SHORT COMMUNICATION

Multiple sclerosis associates with LILRA3 deletion in Spanish patients

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The genetic susceptibility to multiple sclerosis (MS) is only partially explained, and it shows geographic variations. We analyse here two series of Spanish patients and healthy controls and show that relapsing MS (R-MS) is associated with a gene deletion affecting the hypothetically soluble leukocyte immunoglobulin (Ig)-like receptor A3 (LILRA3, 19q13.4), in agreement with an earlier finding in German patients. Our study points to a gene-dose-dependent, protective role for LILRA3, the deletion of which synergizes with HLA-DRB1*1501 to increase the risk of R-MS. We also investigated whether the risk of suffering R-MS might be influenced by the genotypic diversity of killer-cell Ig-like receptors (KIRs), located only ~400 kb telomeric to LILRA3, and implicated in autoimmunity and defence against viruses. The relationship of LILRA3 deletion with R-MS is not secondary to linkage disequilibrium with a KIR gene, but we cannot exclude some contributions of KIR to the genetic susceptibility to R-MS. Genes and Immunity advance online publication, 7 May 2009; doi:10.1038/gene.2009.34

Keywords: chromosome 19; gene deletion; killer-cell Immunoglobulin-like receptors; leukocyte immunoglobulin-like receptors; multiple sclerosis; susceptibility

Introduction

Multiple sclerosis (MS) is a demyelinating disease in which inflammation associates with progressive degeneration of the central nervous system. Its most common clinical form, relapsing-remitting MS (RR-MS), is characterized by episodes of inflammatory lesions in the central nervous system (relapses or exacerbations), followed by the remission of symptoms. In most cases, RR-MS progresses into secondary progressive MS, in which established demyelination and axonal loss cause permanent and progressive disability. RR-MS and secondary progressive MS are considered two stages of the same disease, to which we will refer here as relapsing MS (R-MS). The aetiology of MS is unknown, but this autoimmune disease is believed to be triggered, in a predisposing genetic context, by infection.1

The strongest and the best characterized predisposing genetic factor for MS lies, as in many autoimmune diseases, in the major histocompatibility complex of chromosome 6: the HLA-DRB1*1501 allele.2,3 Polymorphisms of other genes related to the immunological response, namely IL7R and IL2RA, also contribute, albeit more modestly, to the risk of suffering MS (odds ratio (OR) values: 1.18–1.34).4–7 In Germans,8 RR-MS is associated with deficiency of the A3 member of the leukocyte immunoglobulin (Ig)-like receptor family (LILRA3, CD85e), molecule earlier referred to as Ig-like transcript 6 (ILT6), leukocyte Ig-like receptor 4 (LIR-4) or HM43.9–11

The LILRA3 gene maps to the leukocyte receptor complex12 (LRC, 19q13.4), which encodes multiple polymorphic proteins with Ig-like extracellular domains. Those receptors, expressed in leukocytes of both the lymphoid and the myelomonocytic lineages, belong to three families: the LILR (CD85), the leukocyte-associated inhibitory receptors (LAIRs, CD305) and the killer-cell Ig-like receptors (KIRs, CD158). Members of the LILR and the KIR families also share the capacity of transmitting inhibitory or activating signals upon recognition of human leukocyte antigen (HLA) class I molecules.13,14

Among LILRs, which are mainly expressed by myelomonocytic cells, LILRB1 (CD85j) is the best characterized, and it is detected also in T, B and natural killer lymphocytes.11 LILRB1 modulates leukocyte function and survival upon recognition of self HLA class I molecules in target cells. LILRB1 also recognizes the major histocompatibility complex homologue UL18 protein of human cytomegalovirus with ~103-fold greater affinity than that for HLA molecules. The implications of this putative immune-evasion mechanism in the pathogenicity of cytomegalovirus remain ill defined.15–17

Encoded in the central region of the LILR gene complex, LILRA3 diverges from the canonical LILR
structure as it lacks transmembrane and cytoplasmic regions due to a termination codon in the stalk. LILRA3 is likely expressed as a secreted, instead of membrane-anchored, receptor. Some Caucasoids carry a grossly aberrant LILRA3 allele in which the first seven of its eight exons are deleted, a defect that has been associated with RR-MS in Germans. In the same study, French MS patients had an LILRA3 deletion frequency similar to that of German patients, but that frequency was not compared with one of a French healthy control group. Therefore, whether LILRA3 deletion indeed associates with MS in other populations, and whether its association is primary or secondary to linkage to other polymorphic genes in its vicinity, warrants further studies.

Approximately 400 kb telomeric to LILRA3, and only ~10 kb apart from the last LILR complex, maps the KIR complex, which has an enormously variable content of polymorphic genes. Of the 17 KIR genes currently recognized, only the two that mark the 5’ and 3’-ends of the KIR complex are shared by all human genomes, whereas most individuals lack one or more of the other KIR genes. Both the frequency of each KIR gene and the manner in which they combine in haplotypes vary substantially among different populations, variations that have been associated with autoimmune and infectious diseases.

By means of clonally distributed KIRs that recognize different HLA class I molecules, natural killer cells survey alterations in antigen presentation that often take place in infected cells. Best known among pathogens that tamper major histocompatibility complex class I expression are herpesviruses, which can alter T- and natural killer-lymphocyte function by subverting the expression of host major histocompatibility complex molecules, or by encoding viral homologues of these. As all of the polymorphisms of the LRC in chromosome 19, natural killer cells, herpesviruses and HLA class I molecules have been implicated in the susceptibility or pathogenesis of MS, it is also of interest to determine whether the genotypic diversity of KIR is associated with MS and whether such an association could explain, by linkage disequilibrium (LD), the reported relationship between MS and deletion of LILRA3.

Results

Association between LILRA3 deletion and R-MS in Spanish patients

To determine whether the association between the LILRA3 gene deletion and MS previously reported in Germans is also seen in a Mediterranean genetic context, we studied 126 R-MS patients and 174 healthy unrelated controls of Spanish origin (Hospital Universitario Puerta de Hierro, Madrid, Table 1). To simplify and facilitate the analysis of LILRA3 genotypes in a clinical context, we designed a single-tube PCR that amplifies both the wild-type and the deleted LILRA3 alleles, which are then distinguished by their different electrophoretic mobilities in regular agarose gels (Figure 1). This method simplifies further the approach used by Hirayasu et al. by using a single reverse PCR primer.

Using such a method, we found that LILRA3 deficiency is associated with MS also in Spain (Table 2): 31.0% of patients, but only 20.1% of controls, had LILRA3 deleted in at least one chromosome. To further confirm the association between LILRA3 deletion and R-MS, we replicated the study in another series of 99 patients from a different centre (Hospital del Mar, Barcelona, Table 1). These showed a similarly higher frequency of the deletion in comparison with 157 healthy controls from the HUPH and the HdM series, respectively.

Table 1 Characteristics of the patients

<table>
<thead>
<tr>
<th></th>
<th>HUPH</th>
<th>HdM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female-to-male ratio</td>
<td>4.25</td>
<td>2.23</td>
<td>3.13</td>
</tr>
<tr>
<td>Age at onset Mean ± s.d.</td>
<td>26.8 ± 8.3</td>
<td>30.2 ± 9.1</td>
<td>28.4 ± 8.8</td>
</tr>
<tr>
<td>Range</td>
<td>11–65</td>
<td>11–58</td>
<td>11–65</td>
</tr>
<tr>
<td>Age at time of diagnosis Mean ± s.d.</td>
<td>38.9 ± 9.2</td>
<td>42.0 ± 10.5</td>
<td>40.3 ± 9.9</td>
</tr>
<tr>
<td>Range</td>
<td>19–67</td>
<td>23–68</td>
<td>19–68</td>
</tr>
<tr>
<td>Expanded disability status scale Mean ± s.d.</td>
<td>2.92 ± 2.16</td>
<td>2.99 ± 2.29</td>
<td>2.95 ± 2.21</td>
</tr>
<tr>
<td>Range</td>
<td>0–8.5</td>
<td>0–9</td>
<td>0–9</td>
</tr>
<tr>
<td>Multiple sclerosis severity score Mean ± s.d.</td>
<td>3.30 ± 2.59</td>
<td>3.74 ± 2.81</td>
<td>3.51 ± 2.70</td>
</tr>
<tr>
<td>Range</td>
<td>0.05–9.59</td>
<td>0.05–9.79</td>
<td>0.05–9.79</td>
</tr>
</tbody>
</table>

Abbreviations: RR, relapsing-remitting; SP, secondary progressive.

- HUPH: Hospital Universitario Puerta de Hierro (Madrid); HdM: replication series from Hospital del Mar (Barcelona). Only patients with definite RR- or SP-MS diagnosis, according to accepted clinical criteria, were included in the study. Controls were healthy unrelated voluntary donors collected from the same geographical region as each series of patients (174 and 157 healthy controls for the HUPH and the HdM series, respectively).

Analysis of genotypes showed that both homo- and heterozygosity for LILRA3 deletion tended to be more common in R-MS patients than in controls (LILRA3-del/del: 4.0 vs 2.1%; LILRA3-del/wt: 28.9 vs 21.1%, respectively, in the sum of the two series), although the increase of homozygotes separately was only marginally significant and not seen in the replication series. The global divergence in the distribution of genotypes, however, showed a statistically significant linear trend (Table 2). According to that trend, the risk of suffering MS is highest in individuals lacking LILRA3 completely in their genome, intermediate in heterozygotes and lowest in individuals having two full-length LILRA3 alleles. The OR values of homozygosity and heterozygosity for LILRA3 deletion, in comparison with homozygosity for the wild-type LILRA3, were 2.16 and 1.56, respectively.
greater than those reported for IL2RA and ILR7 polymorphisms.\textsuperscript{5,6,35,43} The distribution of genotypes in each series, and in the sum of both, did not diverge significantly from the Hardy–Weinberg equilibrium. The clinical parameters of patients with two complete copies of LILRA3 did not differ significantly from those who carried the deletion (MS severity score $3.42 \pm 2.59$ vs $3.70 \pm 2.92$, respectively), either in homo- or in heterozygosis (not shown).

Analysis of epistatic interaction between deletion of LILRA3 and HLA-DRB1*1501

To determine whether there is epistatic interaction between the associations of R-MS with LILRA3 deletion and the classical risk factor, DRB1*1501, we studied the presence of this HLA allele in patients and controls (41.3 vs 16.6%, respectively, OR 3.54). To enhance the statistical power of all subsequent analyses, the two series of patients and controls derived from each hospital were analysed together (each data set is available upon request). The distribution of positive and negative individuals for each of the LILRA3 deletion and HLA-DRB1*1501 was then determined (Table 3), and the interaction between the two genetic markers was studied as recommended by Svejgaard and Ryder.\textsuperscript{35}

The increase of the LILRA3 deletion was more conspicuous among DRB1*1501-positive than among DRB1*1501-negative patients (OR 3.08 and 1.40, respectively), but in both cases it lacked significance due to loss of statistical power after stratification and correction for multiple comparisons (Table 3). On the other hand, the association of HLA-DRB1*1501 with the disease is even stronger when LILRA3 is deleted (OR 6.57 vs 2.99 in the absence of the deletion), and, in isolation, it was stronger than that of the LILRA3 deletion alone (OR 2.13). Finally, and in line with the earlier findings, having a LILRA3 deletion and HLA-DRB1*1501 together conferred the highest risk (OR 9.2 when compared with the absence of both markers), indicating that they act synergistically to increase the risk of suffering R-MS.

The severity indexes of HLA-DRB1*1501 patients did not differ from those without this HLA allele (not shown), but patients carrying both a LILRA3 deletion and DRB1*1501 had significantly worse disability indexes (MS severity score: $4.76 \pm 3.04$) than patients with other genotypes (MS severity score: $3.32 \pm 2.60$, \textit{P} = 0.0081), including patients having DRB1*1501, but lacking the LILRA3 deletion (MS severity score: $3.32 \pm 2.56$, \textit{P} = 0.0248). The apparent influence of the genotype LILRA3\textsuperscript{del}-DRB1*1501 on the prognosis of R-MS warrants confirmation on larger series of patients.

The relationship of R-MS with LILRA3 deletion is not secondary to an association with a KIR gene

We next searched whether the genotypic diversity of KIRS, which map ~400 kb telomeric to LILRA3, could be a primary risk factor for R-MS and whether the association of LILRA3 deletion with the disease could just reflect LD with a primarily associated KIR gene. We studied by PCR\textsuperscript{42} the presence or absence of each KIR in the genome of 224 R-MS patients and 289 healthy controls, from whom enough DNA was available. Both groups had rather similar frequencies at all KIR genes, except for KIR3DS1, under-represented among R-MS patients (OR 0.69, \textit{P}<0.05, Table 4). The lower frequency of KIR3DS1, however, lost statistical significance after correcting the \textit{P}-value for the number of comparisons, and the difference was not significant when the two series of patients and controls were compared separately (not shown). No significant LD of LILRA3 genotypes with KIR3DS1 was found in either patients or controls; therefore, the deviations in the frequencies of LILRA3 deletion and KIR3DS1 are unrelated to each other.

Analysis of LD between LILRA3 deletion and other KIR genes revealed only modestly positive values in healthy controls, but not in patients, for the KIR2DS2-KIR2DL2 pair and for KIR2DS1 (LD 0.04 and 0.03; relative LD, 0.51 and 0.38, respectively), but they were not significant (corrected \textit{P}-value $>0.05$). This is consistent with the lack of LD between LILRA3 deletion and KIR genes observed by Norman \textit{et al.}\textsuperscript{47} in British Caucasoids. Furthermore, stratification for the presence or absence of the LILRA3 deletion showed again no significant difference between patients and controls in the frequency of any KIR gene or genotype after...
Table 2  Deletion of the LILRA3 gene is increased in Spanish R-MS patients

<table>
<thead>
<tr>
<th>LILRA3 deletion</th>
<th>Patients</th>
<th>Controls</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUPH</td>
<td>31.0% (n = 126)</td>
<td>20.1% (n = 174)</td>
<td>1.78</td>
<td>1.01–3.13</td>
<td>0.016</td>
</tr>
<tr>
<td>HdM</td>
<td>35.4% (n = 99)</td>
<td>26.8% (n = 157)</td>
<td>1.50</td>
<td>0.84–2.67</td>
<td>0.072</td>
</tr>
<tr>
<td>Total</td>
<td>32.9% (n = 225)</td>
<td>23.3% (n = 331)</td>
<td>1.62</td>
<td>1.09–2.40</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Patients Controls

<table>
<thead>
<tr>
<th>&gt;LILRA3 genotype</th>
<th>HUPH</th>
<th>HdM</th>
<th>Total</th>
<th>HUPH</th>
<th>HdM</th>
<th>Total</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/wt</td>
<td>87</td>
<td>64</td>
<td>67.1%</td>
<td>139</td>
<td>115</td>
<td>76.7%</td>
<td>1.00</td>
</tr>
<tr>
<td>del/wt</td>
<td>32</td>
<td>33</td>
<td>28.9%</td>
<td>32</td>
<td>38</td>
<td>21.1%</td>
<td>1.56</td>
</tr>
<tr>
<td>del/del</td>
<td>7</td>
<td>2</td>
<td>4.0%</td>
<td>3</td>
<td>4</td>
<td>2.1%</td>
<td>2.16</td>
</tr>
</tbody>
</table>

Abbreviations: OR, odds ratio; R-MS, relapsing multiple sclerosis.

Table 3  Combined analysis of LILRA3 deletion and HLA-DRB1*1501 as risk factors for R-MS

| LILRA3 deletion | HLA-DRB1*1501* | Patients (n = 225) | Controls (n = 331) | OR   | P-value | P_c
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>32 (14.2%)</td>
<td>8 (2.4%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>42 (18.7%)</td>
<td>69 (20.8%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>61 (27.1%)</td>
<td>47 (14.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>90 (40.0%)</td>
<td>207 (62.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: NS, not significant; OR, odds ratio; R-MS, relapsing multiple sclerosis.

Correcting P-values for multiple comparisons, although deviations of the genes in LD with LILRA3 in healthy controls were apparent (KIR2DS2, KIR2DL2 and KIR2DS1, Table 4). Finally, no differences between MS patients and controls were appreciated in the distribution of ‘A’- and ‘B’-type haplotypes (for example, 29.5% ‘AA’ genotypes in patients vs 24.2% in controls).

In summary, we have confirmed that the deletion of LILRA3 associates with R-MS in Spain, as reported in German patients, and we have shown for the first time that it synergizes with HLA-DRB1*1501 in increasing susceptibility to the disease. On account of the nature of this genetic trait—a 6.7-kb deletion—it could not be detected by the genome-wide scans for susceptibility loci that focused on micropolymorphisms. Furthermore, among the markers that flank the LRC in chromosome 19 and were studied in earlier genomic scans,4,5,39,49 D19S601 and D19S571 are the closest to LILRA3, but they lie more than 1.5 Mb away from it. LD is generally not detectable across such a distance,47,50–52 which may have prevented detecting the association of LILRA3 with MS through secondary association of flanking linked markers. For the same reason, previously reported associations of markers in chromosome region 19q13 with MS (reviewed in Pericak-Vance et al.38 and Yeo et al.49) are possibly unrelated with those of
Abbreviation: R-MS, relapsing multiple sclerosis.

were corrected for multiple comparisons according to Svejgaard and Ryder. The gene order is based on that of the KIR complex—the genes that define the A type of haplotype (KIR2DL3 through KIR2DS4) are represented in the middle, preceded and followed, respectively, by genes characteristic of the centromeric and the telomeric parts of B haplotypes. Framework genes and pseudogenes found in all individuals are not shown. Frequencies were compared using the \( \chi^2 \) or Fisher’s test, as appropriate, and \( P \)-values were corrected for multiple comparisons according to Svejgaard and Ryder.

LILRA3. Finally, scans of markers within the LRC itself have been complicated by the fact that the complex is constituted by highly homologous duplicated genes (LILR and KIR) variably arranged in tightly packed tandems.

The relationship of R-MS with LILRA3 deletion that we have observed is not secondary to association of either with a KIR gene. Nevertheless, an apparent underrepresentation of KIR3DS1 in patients might indicate a minor and independent protective role of KIR haplotypes carrying this gene; or else, it could be a fortuitous result because of the multiple comparisons performed in this study. To circumvent the latter problem and elucidate the possible influence of KIR3DS1 on the susceptibility to MS, specific studies on that gene in additional series of patients are required.

In favour of a primary role for LILRA3 in the protection from MS is its homology to LILRB1, a receptor expressed on the surface of several leukocyte lineages, which recognizes both self- and nonself-HLA class I molecules and the product of at least one pseudogene.80 LILR polymorphisms located in the LRC itself are represented in the middle, preceded and followed, respectively, by genes characteristic of the centromeric and the telomeric parts of B haplotypes. Framework genes and pseudogenes found in all individuals are not shown. Frequencies were compared using the \( \chi^2 \) or Fisher’s test, as appropriate, and \( P \)-values were corrected for multiple comparisons according to Svejgaard and Ryder.

\( \begin{array}{l}
\text{Table 4 Distribution of frequencies (in %) of variable KIR genes in R-MS patients and healthy controls}\text{*} \\
\hline
\text{Total} & 2DS2 & 2DL2 & 2DS3 & 2DL3 & 2DP1 & 2DL1 & 3DL1 & 2DS4 & 3DS1 & 2DL5 & 2DS5 & 2DS1 \\
\hline
\text{Patients} & N = 224 & 58.0 & 58.7 & 33.5 & 84.8 & 95.5 & 95.5 & 94.2 & 94.2 & 34.8* & 53.6 & 29.0 & 37.9 \\
\text{Controls} & N = 289 & 59.2 & 59.5 & 32.2 & 88.6 & 96.2 & 96.2 & 97.2 & 97.2 & 43.6* & 57.1 & 31.5 & 44.3 \\
\hline
\text{Stratified for deletion of LILRA3} & & & & & & & & & & & & \\
\text{Positive for LILRA3del} & & & & & & & & & & & & \\
\text{Patients} & N = 74 & 52.7* & 52.1* & 37.8 & 86.5 & 98.6 & 98.6 & 87.8 & 87.8 & 37.8 & 52.7 & 29.7 & 37.8* \\
\text{Controls} & N = 67 & 70.1* & 70.1* & 40.3 & 92.4 & 98.3 & 98.5 & 95.5 & 95.5 & 53.7 & 64.2 & 34.3 & 55.2* \\
\hline
\text{Negative for LILRA3del} & & & & & & & & & & & & \\
\text{Patients} & N = 150 & 60.7 & 62.0 & 31.5 & 83.9 & 94.0 & 94.0 & 97.3 & 97.3 & 33.3 & 54.0 & 28.7 & 38.0 \\
\text{Controls} & N = 222 & 55.9 & 56.3 & 29.7 & 87.8 & 95.5 & 95.5 & 97.7 & 97.7 & 40.5 & 55.0 & 30.6 & 41.0 \\
\hline
\end{array} \)

Abbreviation: R-MS, relapsing multiple sclerosis.

*Uncorrected \( P \)-value < 0.05; \( P \)-value not significant after correcting for multiple comparisons.

*Presence or absence in the genome of each KIR gene was determined as described earlier.89 The gene order is based on that of the KIR complex—the genes that define the A type of haplotype (KIR2DL3 through KIR2DS4) are represented in the middle, preceded and followed, respectively, by genes characteristic of the centromeric and the telomeric parts of B haplotypes. Framework genes and pseudogenes found in all individuals are not shown. Frequencies were compared using the \( \chi^2 \) or Fisher’s test, as appropriate, and \( P \)-values were corrected for multiple comparisons according to Svejgaard and Ryder.

\text{Conflict of interest} \n
The authors declare no conflict of interest.

\text{Acknowledgements} \n
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