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ARTICLE

Association and linkage analysis of candidate chromosomal regions in multiple sclerosis: indication of disease genes in 12q23 and 7ptr–15

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Four recent genome-wide screen studies in multiple sclerosis (MS) identified a number of candidate regions for susceptibility genes in addition to the HLA complex in 6p21. However, none of these regions provided formally significant evidence for genome-wide linkage. We have investigated such regions in 46 Swedish multiplex MS families, 28 singleton families, 190 sporadic MS patients and 148 normal controls by parametric and nonparametric linkage and association analysis. One microsatellite marker, in 12q23, provided evidence for association in addition to suggestive transmission distortion and slightly positive linkage. In addition, a marker in 7ptr–15 showed a significant transmission distortion as well as a highly significant score in affected pedigree member analysis, but not quite significant deviations in association analysis. One of three markers in 5p, a region implicated in all four previous studies, showed a weakly positive lod score, but no other evidence of importance. Markers in 2p23, 5q11–13, 6q25, 7q21–22, 11q21–23, 13q33–34, 16p13.2, 18p11.32–23, Xp21.3 provided little or no evidence of importance for MS. In summary, these data support the importance of genome-wide screens in the identification of new candidate loci in polygenic disorders.

Keywords: multiple sclerosis; candidate regions; linkage analysis; genetic susceptibility

Introduction

Multiple sclerosis (MS), a chronic, inflammatory, autoimmune, demyelinating disorder of the central nervous system, is the most common cause of acquired neurological dysfunction arising in the second to fourth decades of life.[1,2] Knowledge of the pathophysiological basis of MS is incomplete. However, genetic epidemiology clearly indicates that genetic factors are of importance for the risk of MS, and a polygenic inheritance is most likely.[3,4] Although the *HLA* gene complex has been clearly identified as important, many patients fail to carry the associated haplotype and estimations suggest that this locus only contributes a minor part of the overall genetic susceptibility.[5] Recently, four groups[6–9] have completed full genomic screens using large numbers of microsatellite markers in high numbers of affected sibling pairs. Each screen used a different set of markers and identified mostly different chromosomal regions as being of possible importance. However, all studies confirmed the *HLA* gene complex in 6p21 and, in addition, suggested that a locus in 5p might be of importance. Thus, these data give some idea

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of where chromosomal regions suitable for hypothesis generation and further testing are located.[10] The aim of this study was to investigate whether a number of these non-HLA loci play any role for the risk of MS in the Swedish ethnic group, by applying a combination of statistical analyses on familial and non-familial MS.

Materials and Methods

Family Materials

DNA samples were obtained from 286 individuals, including 207 individuals from 46 multiplex MS families and 79 individuals from 28 singleton MS families (Table 1). MS was diagnosed on the basis of clinical and paraclinical investigations.[11] In cases where one or both parents were unavailable, DNA was obtained from unaffected siblings in order to allow derivation of parental haplotypes.

Case-control Materials

We investigated 190 unrelated Swedish patients with clinically define MS[12] and 148 ethnically matched healthy controls in association analyses (Table 1). Most of patients with MS showed signs of intrathecal immunoglobulin production in cerebrospinal fluid analysis. Swedish ethnicity of MS patients determined the clinical setting. As healthy controls only individuals with Swedish names were accepted.

Microsatellite Markers

Sixteen microsatellite markers were selected (based on their location within or close to regions in 2p23, 5p15, 5q11, 6q25, 7ptr, 7q21, 11q21, 12q23–24, 13q33, 16p13.2, 18p11, Xp21) that showed varying degrees of evidence for linkage in at least one of four previous genomic screen studies of MS affected sibling pairs.[6–9] The markers chosen were either identical to ones used in the previous studies or mapped closely to the markers used (Table 2). Of the present markers, 11 contained dinucleotide repeats, four were tetranucleotide repeats and one was a trinucleotide repeat. The locations of markers were obtained from public domain databases (http://www.hgmp.mrc.ac.uk, http://www.cephb.fr/ceph-généthon-map.html and http://cedar.genetics.soton.ac.uk/pub/).

Genotyping

All 624 individuals were genotyped for the 16 selected markers. Genomic DNA was extracted from peripheral blood leukocytes using a modified salting-out protocol.[13] Polymerase chain reaction (PCR) was employed in 7 μl reaction volumes. Primers were synthesised by Scandinavian Gene Synthesis AB and forward primers for each primer pair were labelled with 5'-FAM, HEX, or TET phosphoramidites (Köping, Sweden). Optimal PCR condition were usually 95°C for 1 min, 55°C for 40 s and 72°C for 1 min with 30 cycles, by a PCR cycler Gene Amp 9600 (Perkin Elmer), but varied somewhat among the markers. Genotyping was carried out in the semiautomatic GENESCAN/GENOTYPER system on Applied Biosystems 377 DNA sequencing equipment.[14] All samples of PCR product were diluted accordingly and 1.5 μl was combined with 0.5 μl internal size standard (Genescan –350 or –500 TAMRA, Applied Biosystems). The accuracy and reproducibility of automated sizing of fragments were further confirmed by randomly chosen repeated analyses of identical samples and two independent individuals reading data. Genotypes that were found to be inconsistent with inheritance or that were discordant between two analyses were either repeated or discarded.

Statistical Analysis

Two-point linkage analysis was performed with MLINK, version 5.10 of the LINKAGE package and FASTLINK, version 3.0.[15,16] Equal recombination fractions were used for males and females. The age-dependent penetrance values were 0.7, 0.35 and 0.1 and the disease gene frequency was 0.001.

Since the mode of inheritance for MS remains unclear and parametric linkage analysis can be highly sensitive to misspecification of the linkage model, several non-parametric analysis were performed:

1) Affected pedigree member (APM) analysis was performed by the APM program, version 2.10.[17] Marker allele frequencies were estimated from 148 normal controls. Heterozygosity for each marker was estimated from these frequencies (see Table 2);
2) Non-parametric linkage (NPL) analysis of the GENEHUNTER package, version 1.1;[18] and
3) ETDT (extended transmission disequilibrium test) was carried out according to the method described by Sham and Curtis *et al*[19] using the software package ETDT version 1.4.

Association Analysis

Comparisons of allele frequencies between cases and controls were calculated by the χ^2 test for a $2 \times k$ contingency table and Fisher exact tests. For calculations of full allele distribution tables, rare alleles (expected numbers less than 5) were combined into a separate class. As this was an exploratory

Table 1 Characteristics of the investigated MS families and sporadic patients

	No. of families	*Total ind.*	*Affected ind.*	*aSP*	*aAN*	*aPC*	*aCP*	*aGG*	*aHSP*		*No. of ind.*
Families										Population	
multiplex	46	207	104							Sporadic MS patients	190
singleton	28	79	28							Healthy controls	148
Total	74	286	132	26	19	12	5	4	2	Total	338

ind: individuals; aSP: affected sibpairs; aAN: affected aunt (uncle)–nephew (niece) pairs; aPC: affected parent–child pairs; aCP: affected cousin pairs; aGG: affected grandparent–grandchild pairs; aHSP: affected half-sibpairs.

study, no corrections of *P* values due to multiple testing have been performed. Thus, the interpretation of *P* values should be made with the large number of comparisons in mind.

Results

Identification of Marker Allele Frequencies

The allele frequencies for the 16 polymorphic markers were estimated and average observed heterozygosity rates were 0.795 from 148 Swedish controls (Table 2). In comparison with database information, a number of additional alleles were observed. Heterozygosity rates were generally higher than previously reported.

Linkage and Association Analyses

The results of APM, ETDT, χ^2, two-point lod score and NPL analyses for the 16 markers are shown in Tables 3, 4, 5. As shown, four candidate loci, 5p15, 7ptr–15, and 12q23, each represented by a single marker, scored positively in various ways.

12q23

The marker D12S1052, mapped to 12q23, a locus which scored slightly positive (lod score = 1.48) for linkage in the report by Haines *et al*,[7] scored slightly positive (NPL score = 0.95) in the non-parametric linkage analysis. In addition to a slight transmission distortion ($P < 0.04$), the strongest evidence for an importance in MS was obtained from the association study (Table 4). In addition to a significant deviation for the full contingency table, ($\chi^2 = 22.5$, $P < 0.0004$) the most common allele was significantly increased in frequency in MS patients (41.2% *vs* 27.7%, $P < 0.01$).

7ptr–15

Four published genomic screens in MS reported slightly positive lod scores, between 0.80 and 1.11, in the 7ptr–15 chromosomal region.[6–9] In our analysis, the D7S513 marker showed a similarly weak NPL score of 0.73. A more significant finding, however, may be the significant statistic of 6.92 ($P < 0.00001$, Table 3) provided by the APM analysis. On the other hand, the APM analysis is sensitive to incorrectly assigned allele frequencies. Thus, under or overestimated allele frequencies can lead to a bias in favour of sharing and produce false positive or false negative results.[20] In addition, an increase in frequency of D7S513-allele 2 in MS patients (13.8%) compared with controls (5.32%) ($\chi^2 = 6.91$, $P < 0.01$, Table 4).

5p

The most consistent finding in the four previous genomic screens, apart from positive scores for the HLA complex in 6p21, was the observation of a putative susceptibility gene locus in 5p.[6–9] However, this region was loosely defined, and the lod score peaks in four studies did not clearly overlap. In the present study, one of three selected markers showed a slightly positive NPL score (0.95, Table 3) of the GENEHUNTER package. Since this analysis is known to be

Table 2 Number of alleles, range of sizes and heterozygosity of markers used as observed in 148 Swedish healthy controls

Locus	*Chromosome location*	*No. of alleles*	*Repeat type*	*Size range (bp)*	*Hetero-zyg. rate*
D2S131	2p25–22	14	di	227–257	85
D5S406*	5p15.31	14	di	157–185	79
GATA84E11*	5p15.31	9	tetra	250–282	80
D5S407*	5p15.1	14	di	100–128	75
D5S427	5q11–13	20	di	271–327	81
D6S305	6q25.2	17	di	193–235	84
D7S513	7ptr–p15	17	di	175–211	82
D7S554	7q21–22	11	di	216–238	71
D11S2000	11q21–23	20	tetra	199–239	87
D12S1052	12q23	7	di	142–174	72
D12S392	12q24–qter	6	tetra	136–156	79
D13S285	13q33–34	9	di	90–112	81
D16S748	16p13.2	10	tri	182–214	82
D18S59	18p11.32–23	10	di	147–169	75
DXS1086*	Xp21.3	11	di	245–273	82
DXS1068*	Xp21	7	tetra	243–265	79

The locations of markers were obtained from http://cedar.genetics.soton.ac.uk/pub/
*The distance between the markers D5S406 and GATA84E11 is 4.7cM; between GATA84E11 and D5S407 37.5cM; between DXS1086 and DXS1068 11.1cM.

Table 3 Linkage analysis of putative candidate regions on chromosomes 2, 5, 6, 7, 11, 12, 13, 14, 16, 18, and X

		Present study								
Candidate		*APM*	*A-W TDT*[a]	*Case control*		*NPL*	*Highest MLS value in the region*			
regions	*locus*	P *value*	P *value*	χ^2	*Score*	P *value*	*American*	*British*	*Canadian*	*Finnish*
2p22–25	D2S131	0.18	0.2	0.88	–0.48	0.73	1.71	–	(D2S119) 1.24	–
5p15.3	D5S406	0.32	0.48	0.34	0.21	0.40	–	–	4.24	(D5S416) 3.4
5p15.3	GATA84E11	0.4	0.3	**0.0009**	–0.03	0.51	–	–	–	(D5S1991) 2.5
5p15.1	D5S407	0.65	**<0.02**[b]	0.14	**0.98**	0.14	–	1.9	–	(D5S1992) 2.5
5q11–13	D5S427	0.06	0.08	0.13	0.26	0.38	(D5S815) 1.14	2.7	0.2	–
6q25.2	D6S305	**0.03**	**<0.002**[b]	0.43	0.09	0.45	(D6S1693) 0.64	2.4	–	–
7ptr–15	D7S513	**<0.000001**	**0.01**	0.08	**0.73**	0.21	(D7S523) 1.11	0.8	0.87	–
7q21–22	D7S554	0.09	0.88	0.64	0.61	0.75	2.86	(D7S527) 0.3	–	–
11q21–23	D11S2000	0.23	0.33	0.14	0.03	0.48	(D11S922) 1.13	(D11S925) 0.2	1.38	–
12q24–qter	D12S392	0.87	0.12	n.d.	–0.61	0.75	1.71	–	–	–
12q23	D12S1052	0.29	**<0.04**[b]	**0.0004**	**0.95**	0.13	1.48	–	–	–
13q33–34	D13S285	0.79	0.18	0.09	0.00	0.50	0.87	–	–	–
16p13.2	D16S748	0.23	0.24	0.29	0.00	0.5	1.75	(D16S287) 0.6	–	–
18p11.32–23	D18S59	0.99	0.5	0.93	–1.25	0.92	(D18S66) 0.93	–	0.56	–
Xp21.3	DXS1086	0.81	0.41	0.97	–0.75	0.81	–	–	–	–
Xp21	DXS1068	0.13	0.48	0.68	0.57	0.21	–	(DXS991) 1.8	1.85	–

[a]χ^2 for allele-wise TDT; [b]see Table 5 for details; '–' indicates no evidence for linkage

Table 4 Distribution of allele frequencies (%) of markers of four potential important regions in MS patients and healthy controls

	Different alleles in each locus															
Loci	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
D5S406																
MS (%)	1.6	1.2	27.4	12.9	9.1	42.5	0.5	1.1	1.1	1.6	1.1					
Control (%)	1.6	1.6	31.1	16.3	11.1	31.6	0	2.1	2.6	1.6	0.5					
GATA84E11[c]																
MS (%)	2.7	11.8	9.6	12.3	8.6	2.3	4.1	25[b]	18.2	5.5						
Control (%)	4.3	7.7	7.1	7.4	4.9	2.9	1.4	40.6	18.6	5.1						
D5S407																
MS (%)	1.5	14.7	1.5	9.6	8.8	7	4.4	15.8	23.5	9.6	1.1	0.7	1.5	0.4		
Control (%)	0.6	18.8	1	12.7	8.9	4.5	4.5	15	18.5	12.1	0.6	1.3	0.3	1.3		
D6S305																
MS (%)	1.1	22.3	1.7	1.1	2.3	1.7	15.4	7.4	4.6	6.9[a]	5.1	16	5.7	3.4	0.6	4.6
Control (%)	1.1	21	1.1	1.1	2.2	1.7	10.2	5.1	6.8	15.3	8	15.9	2.8	2.3	2.3	2.8
D7S513																
MS (%)	5.8	21.3	13.8[b]	2.9	4	9.2	5.2	21.3	3.5	9.8	3.5					
Control (%)	12.2	20.2	5.3	6.4	3.2	8	9	21.3	3.2	7.5	3.7					
D12S1052[d]																
MS (%)	15.7	41.2[a]	27.2	10.1	3.7	1.4[b]										
Control (%)	12.3	27.7	34.6	9.6	8.2	7.7										

[a]P <0.02; [b]P <0.01; [c]overall distribution of alleles in MS *vs* controls differ (P<0.001); [d]overall distribution of alleles in MS *vs* controls differ (P<0.0005); D12S1052 is located on 12q23; D5S406, GATA84E1 and D5S407 are located in 5p15; D6S305 is located in 6q25.2; D7S513 is located on 7ptr–15.

conservative as a single point test,[21] we have in addition applied multipoint analysis to these three markers. However, since the middle marker scored less positively, this procedure did not improve the evidence for linkage. On the other hand, this marker showed promising signs of allelic association as given in Table 4. In addition, D5S407-allele 1 showed transmission to affected offspring more often than expected (P < 0.02, Table 5).

Other markers

No evidence of linkage or association were found for the markers D2S131 (2p23), D5S427 (5q), D6S305 (6q25), D7S554 (7q), D11S2000 (11q21–23), D12S392 (12q24–qtr), D13S285 (13q33–34), D16S748 (16p12), D18S59 (18p11) and DXS1086/DXS1068 (Xp21) in the 74 Swedish MS families with parametric, non-parametric linkage analyses, transmission disequilibrium and case-control association analyses. In fact, when we evaluated these 12 markers under different inheritance models by classic two-point linkage analyses, all observed lod scores were negative. We conclude that there is no evidence of significant MS in these regions in the Swedish population.

Discussion

The role of genetic factors in the aetiology of MS has been clearly demonstrated, but the loci determining susceptibility to this disease remain largely unidentified. Recently, genome-wide scans of large numbers of MS-affected sibling pairs[6–9] have greatly accelerated the search for additional susceptibility loci in MS. Although these studies failed to identify any locus of significant importance, in addition to the *HLA* gene

Table 5 Transmission disequilibrium test of markers in three candidate loci in multiplex MS families

Allele	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
D5S407 (5p15)													
Transitted	17[a]	0	11	4	2	5	5[a]	15	10	0	1		
Not transitted	6	1	7	5	2	3	14	17	12	1	2		
D6S305 (6q25.2)													
Transitted	16	1	1	0	23[c]	3	5	10	7	6[b]	6	4	0
Not transitted	14	0	0	2	12	5	4	9	6	22	6	1	1
D12S1052 (12q23)													
Transitted	14	24	24	6[d]	5	1	1						
Not transitted	16	21	16	15	7	0	0						

[a]P <0.02; [b]P <0.002; [c]P value is on the borderline of significant; [d]P=0.039.

complex, they did reveal multiple loci (2p23, 5p, 5q, 6q, 7p, 7q, 11q, 12q, 13q, 16p, 18p, Xp21) worthy of further study. In this study, we have investigated some of these putative candidate regions in 74 Swedish MS families and unrelated MS patients and controls using 16 microsatellite markers within or close to previously identified regions of possible importance. We observed suggestive evidence for importance of the candidate regions in 5p15, 6q25, 7ptr–15 (Table 3), and 12q23 (Tables 3, 4, 5). Especially, alleles of markers D12S1052 (12q23) showed significant association with MS ($P = 0.0004$). However, there were only weak, usually non-significant, findings by classic linkage analysis, NPL and extended transmission analysis (ETDT). Nevertheless, our findings indicate that it may be possible to find association in only weakly linked loci. The GENEHUNTER is known to be very conservative as a single point test.[23] We had applied multipoint analysis to three markers in 5p.

The application of linkage analysis in polygenic diseases like MS and diabetes mellitus is problematic for several reasons. For instance, ordinary two-point linkage analysis is hampered by the difficulty in defining a genetic model that adequately explains the observed inheritance pattern. In order to limit this problem, we additionally applied the model-free APM method,[17] the non-parametric linkage (NPL) analysis of the GENEHUNTER package,[18] and the extended TDT analysis.[19]

Linkage analysis has been a successful tool in the identification of major genes in mono- or oligogenic disorders. However, for complex diseases, the power of linkage analysis may be too limited to detect genes of modest effect. Thus, large genomic scans in diseases such as insulin-dependent diabetes mellitus (IDDM) and MS have usually only provided slight evidence for an importance of the different loci, only occasionally fulfilling stringent criteria for genome-wide significance.[22] In fact, association analysis, as in transmission disequilibrium text or case-control analysis, is likely to be more powerful and may identify more circumscribed chromosomal regions than linkage. Thus, it has been suggested that the successful genetic analysis of complex diseases will be performed as large-scale testing by association analysis.[9] For the same reasons, it is also relevant to study weakly defined loci originating from genome-wide linkage analysis in independent data sets. In this study, we find that a few previously identified loci may indeed be of importance also in the present population, corroborating their possible importance.

For a marker in 12q23, we report the presence of association, mainly in the case-control analysis ($P < 0.0004$), but also slightly in the transmission analysis ($P < 0.04$), in spite of weak evidence for linkage (an NPL score of 0.95) by NPL and ETDT (Tables 3, 4 and 5). For the marker in 7ptr–15, the evidence for linkage was similarly weak and somewhat variable. On the other hand, as for 12q23, there were also indications of association, although somewhat less clear-cut. The presence of association without significant linkage might signify that the studied alleles explain only a minor proportion of the variance of a trait. Thus, certain alleles may occur more often in affected individuals but are less useful in predicting disease status within pedigrees.[23] On the other hand, a modest association with a common allele may in fact contribute significantly to the prevalence of disease in the population. In addition, an important benefit of an observed association is that it provides a tool to further delineate the relevant chromosomal region, which greatly facilitates the eventual identification of the responsible genetic factor.

In addition, we observe both indicative linkage and possible association with markers in 5p (Tables 3, 4). Thus, our findings add to the general impression that this locus may indeed be of importance in MS.

The data from our study provide no significant evidence in favour of linkage or association with MS for the putative candidate regions on chromosomes 2p23, 5q11–13, 6q25, 7q21–22, 11q21–23, 13q33–34, 16p13.2, 18p11 and Xp21.3. This may be explained in a number of different ways:

1) There is a heterogeneity in the genetically determined susceptibility to MS between the Swedish population and the populations studied in the previous scans;[6–9] or
2) the present material is too small to give the analysis adequate statistical power; or
3) these loci were ‘false positives’, and lack importance in all populations.

Only further analysis in different populations will eventually give the answer to this question.

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