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ORIGINAL ARTICLE

Follow-up investigation of 12 proposed linkage regions in multiple sclerosis

BM Herrera[1,2], MZ Cader[1,2], DA Dyment[1,2], JT Bell[1], SV Ramagopalan[1,2], MR Lincoln[1,2], S Orton[1,2], MJ Chao[1,2], AD Sadovnick[3] and GC Ebers[1,2]

[1]*The Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK;* [2]*Department of Clinical Neurology, Radcliffe Infirmary, Oxford, UK and* [3]*Department of Medical Genetics and Faculty of Medicine Division of Neurology, University of British Columbia, UBC Hospital, Vancouver, British Columbia, Canada*

*Multiple sclerosis (MS) is an autoimmune disease with overwhelming evidence for genetic determination, and for which a maternal parent-of-origin effect has been reported. As with many complex diseases, multiple suggestive linkage signals have been observed. However, the only unambiguous association and linkage identified to date is with alleles of the human lymphocyte antigen (HLA) class II region. We have now carried out high-density microsatellite genotyping for 12 of the most promising regions (1p, 1q, 2q, 4q, 5p, 9q, 10p, 11p, 12q, 17q, 18p, 19p) from a whole-genome scan in 552 affected sibling pairs. This has been carried out in 194 families containing avuncular pairs. These permit examination of parent-of-origin effects in non-colineal pairs when divided into likely maternal and paternal trait transmission. The results do not confirm any non-major histocompatibility complex linkage in the overall subset nor in the maternal, paternal or HLA-DRB1*1501 subsets. We were able to establish exclusion for a locus with $\lambda_{AV} \geqslant 1.3$ for all the previously suggested regions. These results again raise the possibility that the paradigm of multiple genes of small individual effect used to justify genome searches in MS is incorrect.*
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Introduction

Genetic susceptibility to multiple sclerosis (MS) is strongly implied by both epidemiological and familial studies. Family studies assessing risks to twins, adoptees, half-siblings, step siblings, conjugal pairs and offspring of conjugal matings of patients with MS have demonstrated marked familial aggregation of the disease, which cannot be attributed to the familial environment. The successive addition of environmental sharing at all ages cannot be demonstrated to add any risk.[1] In contrast, the risk of the disease increases with each successive addition of genetic sharing.[2–4]

For example, the age-adjusted recurrence risk for a half-sib is 1.89%,[3] 3.11% for a full-sib and 3–5% for dizygotic twins, in contrast to 20–30% for monozygotic twins.[5]

Although the aetiology of the disease is still unknown, the evidence that MS risk follows a complex non-Mendelian inheritance pattern is considerable. Although there is no ambiguity about a major histocompatibility complex (MHC) class II association which lies primarily, if not entirely, with alleles at human lymphocyte antigen (*HLA*)-*DRB1* and *HLA-DQB1*, the involvement of these loci is likely to be complex.[6] The overall contribution of HLA class II now needs to be reconsidered in this new light. Importantly, in terms of pathogenesis, the general pattern of MHC association follows that of other diseases in which the evidence for an autoimmune pathogenic component is even stronger, for example, type I diabetes. Analogizing with spontaneous and induced autoimmune disorders in experimental animals, the expectation might be that many genes would influence the complex processes of immune tolerance, inflammation, repair and others. In anticipation of the existence of a large number of genes, many linkage and association studies have been carried out. Conforming to this expectation, many suggestive linkages have been reported. This is well exemplified by published evidence for linkage and association on all chromosomes (Table 1), although the low level of confirmation has aroused appropriate concerns.[7] At present, there are no generally agreed-upon linkages outside the MHC. If haplotype sharing in affected relative pairs is used as a measure of the degree of contribution of a given locus, the MHC association accounts for only 14% of the observed familial aggregation of MS.[8] Recent data suggest that complexity at this locus may make this calculation erroneously low.[6]

In MS, as in other complex diseases, whole-genome linkage scans have been employed in order to discover genes of small effect that determine susceptibility.

Correspondence: Professor G Ebers, Department of Clinical Neurology, Radcliffe Infirmary, Woodstock Road, University of Oxford, Oxford OX2 6HE, UK.
E-mail: george.ebers@clneuro.ox.ac.uk
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Table 1 Previous findings from other large-scale linkage studies in MS

Chromosome	*Regions of interest identified in previous linkage scans*
1	1p,[24–26]1p34,[27]**1p31**,[28]1p21,[29]1cen DR15(−),[24] 1q,[26]1q11–24,[30]1q31,[31]**1q42**,[32]1q43–44,[33]1q44,[32] [16]
2	2p23,[34] 2p16.3,[35] 2p13,[33] 2p11,[36] 2q,[26] 2q24–32,[30]2q27,[28] ,2q33,[36] **2q36**,[32] **2q37**,[28]
3	3p26.3,[30] 3p21,[37] 3p14,[27]3p11,[36] 3pDR15(−),[24,26] 3q13–24,[33]3q21,[36]3q21.1,[30]3q22.2,[35]3q22–24[34]
4	4cen,[38]4q12,[30]4q24,[33]4q26–28,[33]**4q31–34**,[33] 4q31-qter[34]
5	5p15.3,[35] 5p15,[36,28]5p14–p12,[38] 5p14,[28] 5p11,[36] 5p,[39,40] 5q,[26]5q11,[29,37]5q11–13,[33]5q12,[36] 5q13–23,[34] 5q14[36], 5q33,[32] [17]
6	6pter,[32]6p25.3,[30]6p22.1,[35]6p21–22,[30,29,34,38,36,39,37,15] follow-up to,),[34,28,17] [28]6p,[25]6p,[26] 6q21,[30] 6q22,[32] 6q25,[40] 6q26,[33] 6q27,[34,33,15] (follow-up to),[34]6qtel[37]
7	7p15,[36,29]7pDR15(−),[24] 7q11-q21,[34] 7q21-22,[15,34](follow-up to),[34] 7q32–34,[34] 7q33–35,[33]7q34–36[41]
8	8p23–21[33]
9	9pter-p22,[34]9q,[26]**9q21**,[33,28] ,9q22.1,[42]9q34.3,[30,34]
10	**10p15**,[30,43] 10p12–13,[30]10cen,[32] 10q21–q22,[34]10q23[31]
11	**11p15**,[34,31] 11p15.5,[30]11q22.3,[35][11]tel[38]
12	12p13–12,[41] 12p13,[37]12p12,[29,44] **12q21.3**,[30]12q23,[34,40] 12q23–24 DR15+,[15](follow-up to),[34]12q24-qter,[34]
13	13q,[26] 13q31–32,[33]13q33–34,[34]DR15+,[15](follow-up to)[34]
14	14qDR15(−),[24] [25]
15	15q21[32]
16	16p13.3,[30] 16p13,[37,33]16p13 DR15+,[15] (follow-up to),[34]16p11,[33]16p,[26]16p13-cen,[34]16q,[26]16q23–24[33]
17	17p13,[33]17q,[24] (follow-up to),[29] [25]17q11,[37]17q12,[36] 17q21meta-analysis,[45] **17q22**,[29,37,28],**17q22–24**,[38] 17q22–q24,[46] (follow-up to),[38]**17q23**,[17] **17q24.2**,[28] **17q25.3**[30]
18	**18p11**,[34,33,28] 18p,[26]
19	**19p13**,[17,34] 19q13 DR15+,[15](follow-up to),[27,34]
20	20p12–11[33]
21	None
22	22q,[26]in DR15(−),[24] (follow-up to,)[29]22q12–13,[30] 22q13,[29] meta-analysis,[45]22q13.1[36]
X	X,[24] X marker,[42] Xp22.3,[30]Xp21–11,[33]Xp11.4,[35,29,36,33] Xq[25]Xq21,[29]Xq23–28,[33]

Abbreviation: MS, multiple sclerosis.
The regions investigated in this study are emphasized in bold typeface.
Key to references: [35]Ebers *et al*. (1996), [29] Sawcer *et al*. (1996), [34]Haines *et al*. (1996), [38]Kuokkanen *et al*. (1997), [24]Chataway *et al*. (1998), [36]D'Alfonso *et al*. (1999), [39] Oturai *et al*. (1999), [40]Xu *et al*. (1999), [37]Transatlantic-Multiple-Sclerosis-Genetics-Cooperative (2001), [32]Broadley *et al*. (2001), [31] Coraddu *et al*. (2001), [28] Dyment *et al*. (2001), [41]Xu *et al*. (2001), [30]Akesson *et al*. (2002), [33]Ban *et al*. (2002), [15]Haines *et al*. (2002), [46]Saarela *et al*. (2002), [44]Vitale *et al*. (2002), [42]Modin (2003), [27] Pericak-Vance *et al*. (2004), [25]Hensiek *et al*. (2003), [45]GAMES and Cooperative (2003), [43]Akesson *et al*. (2003), [26]Kenealy *et al*. (2004), [9]Dyment *et al*. (2004), [17] Sawcer *et al*. (2005), [16]Kenealy *et al*. (2005).

Recently, we carried out a two-stage genome scan of 552 sibling pairs from 442 families using 524 microsatellite markers. Regions on chromosome 1p31, 2q27, 5p15, 9q21, 17q and 18p11 showed suggestive linkage in the replication analysis.[9] A number of epidemiological studies have described parent-of-origin effects operating in MS. In a study of MS patients with half-siblings (one parent in common), it was observed that maternal half-siblings are at significantly increased risk compared to paternal half-siblings.[3] The risk for maternal half-sibs was estimated to be 2.35% compared to 1.31% for paternal half-sibs. These observations show a maternal effect that had also previously been indicated in a data set of affected parent–child pairs where father–son transmissions were underrepresented.[10] A paternal parent-of-origin effect has also been suggested.[10,11] One underutilized approach to the study of parent-of-origin effects is the use of affected avuncular pairs (aunt/uncle–niece/nephew – AUNN). Here, maternal or paternal transmission can be studied by the avuncular relationship to the affected proband.

In light of these observations, we have carried out dense genotyping of 12 promising linkage regions (on chromosomes 1p, 1q, 2q, 4q, 5p, 9q, 10p, 11p, 12q, 17q, 18p and 19p) suggested by our own and other genome scans (see Table 1). Genotypes were collected from a novel cohort of AUNN ($n=194$) to assist in fully evaluating parent-of-origin effects in genetic linkage.

Results

Genotypes from 194 families containing affected AUNN pairs were analysed for 12 genomic regions (average marker spacing of 4.19 cM). No statistically significant allele frequency differences between maternal and paternal families were found, or between the carrier fathers and the carrier mothers. Linkage analysis with single-point and multipoint methods within these 12 regions of interest did not provide any evidence for linkage exceeding a threshold set to logarithm of odds (LOD) >3.7 [12] (Table 2). No regions were suggestive of linkage for this data set (LOD$\geqslant$2.3). Stratification of the families according to a maternal/paternal route of transmission produced a suggestive region of linkage; the paternal subset for chromosome 2q36.3–37.3 produced a single-point NPL $=3.21$ ($P_{\text{uncorrected}}=0.00006$) for marker D2S2204. However, multipoint analysis reduced this to a modest Non-parametric lincake (NPL) of 0.87 ($P=0.02$), and in the maternal families, the multipoint NPL for this region was -0.07. Furthermore, correction for multiple testing removes statistical significance for linkage.

Stratification of the families based on the presence of the *DRB1*15* allele produced only modest NPL scores. In single-point analysis, the peak NPL was 0.87 ($P=0.002$) for 18p11.32–21; however, the multipoint analysis reduced this score to 0.14. Multipoint analysis of chromosome 10p15.3–15.1 resulted in an NPL of 0.65 ($P=0.04$).

Table 2 Peak non-parametric LOD scores

Chromosome	*Region*	*Markers*	*Average spacing (cM)*	*All families* (n = 194)		*Maternal* (n = 107)		*Paternal* (n = 87)		*HLA-DRB1*1501+* (n = 67)		
				Peak two-point NPL	*Peak multipoint*	*Peak two-point NPL*	*Peak multipoint*	*Peak two-point NPL*	*Peak multipoint*	*Peak two-point NPL*	*Peak multipoint*	*Exclusion* λ_N
1	p32.2–p31.1	6	3	−0.07	−0.69	−0.18	−0.5	0	0	−0.04	−0.16	1.28
1	q42.12–q43	6	3.31	0.01	−0.04	0	−0.17	0.13	0.04	0.16	0.04	1.45
2	q36.3–q37.3	10	4.2	0.55	0.11	−0.15	0	3.21	0.87	0	0	2
4	q32–q34.3	5	4.08	0.03	−0.35	0.15	0.02	−0.02	−0.45	0.13	0.08	1.6
5	p15.33–p15.2	8	2.51	0.07	−0.02	0.49	0.16	0.04	−0.14	0.05	0.03	2.2
9	q21.11–q21.32	5	3.73	0.2	0	0.05	−0.01	0.3	0.06	0.14	−0.11	1.7
10	p15.3–p15.1	4	4.4	0.01	0.02	−0.01	0	0.3	0.02	0.34	0.65	1.65
11	p15.4–p15.1	5	1.97	0.19	−0.06	0.03	−0.12	0.14	0.17	0	0.01	1.47
12	q21.31–q23.1	5	13.19	0.35	−0.03	0.12	−0.14	0.56	0.16	0.7	0.29	1.5
17	q21.33–q25.3	12	3.1	0.03	−0.14	0.09	−0.08	0.26	0	0.06	−0.02	1.65
18	p11.32–p11.21	8	5.43	0	−0.22	0.21	−0.08	0.16	0.14	0.87	0.14	1.4
19	p13.3	4	1.42	0.07	0	−0.01	−0.12	0.19	0.28	0.54	0.08	1.6

Abbreviations: LOD, logarithm of odds.
Peak linkage scores in avuncular pairs in all families ($n = 194$), maternal ($n = 107$), paternal ($n = 87$), HLA-DRB1*1501 ($n = 67$). The peak score for region 2q36-3-37-3 is for marker D2S2204.

Exclusion mapping of our regions using EX-PAIRS was carried out and we were able to obtain exclusion LOD < -2 for all the regions for λ_{AV} values >1.3 (Table 2).

Discussion

The rationale for genetic linkage searches in MS is based on solid footing. The genetic epidemiology shows unambiguously that familial aggregation is genetic, although the penetrance in populations can be strongly influenced by broadly acting factors, which are likely climate related.[13] The twin data originally suggested oligogenic inheritance by virtue of the steep drop in concordance between monozygotic and dizygotic twins, but comparable population-based data on more distant relatives has remained relatively unavailable. MS is likely autoimmune in nature and the biological processes involved are under genetic control by numerous loci. By analogy, in experimental animals with spontaneous autoimmune disease, there are a number of potential targets where genetic regulation might exert an effect.

These considerations aside, the inability to find any clear linkages outside the MHC is unsettling.

Failure to clearly replicate linkage may be attributed to confounding factors such as the genetic and the clinical heterogeneity of the disease. The assumption generally made in genomic screens in MS is that all families have genetic susceptibility determined by the same genes. This reflects the failure to firmly establish earlier reports of two MS populations, linked and unlinked, and the inability to readily distinguish among families phenotypically. The clinical heterogeneity within pedigrees is hardly exceeded by that outside them.[14] However, many sources have shown that genetic complexity exists,[8,15] a very clear example being the MHC region where it has been shown that alleles are operating epistatically to modify the risk of MS.[6] Additionally, if the possibility of epistatic interactions between different loci throughout the genome is taken into consideration, the potential genetic complexity of MS becomes overwhelming. One way of tackling the heterogeneity challenge is to study phenotypically homogeneous groups; however, this approach significantly reduces sample size and therefore statistical power.

Our follow-up study, which to our knowledge is the first study investigating linkage to MS with respect to parent of origin, did not yield significant linkage to any of the 12 regions investigated. Analysis of the AUNN families after stratification by the presence/absence of the associated *HLA-DRB1*1501* allele did not result in significant or suggestive linkage. However, investigation of the paternal subset of our AUNN families resulted in a modest multipoint linkage (NPL = 0.87) for chromosome 2q36.3–37.3 (D2S2204). This marker also generated a peak single-point score, which was considerably higher. This finding is not unexpected, as two-point linkage analysis is known to be more prone than multipoint analysis to inflated or deflated LOD scores. Marker information content at D2S2204 was 32% in the single-point analysis. This was much lower than the marker information content of 0.63 that was reached for this position in the multipoint analysis. When partially linked or unlinked pedigrees become uninformative, the LOD score becomes deflated or inflated, respectively. This

problem is largely responsible for the phenomenon of having strong evidence for linkage to a single marker, without evidence, or with much weaker evidence, for the flanking markers. The peak LOD score obtained for this region in our whole-genome scan of 552 sibling pairs was an multipoint parametric LOD score (MLOD) = 2.27 for marker D2S2338. This marker is adjacent to the 2q35 region investigated in the American follow-up study,[16] using a very dense coverage of this region single-nucleotide polymorphism (SNP) markers in 173 multiplex families, although they also found no significant linkage to this region.

Although our linkage study failed to confirm linkage, the negative results using this novel AUNN data set have a twofold significance; first of all, they suggest that the regions studied do not harbour major susceptibility loci for MS that can be observed using a map of this resolution (~4.19 cM). Findings from the linkage study by the International MS Genetics Consortium,[17] which used a panel of 4506 SNPs across the genome in 730 multiplex families, and is so far the study with the highest marker resolution for MS susceptibility, failed to identify significant linkage outside of the MHC (maximum LOD score (MLS) = 11.66). This study provided suggestive MLS scores for the regions around 3p26, 5q33, 17q23 and 20p12, but these findings are not independent of their previous results. Secondly, these results show that the observed parent-of-origin effect in MS does not appear to operate through the 12 genomic regions investigated, although we are not able to exclude an important biological mechanism like imprinting. Furthermore, the potential theoretical advantage of using non-colineal relatives for linkage as in the AUNN group studied here was not realized. It might be assumed that common environmental influences would be less in an AUNN pair than in cohabiting sibling pairs.

Negative findings from these data and from other higher density linkage studies are unable to confirm any linkages for locations outside the MHC. These observations raise the possibility that the paradigm that has been used to justify genome searches may be incorrect for MS; it therefore remains possible that few genes or perhaps a single locus with much complexity may be operating in MS susceptibility. The use of whole-genome association scans and admixture studies of large clinically homogeneous cohorts may prove to be more fruitful in elucidating the genetic bases of MS. Future studies should also take into account observations related to the variable risk conferred from genotypes at the HLA class II region.[6] An alternative approach may necessitate a move beyond Mendelian genetics to directly investigate important epigenetic processes including imprinting and epistasis, which may well play a crucial role in complex disease but have so far been overlooked.

Materials and methods

Two hundred and fifteen families with affected AUNN pairs from the Canadian Collaborative Project on the Genetic Susceptibility to Multiple Sclerosis (CCPGSMS) database[18] were genotyped for this study. The unique structure of these pedigrees allow for the investigation of putative maternal and paternal routes of transmission by subdividing the families according to the sex of the parent that connects the proband to the affected aunt or uncle. Within these families, 107 were classified as being maternal, whereas 87 were classified as paternal. The remainder of the families ($n = 21$) included both maternal and paternal affected avuncular pairs and were therefore removed from the analysis in order to minimize confounding factors.

Automated genotyping of 78 fluorescent microsatellite markers, with an average marker heterozygosity of 73%, was carried out by DeCODE. Map distances were obtained from the DeCODE map with an average marker spacing of 4.19 cM. Pedigree checking was carried out using PEDCHECK.[19] Mendelian errors were investigated using PEDSTATS[20] and were dealt with on a case-by-case basis. Files were prepared for analysis using MEGA2 3.0.[21] Allele frequencies were estimated from all the genotyped individuals and Hardy–Weinberg calculations were performed for each marker. If parental genotypes were unavailable, MERLIN would calculate identical by descent (IBD) probabilities on the basis of population allele frequencies. Therefore, it was important to check whether there were significant differences between the maternal and the paternal families. Allele frequency heterogeneity between the different families and allele differences between carrier mothers vs carrier fathers were investigated using χ^2 tests.

Low-resolution *HLA-DRB1* genotypes were available for a subset of families. However, for the newly recruited AUNN families and families that had previously failed low-resolution typing, high-resolution *HLA-DRB1* typing was carried out. High-resolution genotyping requires 72 PCR reactions to be carried out per sample to amplify allelotypes corresponding to *DRB1*01*, *04*, *07–18* as well as amplicons for the *DRB3*, *DRB4* and *DRB5* genes. Each reaction contained primers to amplify a non-polymorphic region that served as a control segment (details of primer sequences and thermocycling conditions are available upon request). The amplified products were electrophoresed in 2% agarose gels containing ethidium bromide and visualized under UV light. Each gel was scored twice by independent observers.

Statistical analysis

Single-point and multipoint non-parametric linkage analysis was conducted using MERLIN.[20] MERLIN uses sparse inheritance trees for pedigree analysis. The analysis of dichotomous trait data implemented in MERLIN is essentially a model-free approach, the Kong and Cox[22] LOD score-type statistic is calculated on the basis of allele sharing.

Transmission disequilibrium test (TDT) analysis was performed on parent–child triads from the data set using the SIB_TDT facility from ASPEX.[23] The structure of the AUNN data set allowed us to subdivide the families into likely maternal and likely paternal transmission. Therefore, the data set was subdivided into smaller cohorts depending on the putative parental transmission (maternal = 107, paternal = 87). In order to test for a possible influence of the *HLA-DRB1*1501* allele, a third subset of families was created. HLA-DRB1*1501^{positive} families qualified for this subset when all the members of the avuncular pair carried the *HLA-DRB1*1501* allele; there were 67 families in this category.

Exclusion mapping was carried out using a novel programme developed especially for this type of

pedigree: EX-PAIRS (available upon request). This programme uses IBD output files generated by MERLIN[20] to compare the observed vs expected IBD probabilities for each of the possible avuncular pairs.

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