Epigenetics in multiple sclerosis susceptibility: difference in transgenerational risk localizes to the major histocompatibility complex

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Multiple sclerosis (MS) susceptibility demonstrates a complex pattern of inheritance. Haplotypes containing HLA-DRB1*1501 carry most of the genetic risk. Epidemiological evidence implicating epigenetic factors includes complex distortion of disease transmission seen in aunt/uncle–niece/nephew (AUNN) pairs. Unexpectedly, in AUNN families we found that allele frequencies for HLA-DRB1*1501 were different between the first and second generations affected. Affected aunts had significantly lower HLA-DRB1*15 frequency compared with their affected nieces ($\chi^2 = 9.90, P = 0.0016$), whereas HLA-DRB1*15 frequency in affected males remains unaltered across the two generations ($\chi^2 = 0.23, P = 0.63$). We compared transmissions for the HLA-DRB1*15 allele using a family-based transmission disequilibrium test approach in 1690 individuals from 350 affected sibling pair (ASP) families and 960 individuals from 187 AUNN families. Transmissions differed between the ASP and the AUNN families ($\chi^2 = 6.92; P = 0.0085$). The risk carried by HLA-DRB1*15 was increased in families with affected second-degree relatives (AUNN: OR = 4.07) when compared with those consisting only first-degree relatives (ASP: OR = 2.17), establishing heterogeneity of risk among HLA-DRB1*15 haplotypes based on whether collateral parental relatives are affected. These observations strongly implicate gene–environment interactions in susceptibility and more specifically, that epigenetic modifications differentiate among human leukocyte antigen class II risk haplotypes and are involved in the determination of the gender bias in MS. These data strongly suggest that the female-specific increasing risk of MS is mediated through these alleles or adjacent variation. The comparison of transmission of the same allele in vertically affected pedigrees (AUNN) to collinear sibling pairs (ASP) may provide a useful screen for putative epigenetic marks.

INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory and demyelinating neurological disease affecting the central nervous system (1). The precise mode of inheritance of MS is not yet well established. Approximately 15–20% of MS patients have one or more affected relatives, and first, second- and third-degree relatives are more likely to have the disease than the general population (2,3). A multifactorial aetiology is implied, which includes interactions among genetic, environmental and stochastic factors (4). The decreasing risk in families by increasing genetic distance from the MS proband is consistent with a complex model of inheritance of risk. However, this decrease is irregularly proportional to genetic sharing and similar risks for children and for siblings of probands seem at odds with an unexpectedly high risk (asymmetric by parent-of-origin) for half-siblings compared with full siblings from the same families (5,6).

The genetic association of the human major histocompatibility complex (MHC) region in conferring susceptibility to MS is well-documented (7,8). Susceptibility may be mediated...
by the human leukocyte antigen (HLA) class II genes involving HLA-DR, HLA-DQ or both (9). These genes encode key molecules in antigen presentation and T-lymphocyte repertoire determination (10). The contribution of HLA-DRB1*15 extended haplotypes (HLA-DRB1*1501-DRB5*0101-DQA1*0102-DQB1*0602) to MS risk has been replicated and confirmed in most population-based studies. Additional involvement of MHC loci outside the HLA class II region has not been supported (9,11,12), but powerful epigenetic and suppressor effects are now well-documented (8,13).

The MHC association in MS susceptibility has been investigated using either case–control studies of unrelated individuals or utilizing family-based association studies. The most commonly used approach of a case–control design readily generates large study samples. However, association findings with this design can arise as a result of population stratification (14) with low likelihoods of replication using this method (15). Reliability is increased with the application of family-based approaches (16). These are demonstrably resistant to population heterogeneity. Furthermore, families are more homogeneous regarding exposure to environmental factors, which loom large in susceptibility (17). Case–control designs have no capacity to identify patterns of inheritance, suggesting epigenetic influences. However, the need to gather available parents as well as additional family members and accumulation of an adequate sample size can be challenging. Strong convictions have been expressed on the preference for family-based studies (14,17).

Family-based transmission disequilibrium test (TDT) approaches assess evidence for preferential transmission of one allele over the other while also testing linkage (18). In addition to the analysis of parent–child trios, the TDT can also be extended to other family pedigrees, including affected sibling pairs (ASPs) and multiplex families, and would remain valid for both association and linkage (19).

We have applied this approach in previous studies investigating the association of HLA-DRB1 locus in MS susceptibility in 4347 individuals from 873 multiplex families. Complex interactions at the HLA-DRB1 were identified including a hierarchy of resistance and susceptibility alleles interacting in cis and trans to influence overall risk (8). Borrowing the same design, these findings have been replicated (20). More recently, in an expanded cohort of 7093 individuals from 1432 MS families, two different types of HLA-DRB1 resistance alleles were reported (13). These studies included a variety of MS family types consisting of ASP, aunt/uncle–niece/nephew (AUNN) pairs, parent–child pairs, cousin pairs, sporadic and multiplex families. The novel observations demonstrated by the aforementioned studies were made possible by the application of family-based TDT methods with large cohorts of MS families.

We have reported several lines of evidence which raise the possibility of epigenetic mechanisms in MS. The concordance rate in monozygotic (MZ) twins demonstrates that genetic factors are important, but most MZ twins are discordant, and concordant pairs are almost entirely female (21). The female excess characterizes the total MS population, despite regional variation. In Canada, the female-to-male ratio has been increasing for at least 60 years and now surpasses 3:2:1, whereas in Scotland, this ratio was around 1:1 almost five decades ago, currently matches or exceeds Canada’s gender ratio (22).

Our genetic-epidemiological studies have demonstrated several findings implicating epigenetic influences. Initial observations had found a higher number of mother–daughter pairs and few father–son pairs (23,24). The gender of the affected parent clearly influences the risk for subsequent children to develop MS, and this risk is increased when the mother is affected (25). Furthermore, maternal half-siblings (2.35%) are at a greater risk of developing MS when compared with paternal half-siblings (1.31%) (5). These observations indicated a maternal effect in MS susceptibility and recently, in some 7000 individuals from more than 1500 MS families, significant over-transmission of the maternal HLA-DRB1*15 allele was observed (26). The latter findings suggested that the gender predilection in MS in general and in the specific sub-cohorts mentioned could be mediated by variation within the MHC. An epigenetic mechanism had been specifically suggested previously (5,6) to explain the maternal effect in half-sibling data and in MHC transmission by parent-of-origin (26).

Here, we compare the transmission of HLA-DRB1*15 alleles in ASP families versus AUNN families. Although ASP families have been commonly used for finding linkage in complex traits, a recent study has highlighted the value of the AUNN families in the identification of distorted disease transmission (27). We hypothesized that the risk carried by HLA-DRB1*15 may be different in families with affected first-degree relatives (ASP) when compared with families consisting additional affected second-degree relatives (AUNN). This was hinted at indirectly by the fact that alleles which were not associated with the disease nevertheless show increased sharing within affected sib pairs (28). This finding itself we interpreted early on as implying epigenetic modification within the MHC.

Here we confirm the prior hypothesis of differential transmission by family type for HLA-DRB1*15, the main risk allele in MS susceptibility, in two large cohorts of ASP and AUNN families, on the one hand collinear and on the other intergenerational.

RESULTS

TDT in ASP families

In the ASP families (n = 350), HLA-DRB1*15 was transmitted 213 times and not transmitted 98 times (OR = 2.17; x^2 = 43.55; P = 4.13 x 10^-11). Significant over-transmission of HLA-DRB1*15 was accompanied by significant under-transmissions of HLA-DRB1*09 (OR = 0.41; x^2 = 4.30; P = 0.038), *11 (OR = 0.63; x^2 = 5.09; P = 0.024) and *14 (OR = 0.32; x^2 = 9.19; P = 0.0024) (Table 1).

TDT in AUNN families

In the AUNN families (n = 187), HLA-DRB1*15 was transmitted 118 times and not transmitted 29 times (OR = 4.07; x^2 = 57.78; P = 2.93 x 10^-14). Significant over-transmission of HLA-DRB1*15 was accompanied by significant under-transmissions of HLA-DRB1*04 (OR = 0.62; x^2 = 4.50;
**DISCUSSION**

Human MHC genes have been associated with most known autoimmune diseases even though these have complex modes of inheritance. These associations have provided powerful tools for studying the inheritance of susceptibility (29). Family-based TDT study designs have profitably explored the transmission of MHC genes in familial MS. Designs have included single affected children and ASPs with parents (16). Numerous family-based association studies of varying type and size show the *HLA-DRB1* extended haplotype consistently linked to MS susceptibility, especially in Northern Europeans (8, 9, 11–13).

In addition to the *HLA-DRB1* association, family-based studies have shown allelic heterogeneity at the *HLA-DRB1* locus and complex interactions between different *HLA-DRB1* alleles in *cis* and *trans* which influence overall risk (8, 13). These studies included a variety of MS families and investi-
The genetic epidemiology of MS has been studied extensively and profitably and has been recently reviewed (30). This novel approach highlighted the effectiveness of AUNN families in dissecting the complex susceptibility of MS (27). By comparing risks in the two generations spanned by AUNN families, this study was able to independently confirm the maternal parent-of-origin effect in MS (5,23–27) and also the increasing rate of MS in females (22). The number of affected aunts was much less than the number of nieces in population-based data while the reverse was expected given the age of onset curve for the disease. This ensures that aunts will have passed much more of the disease-at-risk period than nieces or nephews. AUNN families provided an innovative method of revealing distorted disease transmission, emphasizing the value of family-based cohorts that can look beyond the more commonly used ASP approach (27). Ascertainment bias is a common confounder in epidemiological studies. We have recently reviewed the importance of a standardized method for ascertainment of cases and their families to minimize any potential bias (30).

Since MHC alleles are by far the most powerful genetic disease susceptibility factors, we reasoned that an AUNN cohort could help determine if the pattern in this type of family with affected second-degree relatives is mirrored by allelic transmissions at the MHC. Here we provide further evidence for differential risk carried by \( HLA-DRB1^{*15} \), by

### Table 2. Showing the \( HLA-DRB1^{*15} \) frequency across two generations of AUNN families

<table>
<thead>
<tr>
<th>Maternal Count</th>
<th>Frequency (%)</th>
<th>Paternal Count</th>
<th>Frequency (%)</th>
<th>Total Count</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aunt–niece</td>
<td>89/268</td>
<td>33.21</td>
<td>58/190</td>
<td>30.53</td>
<td>147/458</td>
</tr>
<tr>
<td>Aunt–</td>
<td>37/130</td>
<td>28.46</td>
<td>21/92</td>
<td>22.82</td>
<td>58/222</td>
</tr>
<tr>
<td>–Niece</td>
<td>52/138</td>
<td>37.68</td>
<td>37/98</td>
<td>38.95</td>
<td>89/236</td>
</tr>
<tr>
<td>Aunt–nephew</td>
<td>21/74</td>
<td>28.38</td>
<td>15/60</td>
<td>25.00</td>
<td>36/134</td>
</tr>
<tr>
<td>Aunt–</td>
<td>10/38</td>
<td>26.32</td>
<td>5/30</td>
<td>16.67</td>
<td>15/68</td>
</tr>
<tr>
<td>–Nephew</td>
<td>11/36</td>
<td>30.56</td>
<td>10/30</td>
<td>33.33</td>
<td>21/66</td>
</tr>
<tr>
<td>Uncle–niece</td>
<td>26/86</td>
<td>30.23</td>
<td>21/54</td>
<td>38.89</td>
<td>47/140</td>
</tr>
<tr>
<td>Uncle–</td>
<td>12/38</td>
<td>31.58</td>
<td>9/28</td>
<td>32.14</td>
<td>21/66</td>
</tr>
<tr>
<td>–Niece</td>
<td>14/48</td>
<td>29.17</td>
<td>12/26</td>
<td>46.15</td>
<td>26/74</td>
</tr>
<tr>
<td>Uncle–nephew</td>
<td>8/36</td>
<td>22.22</td>
<td>5/28</td>
<td>17.86</td>
<td>13/64</td>
</tr>
<tr>
<td>Uncle–</td>
<td>2/18</td>
<td>11.11</td>
<td>3/14</td>
<td>21.43</td>
<td>5/32</td>
</tr>
<tr>
<td>–Nephew</td>
<td>6/18</td>
<td>33.33</td>
<td>2/14</td>
<td>14.29</td>
<td>8/32</td>
</tr>
<tr>
<td>Aunt</td>
<td>47/168</td>
<td>27.98</td>
<td>26/122</td>
<td>21.31</td>
<td>73/290</td>
</tr>
<tr>
<td>Uncle</td>
<td>14/56</td>
<td>25.00</td>
<td>12/42</td>
<td>28.57</td>
<td>26/98</td>
</tr>
<tr>
<td>Niece</td>
<td>66/186</td>
<td>35.48</td>
<td>49/124</td>
<td>39.52</td>
<td>115/310</td>
</tr>
<tr>
<td>Nephew</td>
<td>17/54</td>
<td>31.48</td>
<td>12/44</td>
<td>27.27</td>
<td>29/98</td>
</tr>
<tr>
<td>Total aunt/uncle</td>
<td>61/224</td>
<td>27.23</td>
<td>38/164</td>
<td>23.17</td>
<td>99/388</td>
</tr>
<tr>
<td>Total niece/nephew</td>
<td>83/240</td>
<td>34.58</td>
<td>61/168</td>
<td>36.31</td>
<td>144/408</td>
</tr>
</tbody>
</table>

### Table 3. Intergenerational comparison of \( HLA-DRB1^{*15} \) frequency matched by gender in AUNN families

<table>
<thead>
<tr>
<th>Group a</th>
<th>Count</th>
<th>Frequency (%)</th>
<th>Group b</th>
<th>Count</th>
<th>Frequency (%)</th>
<th>Comparison</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>First generation a versus second generation b</td>
<td>99/388</td>
<td>25.52</td>
<td>144/408</td>
<td>35.29</td>
<td>8.97</td>
<td>0.0027</td>
<td></td>
</tr>
<tr>
<td>Aunt c versus niece d</td>
<td>73/290</td>
<td>25.17</td>
<td>115/310</td>
<td>37.10</td>
<td>9.90</td>
<td>0.0016</td>
<td></td>
</tr>
<tr>
<td>Uncle c versus nephew d</td>
<td>26/98</td>
<td>26.53</td>
<td>29/98</td>
<td>29.59</td>
<td>0.23</td>
<td>0.63</td>
<td></td>
</tr>
</tbody>
</table>

Group a may include first generation, aunt or uncle. Group b may include second generation, niece or nephew.
demonstrating that the inheritance of HLA-DRB1*15 in ASP families versus AUNN families is unequivocally different. As per the prior hypothesis, we found that the AUNN transmission distortions from the genetic epidemiological study (27) were paralleled by the transmission of HLA class II alleles.

significant over-transmission of HLA-DRB1*15 in both ASP and AUNN families was observed (see Table 1). However, the risk carried by HLA-DRB1*15 is demonstrably greater in families with additional affected second-degree relatives when compared with families consisting only affected first-degree relatives (AUNN: OR = 4.07; ASP: OR = 2.17; Comparison: $\chi^2 = 6.92$, $P = 0.0085$; see Table 1). Although HLA-DRB1*15 haplotypes contributed to risk in both family types, the degree of contribution was not the same. These data show that differential risk for transmission of the main MS susceptibility allele is dependent on family structure and the MS status of the relatives of the transmitting individual. This difference between ASP and AUNN families provides a useful tool of screening for parental modifications in disease-associated haplotypes. The exact reason for this difference is yet to be determined; however, gene–environment interactions and epigenetic modifications are implied. We believe that this variation in the transmission of HLA-DRB1*15 will provide important insights in our understanding of the complex genetic inheritance of MS. The distorted transmissions may well be applicable to other non-HLA-DRB1*15 alleles shown to be involved in susceptibility or resistance (see Table 1). This will require much larger numbers to establish than for the frequent HLA-DRB1*15 allele. It does raise the possibility that the allelic associations in MS are influenced by the relative predilection of individual haplotypes which carry them for epigenetic modification.

Our results further highlight the usefulness of AUNN families. When matched by gender, we show that the HLA-DRB1*15 frequency is significantly lower in the first generation affected females when compared with second generation affected females (aunts versus nieces: $\chi^2 = 9.90$, $P = 0.0016$; see Tables 2 and 3), whereas the HLA-DRB1*15 frequency in the affected males remains unchanged across the two generations (uncles versus nephews: $\chi^2 = 0.23$, $P = 0.63$; see Tables 2 and 3). These findings suggest additional MS heterogeneity based on MHC requirement, which can be further explored clinically. AUNN pairs across two generations emphasize the possibility of intergenerational epigenetic effects. Moreover, these observations demonstrate that the human MHC is directly involved in the increasing MS incidence in females, suggesting that the MHC is the site of a gene–environment interaction central to disease susceptibility.

**MATERIALS AND METHODS**

**Subjects**

We selected 1690 individuals from 350 ASP families, and 960 individuals from 187 AUNN families as part of the ongoing Canadian Collaborative Project on the Genetic Susceptibility to MS (CCPGSMS), for which the methodology has been described previously (31). All families were Canadian and of European descent. We have not included any ASP families with affected second-degree relatives for this study.

**HLA typing**

The genotyping of HLA-DRB1 was performed using either a low- or high-resolution allele-specific polymerase chain reaction (PCR) amplification method (8). Low-resolution HLA-DRB1 genotypes were obtained by a combination of 24 PCRs, and high-resolution HLA-DRB1 genotypes were obtained with an additional 48 PCRs. In each individual reaction, positive control primers were designed to amplify a second non-polymorphic genomic control segment. Amplified products were separated by electrophoresis in 2% agarose gels containing ethidium bromide after the addition of loading buffer and visualized using ultraviolet illumination.

**Statistical methods**

The family pedigree files were first tested using the PEDCHECK program (32) for the presence of errors in Mendelian transmission. TDT was performed using the TDTPHASE program of the UNPHASED software package (33). Families had been selected for those with parents typed, but in a minority, parents who were not genotyped were reconstructed whenever possible from unaffected siblings only. In cases when one parent was unknown, only in those instances where both the genotyped parent and the affected offspring were heterozygous for different alleles were the transmissions counted in order to avoid directional bias.

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