$) and the NPL score 1.89 ($P=0.029$) (Table 2). In the multipoint NPL analysis the maximum lod score was 1.71 ($P=0.043$) near D3S2432. As the family composition did not represent a pure sib-pair material, the lod score in affected sib-pair analysis (ASP) for the total study sample (lod score=1.48; $P=0.0045$) was slightly less significant. The marker D3S1619, approximately 3 cM telomeric to D3S2432, gave a maximum lod score of 0.98 ($P=0.017$) in the recessive pseudomarker linkage analysis. Stronger evidence for linkage was obtained in the

mixed group where the lod score was 1.47 ($P=0.0047$, Table 3). In the UC group at D3S1619, the haplotype relative risk test gave a P -value of 0.019 (0.057 for the total family cohort and 0.03 for the UC-mixed group), which reflects a minor overrepresentation of the allele 9 in the affected chromosomes (23%) compared to control chromosomes (10%).

The analyses carried out for subgroups defined by disease onset (age <30 or <22 years) or parents' birth places (Southwestern vs Northeastern Finland) did not give any more significance to the linkage analysis (data not shown). Multipoint non-parametric analyses did not add information content on the families that we studied: for 3p21 the highest two-point NPL score was 1.89 whereas the highest multipoint NPL score was 1.71 near D3S2432. In the disease-specific analyses, CD families did not seem to contribute to the linkage evidence (Table 3), which was also evident in the multipoint NPL analyses on 3p21 (Figure 1). On chromosome 16, in the affecteds-only analysis the marker D16S407 gave a maximum two-point lod score of 0.93 ($P=0.019$) and a NPL score of 1.63 ($P=0.049$) for the CD group consisting of 16 families with two affected sibs (Table 2). Here also, the multipoint NPL analysis did not give any more significance to the results (data not shown). In other groups, and in the total study group, the lod scores for chromosome 16 markers remained non-significant. The non-significant results for other loci on chromosomes 1, 7, 12 and 14 are presented in Table 2.

Association analyses of the functional variants of CCR5 and IL4RA

The genotype or allele frequencies of the CCR5 insertion/deletion polymorphism did not differ significantly between the unrelated inflammatory bowel disease (IBD) patients and controls (Table 4). Furthermore, no significant differences in these frequencies emerged, when calculations were carried out in different disease types, or when sporadic IBD was compared to familial IBD. The IL4RA allele (A1902G) frequencies were similar in the IBD group and the control group, but the genotype frequencies showed minor differences in these two groups (Table 4). There was no significant deviation of the observed genotype frequencies from the Hardy-Weinberg's equilibrium in the patient or control groups ($P > 0.10$). In the IBD group, the homozygous genotype for the variant allele 1902G was less frequent (0.019 vs 0.049, $P=0.038$) and the heterozygous genotype more frequent (0.36 vs 0.28) than the corresponding frequencies in the controls. No excess transmission of either of the IL4RA alleles to affected individuals was observed in the HRR-test of the whole family cohort ($P=0.33$).

Discussion

The present study supports the assumption that one of the genes conferring increased risk of IBD is located on

Table 2 Two-point linkage analysis of the candidate IBD loci

Chr	Marker	cM	Z_{max}			NPL score
			Rec	Dom	ASP	
1	D1S552	45.3	0.52	0.90 (0.021)	0.31	1.09
	D1S234	55.1	0.00	0.06	0.00	-0.29
3p	D3S2396	46.5	0.07	0.00	0.03	0.02
	D3S1609	49.5	0.10	0.20	0.30	0.32
	D3S2432	51.0	1.68 (0.0027)	1.12 (0.012)	1.48 (0.0045)	1.89 (0.029)
	D3S1619	55.4	0.98 (0.017)	0.50	0.60 (0.048)	1.34
	D3S3521	58.0	0.09	0.21	0.03	0.37
	D3S2304	63.0	0.02	0.13	0.05	0.16
	D3S1573	67.0	0.23	0.25	0.06	0.51
	D3S1766	74.0	0.15	0.25	0.02	0.41
3q	D3S3053	195.0	0.04	0.23	0.007	0.70
	D3S2427	207.0	0.75 (0.031)	0.57	0.64 (0.043)	1.40
7	D7S484	55.6	0.07	0.24	0.00	0.58
	D7S519	70.0	0.12	0.008	0.05	-0.23
	D7S669	90.9	0.14	0.27	0.09	0.68
	D7S524	98.0	0.29	0.36	0.30	0.84
	D7S527	109.0	0.02	0.26	0.00	0.75
12	D12S368	67.3	0.00	0.00	0.00	-0.002
	D12S1586	70.0	0.00	0.00	0.00	-0.90
	D12S1662	76.4	0.0004	0.00	0.00	0.09
	D12S83	76.5	0.00	0.01	0.00	-0.25
	D12S1655	76.6	0.00	0.00	0.00	-0.45
	D12S43	86.0	0.00	0.00	0.002	-0.20
	D12S95	98.0	0.01	0.00	0.00	0.16
14	D14S261	6.5	0.02	0.00	0.00	-0.42
16	D16S407	16.7	0.38	0.30	0.45	1.54
	CD-only		0.93 (0.019)	0.76 (0.031)	1.04 (0.014)	1.63 (0.049)
	D16S403	43.0	0.02	0.16	0.02	0.11
	D16S3131	49.0	0.006	0.11	0.00	-0.25
	D16S411	57.8	0.04	0.13	0.003	0.52
	D16S419	65.6	0.01	0.13	0.00	0.15
	D16S408	72.6	0.0002	0.28	0.09	0.70
	D16S503	81.8	0.34	0.37	0.34	1.17
	D16S516	98.3	0.00	0.00	0.00	-1.44

Note. Maximum two-point lod scores (Z_{max}) obtained by using the pseudomarker analysis with recessive (Rec) or dominant model (Dom) and by affected sib-pair analysis (ASP). Two-point NPL scores are also shown. *P* values < 0.05 are indicated in parentheses. The genetic distances (cM; centiMorgan) are based on human GeneMap (<http://www.ncbi.nlm.nih.gov/genemap>), except the distances for chromosome 1, which are derived from the comprehensive Marshfield genetic map. Only the significant results obtained in the subgroup analysis for disease type are presented. For the disease-specific analyses of chromosome 3 markers see Table 3.

chromosome 3p21. Our supportive linkage assignment overlaps the original linkage region on 3p21 reported by Satsangi *et al.*² So far, only the genome scan by Rioux *et al.* has shown multipoint lod scores over 1.5 on this region.¹⁷ Satsangi *et al.* observed the strongest evidence of linkage in CD families,² while in our material the greatest evidence of linkage seems to be from the mixed families, which may reflect greater average size of the families in this group. The chromosome 3 region has also raised attention in an association study by Pokorny *et al.*³⁰ who reported an increase of a specific haplotype, constructed from the alleles of the marker D3S1611 and the HNPCC (hereditary non-polyposis colon cancer) gene *MLH1*, in IBD patients. The chromosome 3p21 region seems intriguing as it harbours, in addition to *MLH1*, other interesting candidate genes including those encoding a cluster of chemokine receptors, and a G protein subunit α_{12} (GNAI2) the deficiency of which causes an UC-like phenotype in knockout mice.³¹

A striking feature of the composition of our family material was the relative excess of UC families to CD families. The threefold difference in prevalence rates of UC to CD, similar to the situation observed elsewhere, can not wholly explain this. Whether this means that different combinations of susceptibility genes for IBD exist in the Finns in comparison to other populations remains to be studied. Due to the nature of complex genetics and our restricted sample size regarding especially the CD families, failure to replicate previous findings on other chromosomes than 3p21, however, does not exclude the possibility that these regions would harbour Finnish IBD genes. We take our findings on the pericentromeric markers of chromosome 16 as preliminarily interesting as positive lod scores with 16 CD families for the marker D16S407 were observed.

We selected two candidate genes showing common functional variation, one in chromosome 3 (chemokine receptor *CCR5*) and the other in chromosome 16 (*IL4RA*),

Table 3 Disease-specific, two-point linkage analysis of IBD on chromosome 3p21

Marker	Disease type	cM	Z_{max}			NPL score	P value	
			Rec	Dom	ASP		HRR	TdT
D3S2396	UC families	46.5	0.00	0.00	0.00	-0.69	0.50	0.42
	CD families		0.03	0.02	0.04	0.27	0.50	0.34
	Mixed families		0.30	0.09	0.19	0.85	0.50	0.23
	All		0.07	0.00	0.03	0.02	0.50	0.32
D3S1609	UC families	49.5	0.00	0.10	0.06	0.06	0.50	0.073
	CD families		0.00	0.00	0.00	-0.20	0.50	0.37
	Mixed families		0.69	0.32	0.66	0.72	0.50	0.32
	All		0.10	0.20	0.30	0.32	0.50	0.062
D3S2432	UC families	51.0	0.92	0.84	0.92	1.49	0.50	0.43
	CD families		0.08	0.10	0.12	0.63	0.50	0.24
	Mixed families		0.78	0.22	0.46	0.98	0.10	0.0097
	All		1.68	1.12	1.48	1.89	0.50	0.31
D3S1619	UC families	55.4	(0.0027)	(0.012)	(0.0045)	(0.029)		
	CD families		0.43	0.16	0.31	0.82	0.019	0.030
	Mixed families		0.00	0.00	0.00	-0.41	0.50	0.50
	All		1.47	1.56	0.70	1.78	0.50	0.50
D3S3521	UC families	58.0	(0.0047)	(0.0037)	(0.036)	(0.040)		
	CD families		0.98	0.50	0.60	1.34	0.057	0.025
	Mixed families		(0.017)		(0.048)			
	All		0.14	0.12	0.08	0.27	0.50	0.44
D3S2304	UC families	63.0	0.00	0.00	0.00	-0.24	0.50	0.25
	CD families		0.00	0.00	0.00	0.53	0.50	0.40
	Mixed families		0.04	0.31/0.45 ^a	0.0007	0.53	0.50	0.40
	All		0.09	0.21	0.03	0.37	0.50	0.33
D3S1766	UC families	63.0	0.00	0.12	0.00	-0.12	0.50	0.039
	CD families		0.00	0.00	0.00	-0.29	0.50	0.50
	Mixed families		0.63/1.04 ^a	0.06	0.33	0.76	0.50	0.037
	All		(0.044/0.014 ^a)	0.13	0.05	0.16	0.50	0.0074

Note. Maximum lod scores (Z_{max}) for affected sib-pair analysis (ASP) and the pseudomarker analysis with recessive (Rec) and dominant (Dom) model. NPL scores are shown for comparison. *P* values <0.05 are indicated in parentheses. In these complex families TdT is a test for linkage, and the haplotype relative risk (HRR) test measures allelic association.

^aLod score when assumption of heterogeneity gives more significance to the analysis.

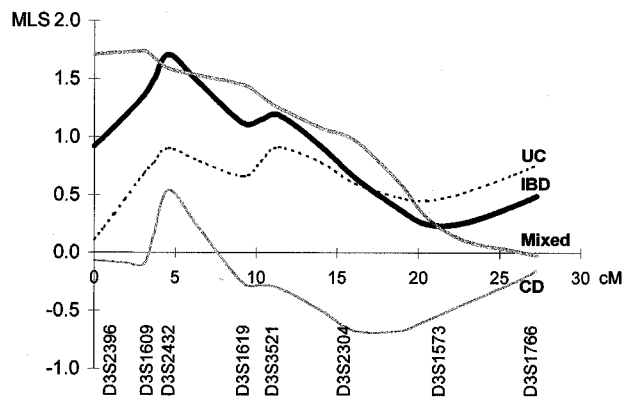


Figure 1 Multipoint non-parametric linkage analysis on chromosome 3p21 for the total family cohort (IBD) and disease-specific subgroups (UC, CD, and mixed families). Multipoint lod scores (MLS) were calculated by using Genehunter 2.0 version beta.

for a genetic association study. *CCR5* has been shown to be abundantly expressed in colon epithelial cell lines and regulate proinflammatory chemokine production.³² A natu-

rally occurring variant of *CCR5* exists, where a 32-bp deletion results in synthesis of a non-functional receptor due to generation of a premature stop codon. This $\Delta 32$ variant was first identified in individuals resistant to HIV-1 infection, and was subsequently associated with reduced risk of asthma,²² possibly pointing to less liability to chronic inflammation. We did not find any significant, positive or negative, association between risk of IBD and the $\Delta 32$ variant of *CCR5*, and there was no specific IBD group with an apparent loss of the $\Delta 32/\Delta 32$ homozygotes (Table 4).

A nucleotide change A1902G of *IL-4* receptor α -subunit, causing the replacement of glutamine (Q576) with arginine (R576), has been reported to result in increased *IL-4* signaling and increased IgE production *in vivo*, and was initially suggested to be associated with atopy.²¹ Interestingly, a defect in responsiveness to *IL-4* has been proposed in IBD.²⁰ Accordingly, we hypothesised that the G1902 allele of *IL4RA* could be less prevalent in IBD patients than in normal controls. Although the G1902 allele frequencies were similar in the IBD group and the controls, the homozygous G1902/G1902 genotype was slightly less common in UC patients than in controls (Table 4). Whether this is an indication that the *IL4RA* G1902 allele, when present in a homozygous form,

Table 4 The frequencies of the *CCR5* ($\Delta 32$) and *IL4RA* (A1902G) genotypes in Finnish IBD patients and controls

Group	n	CCR5 Genotypes			CCR5 Allele frequencies		n	IL4RA Genotypes			IL4RA Allele frequencies	
		wt/wt ^a	wt/ $\Delta 32$	$\Delta 32/\Delta 32$	wt	$\Delta 32$		AA	AG	GG	A	G
Control subjects	172	129	39	4	0.86	0.14	183	122	52	9	0.81	0.19
Familial patients												
UC	104	75	25	4	0.84	0.16	97	66	30	1	0.84	0.16
CD	51	34	16	1	0.82	0.18	54	31	21	2	0.77	0.23
Total	155	109	41	5	0.84	0.16	151	97	51	3	0.81	0.19
Sporadic patients												
UC	90	62	25	3	0.83	0.17	88	50	37	1 ^b	0.78	0.22
CD	190	148	39	3	0.88	0.12	181	113	64	4	0.80	0.20
Total	280	210	64	6	0.86	0.14	269	163	101	5 ^b	0.79	0.21
All patients												
UC	194	137	50	7	0.84	0.16	185	116	67	2 ^b	0.81	0.19
CD	241	182	55	4	0.87	0.13	235	144	85	6	0.79	0.21
Total	435	319	105	11	0.85	0.15	420	260	152	8 ^b	0.80	0.20

^awt denotes for the (wild-type) non-deleted allele.

^bP=0.038 for comparison of genotype distribution between controls and patients.

may exert a weak protective influence against risk of UC in specific individuals awaits additional studies using other data sets. A recent study by Olavesen *et al.* did not, however, reveal any significant association between risk of IBD and several intragenic single-nucleotide polymorphisms of the *IL4RA* gene.³³

In conclusion, our study carried out in the relatively isolated Finnish population further strengthens the linkage to 3p21 reported in more outbred populations and thus supports the assumption that chromosome 3 contains a gene or genes conferring susceptibility to IBD.

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