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ORIGINAL ARTICLE

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Genetic susceptibility to MS: a second stage analysis in Canadian MS families

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Abstract Four published genome screens have identified a number of markers with increased sharing in multiple sclerosis (MS) families, although none has reached statistical significance. One hundred and five markers previously identified as showing increased sharing in Canadian, British, Finnish, and American genome screens were genotyped in 219 sibling pairs ascertained from the database of the Canadian Collaborative Project on Genetic Susceptibility to MS (CCPGSMS). No markers examined met criteria for significant linkage. Markers located at 5p14 and 17q22 were analyzed in a total of 333 sibling pairs and attained mlod scores of 2.27 and 1.14, respectively. The known HLA Class II DRB1 association with MS was confirmed (P<0.0001). Significant transmission disequilibrium was also observed for D17S789 at 17q22 (P=0.0015). This study highlights the difficulty of searching for genes with only mild-to-moderate effects on susceptibility, although large effects of specific loci may still be present in individual families. Future progress in the genetics of this complex trait may be helped by (1) focussing on more ethnically homogeneous samples, (2) using an increased number of MS families, and (3) using transmission disequilibrium analysis in candidate regions rather than the affected relative pair linkage analysis.

Keywords Multiple sclerosis · Linkage analysis · Susceptibility loci

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system. The exact pathogenesis remains unknown, although a role for genetic factors has been clearly indicated by genetic epidemiological studies [1, 2, 3, 4]. Given the Canadian lifetime population risk of 0.2%, the population relative risk for the siblings of MS patients (or λ_{sibs}) can be estimated at 20 [2, 5]. The significant difference between the monozygotic twin concordance rate of 20%–30% and the dizygotic twin concordance rate of 3%–5% suggests that multiple genes are involved [6, 7].

Given the strong evidence for the existence of susceptibility genes in MS, molecular genetic methods have been used to search for candidate genes, largely based on the hypothesis that MS is an autoimmune disorder [8, 9]. Equivocal findings (negative and positive) have been reported for a host of candidates, including the TCR beta locus, the myelin basic protein gene, and the immunoglobulin variable gene segment locus [10, 11].

The major histocompatibility complex locus (MHC) is the only candidate locus to be unambiguously associated with MS [12]. This association has been fine-mapped to the human leukocyte antigen (HLA) class II region containing the DQA1, DQB1, and the DRB1 genes [13]. Nevertheless, given the low relative risk of this extended haplotype and the mild evidence for linkage, the MHC susceptibility locus is thought to have only a mild-to-modest effect on overall risk [14].

Canadian, British, American, and Finnish groups have performed genome-wide screens on MS multiplex families as a means of identifying genetic determinants [15, 16, 17, 18]. The first stage of these screens used 61, 129, 52, and 16 MS multiplex families, respectively. After the initial screens, each group explored regions of potential linkage in additional families and with a higher density of markers [15, 16, 17, 18].

In total, 105 markers were identified for which there was some evidence for nominal or suggestive linkage by at least one of the four research groups. The present investigation expands on these findings. We genotyped these 105 markers in an independent sample of sibling pairs from Canadian MS families.

Materials and methods

Resources

The collection of the MS family material has been described in detail elsewhere [17, 19]. The Canadian family material is arranged in "Datasets" as follows: (1) Dataset 1–97 sibling pairs from 58 families; (2) Dataset 2–44 sibling pairs from 42 families; (3) Dataset 3–78 sibling pairs from 72 families; (4) Dataset 4 –114 sibling pairs from 97 families (Table 1). Together Datasets 1–3 consist of

Table 1 Characteristics of family material[a]

	Dataset 1[b]	Dataset 2	Dataset 3	Datsets 1–3	Dataset 4	Datasets 1–4
Families	58 (61)	42	72	172	97	269
Sib-pairs	49 (52)	41	69	159	90	224
Trios	6 (6)	1	3	10	6	16
4 affected	0	0	0	0	1	1
5 affected	3 (3)	0	0	3	0	3
Effective pairs	97 (100)	44	78	219	114	333
Two parents	22 (23)	34	35	91	74	165
One parent	15 (16)	8	33	56	23	79
Neither parent	21 (22)	0	4	25	0	25
Unaffected sibs	111 (122)	19	76	182	59	265

[a] Numbers in parentheses are the previously published numbers present in the datasets [17]
[b] Since the time of the original publication there have been minor changes to Dataset 1. Two families, upon follow-up diagnosis (once every 6 months over the last 3 years) were determined not to meet clinical criteria for clinically definite MS [34, 35]. *Sib_kin*, the Aspex statistical packages program for determining kinship, was also run on the Dataset 1, 2, and 3. It was found that 1 family from Dataset 1, thought to be a concordant dizygous twin pair, was in fact a monozygotic twin pair. These 3 families have been excluded from further analysis

172 families and 219 sibling pairs and Datasets 1–4 consist of 269 families and 333 sibling pairs. Datasets 1–4 are to be considered independent samples for the evaluation of the American, British, and Finnish markers, while for the Canadian markers, only Datasets 2, 3, and 4 are considered independent.

Marker selection

Markers used for this investigation were from the initial four published genome screens [15, 16, 17, 18]. Each study used different criteria for nominal evidence of linkage, and as such the respective markers were selected by different methods. In total, 105 markers were selected from the four studies for use in the present study. Of these, 10 markers overlapped between two genome screens.

Canadian markers

The present study used markers which, in the earlier Canadian screen [17], demonstrated an increased sharing of 56% or more as determined by sibpair analysis with the Aspex statistical package. A total of 29 markers met this minimum requirement (Appendix A).

American markers

The American study criterion for nominal linkage was a significant result in at least two of three statistical tests [16], using three linkage analysis programs – FASTLINK, SimIBD, and SIBPAL. The significance levels were a lod score of 1 for FASTLINK and *P* values of 0.05 or less for SimIBD and SIBPAL. A total of 30 markers were identified and 29 used in the present study (Appendix A). The single exception was marker D7S489, which had a relatively low heterozygosity (Het=0.37). This marker was replaced with D7S672 (Het=0.82).

Finnish markers

The 8 Finnish markers used were those considered significant in the published screen [18]. The 8 selected Finnish markers included the ones that gave an NPL score greater than 1.0 after the initial screen and the subsequent addition of the finer resolution markers (Appendix A).

British markers

From the British study [15], markers were selected that attained at least a significance level of 0.05. If a marker achieved a level of significance in the first stage of the British screen, it was then genotyped in a further 98 families and re-analyzed. Only markers that continued to have a significance level of 0.05 or less after the second stage were selected for the present study. A total of 48 markers met this minimal criterion (Appendix A). One restriction fragment length polymorphism with relatively low heterozygosity was replaced with microsatellite marker D14S826 at chromosome 14qtel.

Microsatellite genotyping

Genomic DNA was isolated and purified from peripheral blood samples by standard protocols. Polymerase chain reaction (PCR) was performed using the TC-1600 (Intelligent Automation System). PCR conditions for microsatellite genotyping were as follows: final volume of 10 µl with 50 ng of genomic DNA, 10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.60 µmol unlabelled primer, 0.12 µmol [γ-^{32}P] dATP-labelled primer, 200 µM of each dNTP, and 0.25 U of Taq DNA polymerase. Cycle conditions were 94 C for 5 min, 30 cycles of 94 C for 1 min, 50–62 C for 1 min, followed by an elongation step for 5 min. at 72 C. Microsatellite PCR products were separated by size on a 6% denaturing acrylamide gel (Sequagel-6, National Diagnostics). Separated products were visualized by autoradiography. Gels were exposed to Kodak XAR-5 film for 6–48 h at –70 C.

HLA DRB1 genotyping

A PCR-based method for typing individuals at the DRB1 locus was used for this study [20]. Sequence-specific primers were used to amplify alleles corresponding to HLA DRB1* 1, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, and 18. The same conditions for the PCR reactions applied as above with the exception that 200 ng of genomic DNA was used as template, and annealing temperatures ranged between 61 and 66 C. Control primers specific for the third intron of the HLA DRB1 gene were also amplified. PCR products were run on 1.5%–2% agarose gels, stained with ethidium bromide, and visualized with UV light.

Statistical analysis

Linkage and transmission disequilibrium analysis was performed with the Aspex Statistical Package (ftp://lahmed.stanford.edu/pub/aspex*)*. The sib_ibd program of the Aspex statistical package was used for a multi-point linkage analysis. Marker distances were taken from the Marshfield Research Group's "build your own map" function at www.marshfield.org/genetics. The sib_tdt program of the Aspex statistical package was used to test for transmission distortion [21]. Unaffected siblings were used to reconstruct any missing parental genotypes.

Results

Analysis of markers in an independent Canadian sample identified by the original Canadian genome screen

Positive findings were originally reported for D6S461 within the MHC and D5S406 of 5p13. These 2 markers, with the additional flanking markers D6S273, D6S276 and D5S405, D5S635 have been examined in the Canadian families, and the positive findings have been previously reported for Datasets 1, 2, and 3 [17]. The remaining 28 markers from the original reported genome screen showed less than 56% sharing in the independent sample of 122 sibling pairs from 114 families (Datasets 2 and 3). The sharing remained below 56% when these sibling pairs (Datasets 2 and 3) were added to the original Dataset 1 for a total of 219 sibling pairs. Allele sharing of 56% was chosen as a cutoff for consistency with the original Canadian genome scan [17].

Further investigation for markers on chromosome 5p

Marker D5S406 was reported as having a multipoint mlod score of 1.60 in Datasets 1, 2, and 3 [17]. For the present study, this region continued to attain the highest mlod score in the Canadian families. As a result, the markers D5S405, D5S406, and D5S635 were further genotyped in an independent set of 114 Canadian sibling pairs (Dataset 4). The multipoint mlods in this indepen-

Table 2 Selected linkage results for candidate markers with over 56% sharing and/or mlod >0.50 in 219 sibling pairs

Marker	Genome scan	Canadian 2-point mlod (% sharing) [17]	UK MLS score [15]	Finnish NPL score [18]	American linkage scores [16]	Multipoint mlod in 218 Canadian Sibling pairs	Percentage (%) sharing observed
D2S169	B		1.25			0.53	55.2
D2S139	B		1.25			0.56	55.3
D5S406[a]	C	1.60 (57.0)				1.55	58.0
D6S461	B		2.80			0.73	55.3
D6S276	B		2.0			0.54	54.3
D6S273	A, B		2.0		1.28/0.12/0.002	0.73	55.0
D6S291	B		1.5			0.71	54.8
DRB1	A,F			1.55	1.46/0.15/0.007	1.31	56.9
D6S286	C	0.72 (57.6)				0.63	55.9
PAH	A				1.56/0.01/0.007	0.74	56.2
D17S787	B		1.2			0.88	56.5

[a] The discrepancy observed with D5S406 is due to the slight alteration in Dataset 1 and the number of flanking markers used in the multipoint analysis between the follow-up and the genome screen

dent sample for D5S405, D5S406, and D5S635 were 0.82, 0.56, and 0.26, respectively. The sharing observed was 57.4%, 55.9%, and 54.1%. When these data were combined with the data from Datasets 1–3, the total multipoint mlods in 333 sibling pairs were 1.72, 2.27, and 1.40, respectively.

Analysis of markers in an independent Canadian sample identified by the American genome screen

Thirty markers highlighted by the original American screen [16] were genotyped in the present Canadian sibling pair sample (Datasets 1–3). Of these, only 3 markers showed an increased sharing greater than 56%. Markers D6S273 and DRB1 of the HLA Class II region of the MHC demonstrated multipoint mlods of 0.72 and 1.31, respectively. PAH at 12q24 also demonstrated an elevated degree of sharing with a multipoint mlod of 0.74 and 56.2% sharing (Table 2).

Analysis of markers in an independent Canadian sample identified by the Finnish genome screen

Two markers identified as nominally and potentially significant in the Finnish study [18] were positive in the Canadian sample. Marker D17S787, highlighted in both the original British and Finnish studies, had a mlod of 0.88 and sharing of 56.5% in the 219 Canadian sibling pairs (Datasets 1–3). The second marker was the MHC Class II DRB1.

Analysis of markers in an independent Canadian sample identified by the British genome screen

Of the 48 markers identified as nominally and suggestively linked in the British study, only 7 showed any evidence of increased sharing over 56% in the Canadian sample. The multipoint mlods were 0.56 and 0.53 for D2S139 and for the adjacent D2S169, respectively. D6S291, D6S273, D6S276, and D6S461 all reside within the MHC and the mlods for these four markers were 0.71, 0.72, 0.54, and 0.73, respectively. Marker D17S787, located at 17q22, had elevated sharing of 56.5%, and the multipoint mlod score of 0.88 was the highest of all the markers tested outside of chromosome 5p and 6p21.

Further investigation for markers on chromosome 17

As the highest mlod outside of 5p and 6p21 was for D17S787, this marker and the adjacent hotspot markers D17S789, D17S798, and D17S807 were further genotyped in 114 sibling pairs from 97 MS families (Dataset 4). In the combined sample of 333 sibling pairs (Datasets 1–4), the multipoint mlod for D17S787 decreased to 0.24 with 52.6% sharing. The adjacent markers D17S789 and D17S807 demonstrated increased sharing in the 114 sibling pairs of Dataset 4 alone; the multipoint mlods were 1.49 and 0.83 with 59.6% and 57.1% sharing, respectively. In the combined sample of 333 affected MS sibling pairs (Datasets 1–4), the mlod for D17S789 was 1.14 and 0.82 for D17S807 (Table 3).

Transmission disequilibrium testing

As HLA DRB1 is considered a candidate for an MS susceptibility locus, transmission disequilibrium testing (TDT) was performed [21]. The known MHC association with DRB1*15 was confirmed in this Canadian sample in Datasets 1–3; the allelomorph was present in 25.7% of the parents and was transmitted 151 times versus 70 times not-transmitted from heterozygous parents (χ^2=29.69, P<0.0001).

Transmission disequilibrium was also tested for markers D5S405, D5S406, D5S635, D17S798, D17S787, D17S807, and D17S789. The markers showed no significant transmission distortion with the exception of D17S789 (Table 4) (χ^2_{sum}=28.54, P=0.0015).

Table 3 Multipoint mlods and percentage sharing for chromosome 17

Marker	DS1	DS2	DS3	DS4	DS1–4
D17S798	0.31 (65.4)	0 (50.0)	0.09 (53.1)	0.01 (51.1)	0.13 (52.0)
D17S787	0 (50.4)	0.02 (51.9)	1.69 (63.6)	0 (50.0)	0.24 (52.6)
D17S807	0.13 (53.9)	0.10 (53.9)	0.01 (50.9)	0.83 (57.1)	0.82 (54.4)
D17S789	0.16 (54.5)	0.06 (53.2)	0 (50.3)	1.49 (59.6)	1.14 (55.3)

Table 4 Transmission data for locus D17S789 in Datasets 1–4

Allele	*n*	%	Transmitted	Not transmitted	χ^2
1	1	0.1	1	1	0
2	177	23.4	93	134	7.41
3	13	1.7	15	3	8.00
4	51	6.7	52	37	2.53
5	189	25.0	145	140	0.09
6	173	22.9	132	108	2.40
7	64	8.5	61	47	1.81
8	56	7.4	40	65	5.95
9	4	0.5	4	4	0
10	28	3.7	21	25	0.35

χ^2_{sum}=28.54; P=0.0015 (df=9)

Discusssion

The present linkage analysis of 219 Canadian sibling pairs failed to demonstrate significant linkage to the markers with increased sharing from the original Canadian genome screen [17]. In fact, the percentage sharing between siblings decreased for each marker examined. Similarly, the markers of potential linkage originally identified in the British [15], American [16], and Finnish [18] genome screens also failed to provide any significant evidence for linkage in the Canadian sample. These disparate linkage results between study groups may suggest genetic differences between MS populations or, more likely, that these original mildly positive findings were type I errors, implying inadequate sample size to detect linkage to the MS susceptibility genes. However, it should be noted that if these original findings were true, the number of families required to replicate a significant finding may be several times the number of families used to detect the original linkage result [22]. This does not mean the results would be completely negative and still be supportive of linkage.

Modest increased sharing was observed for D6S461, D6S291, D6S273, D6S276, and DRB1 within the MHC, where mild evidence for linkage has been well established. The associated DRB1*15 allele was also shown to be in significant disequilibrium in the Canadian sample by transmission disequilibrium testing (χ^2=29.69, P<0.0001). However, the non-significant mlod (<3) emphasizes the notion that the MHC susceptibility gene(s) contributes only modestly to overall MS risk [3, 14].

One interesting observation is the increased sharing observed for D6S461 in the Class I region of the MHC where it has been hypothesized that a second susceptibility locus resides independent of the action of DRB1 [17, 23]. If the MHC is indeed a paradigm for the complexity of susceptibility loci involved in MS genetics, sample sizes greater than the 333 sibling pairs studied in this investigation will be required to demonstrate significant linkage by affected relative pair methods. In fact, testing for disequilibrium may be the only way to demonstrate linkage, given the realistic sample sizes available to most MS researchers [24].

Markers at 5p were initially highlighted in the original Finnish and Canadian studies [17, 18]. More recently, a Swedish sibling pair study observed a lod score of 1.1 at a marker between the two Finnish and Canadian hotspot markers [25]. Given the continued positive findings in additional Canadian sibling pairs, this region continues to be a potential site for a non-MHC susceptibility locus. To date there have not been any extensive candidate gene studies applied to this area. Potential genes of interest may include various complement component genes (6, 7, and 9), the glial cell line-derived neurotrophic factor (GDNF) gene, and the cadherin genes 10 and 12.

The highest scores for the Finnish and United Kingdom studies were for marker D17S807 on 17q22. In the Datasets 1–3, D17S807 showed no evidence of increased sharing. The adjacent marker D17S787 did however show the highest mlod outside of chromosome 5p and 6p21 in the present Canadian sample. When this was re-examined in a separate set of 114 sibling pairs (Dataset 4), the mlods for D17S787 decreased, although the mlods for the adjacent markers D17S789 and D17S807 increased. When Dataset 4 was combined with Datasets 1–3, the overall mlod in 333 sibling pairs was 1.14 for D17S789. A TDT of the markers in this region provided a positive result for D17S789, although more than one allele seemed to be transmitted preferentially (or non-preferentially) to affected offspring (Table 4). Recently a Scandinavian study of 115 families demonstrated a similar linkage result of 0.90 to the same 17q region [26]. Given that this region provided the most-positive multipoint mlod outside of the MHC and 5p, the significant TDT finding for D17S789, as well as the original findings of the British and Finnish investigations, this region may be a potential site for a modest acting susceptibility gene. The candidates 2′,3′-cyclic nucleotide-3′-phosphodiesterase, APOH, PECAM1, and PRKAR1A have been examined within this region, with negative results [27, 28]. However the myeloperoxidase gene (MPO) at 17q22 was found to be positively associated in the United Kingdom population [25]. Additionally there is evidence that MPO knock-out mice have a higher incidence of experimental autoimmune encephalomyelitis [29] and that MPO is differentially expressed in early onset fe-

male MS cases [30], although this latter observation could not be replicated [31].

A different strategy to examine the significance of the various linkage results is to perform a meta-analysis. A combined meta-analysis has the advantage of analyzing a large sample with no extra genotyping required. Drawbacks include the different marker sets used between studies and, perhaps more importantly, the potential differences in susceptibility genes among MS populations. A recent meta-analysis has been performed using the results of the original Canadian, British, and American genome screens [32]. The study's highest NPL scores were for markers located on chromosome 17q11 and chromosome 6p21 (NPL scores of 2.58 and 2.47, respectively). Chromosome 17q22 also had an increased score with an NPL of 2.30. Despite the slight discrepancy in the results for chromosome 17, the conclusions from the linkage data for the present study and the meta-analysis are largely the same, as neither approach provided any significant findings of linkage [33].

Future studies will have to include family material from more than the 219, and even 333, sibling pairs studied here. At least 400–500 if not thousands of families will be required [24]. More ethnically homogeneous populations (for example the French Canadian or Finnish MS population) and the testing of disequilibrium versus tests for linkage using affected sibling pairs should also be considered strategies for future work. Single nucleotide polymorphisms and the high-throughput technology to detect these polymorphisms will play an important role in the examination of candidate regions. Clues from epidemiological and clinical studies may identify specific subsets of families for further studies in this complex disorder.

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Appendix A

Markers showing over 56% sharing in the original Canadian genome scan [17]: D1S201, D2S119, D2S155, D3S1286, D3S1261, D3S1309, D3S1282, D4S431, D4S402, D5S406, D5S424, D6S286, D7S513, D7S484, D7S524, D10S186, D10S212, D11S2000, D14S78, D15S132, D16S411, D18S59, D18S68, D19S47, DXS1060, DXS987, DXS1214, DXS1068, DXS1047.

Markers showing at least two positive scores in three linkage analysis in the American genome scan [16]: D2S131, D2S123, D3S1744, D4S413, D4S1566, D4S415, D5S815, D6S260, D6S273, DRB1, D6S1693, D7S672, D7S554, D7S523, D9S162, D9S1846, D9S171, D9S66, D10S464, D11S922, D12S1052, D12S101, PAH, D12S392, D13S285, D16S748, D16S287, D18S66, APOC2, D19S219.

Markers highlighted in the Finnish genome screen [18]: D2S1391, D4S3248, D5S416, DRB1, D10S1220, D11S910, D17S787, D17S807.

Markers achieving a minimum 0.05 significance in the United Kingdom genome screen [15]: D1S199, D1S216, D1S207, D1S236, D2S169, D2S139, D3S1289, D3S1300, D3S1285, D3S1261, D4S408, D4S1540, D4S426, D5S407, D5S427, D5S424, D5S428, D5S409, D6S260, D6S461, D6S276, D6S273, D6S291, D6S305, D7S493, D7S516, D7S484, D14S826, D14S292, D17S513, D17S786, D17S799, D17S953, NF1, D17S798, D17S787, D17S807, D17S789, D19S49, D19S225, APOC2, D19S246, D21S258, DXS538, DXS991, DXS990, DXS1059, DXS1047.

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