Genetic HLA Associations in Complex Regional Pain Syndrome With and Without Dystonia

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Abstract: We previously showed evidence for a genetic association of the human leukocyte antigen (HLA) system and complex regional pain syndrome (CRPS) with dystonia. Involvement of the HLA system suggests that CRPS has a genetic component with perturbed regulation of inflammation and neuroplasticity as possible disease mechanisms. However, it is at present unclear whether the observed association with HLA-B62 and HLA-DQ8 in CRPS patients with dystonia also holds true for patients without dystonia. Therefore, we tested the possible association with HLA-B62 and HLA-DQ8 in a clinically homogeneous group of 131 CRPS patients without dystonia. In addition, we investigated the possible association with other alleles of the HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ loci.

We showed an increased prevalence of HLA-DQ8 (molecularly typed as HLA-DQB1*03:02; OR = 1.65 [95% CI 1.12–2.42], P = .014) in CRPS without dystonia, whereas no association was observed for HLA-B62 (molecularly typed as HLA-B*15:01; OR = 1.22 [95% CI .78–1.92], P = .458). Our data suggest that CRPS with and CRPS without dystonia may be genetically different, but overlapping, disease entities because only HLA-DQ8 is associated with both. The findings also indicate that distinct biological pathways may play a role in both CRPS subtypes.

Perspective: This study is the first to replicate a specific HLA region conferring genetic risk for the development of CRPS. Moreover, associations of HLA-DQ8 with both CRPS with and CRPS without dystonia, and HLA-B62 only with CRPS with dystonia, suggest that these disease entities may be genetically different, but overlapping.

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Key words: Complex regional pain syndrome, genetics, human leukocyte antigen, major histocompatibility complex, dystonia.
involved in the disease as CRPS may occur in familial form and there seems an increased risk of disease developing in siblings of patients with young-onset CRPS.\textsuperscript{5} Given the important role of inflammation, genetic studies in CRPS mainly focused on components of the human leukocyte antigen (HLA) system conferring susceptibility to develop CRPS. The HLA system is divided into 3 classes (Classes I–III) of genes that play important roles in regulating the immune response.\textsuperscript{15,21}

Early genetic studies revealed significant associations of CRPS with HLA-DQ\textsuperscript{14,30} and HLA-DR\textsuperscript{30} and of CRPS with dystonia with HLA-DR\textsuperscript{13,26,28} However, the observed HLA associations are likely confounded by phenotypic heterogeneity, statistical analysis of unplanned post hoc analyses, and/or underpowered study designs. These limitations were addressed in our previous HLA association study in which we analyzed a large, clinically homogeneous group of 150 CRPS patients with dystonia. In this subset of CRPS patients a genetic association was observed with HLA-B\textsuperscript{62} and HLA-DQ\textsuperscript{8}.\textsuperscript{6} In the present study, we tested whether these associations are also observed in a large clinically homogeneous group of CRPS patients without dystonia. Thus, we provide evidence whether CRPS with and CRPS without dystonia should genetically be considered as different disease entities. Secondly, we screened for other associations with HLA alleles in this group of CRPS patients.

**Methods**

**Recruitment of CRPS Patients**

Between April 2004 and March 2010, 131 Dutch Caucasian CRPS type I patients were recruited at the Departments of 1) Neurology of the Leiden University Medical Center; 2) Anesthesiology of the VU University Medical Center; and 3) Anesthesiology of the Erasmus Medical Center. Patient recruitment was performed within the national Trauma-RElated Neuronal Dysfunction (TREND) consortium (http://www.trendconsortium.nl), which integrates Dutch research on CRPS. CRPS was diagnosed by specialists of the participating centers, who are experienced in diagnosing and treating CRPS patients and in using appropriate tests to exclude other causes of CRPS. Patients were excluded if additional tests revealed a different clinical diagnosis. In TREND, the acquisition of data concerning the clinical diagnosis and other phenotype characteristics is performed in a standardized manner, after which this information is stored in a central internet-based database. This strategy was chosen to obtain a homogeneous group of patients with CRPS, which is very challenging, instead of maximizing the sample size by also including less well-characterized patients. Homogenization of clinical diagnoses was ensured by including only CRPS patients who fulfilled the Budapest Research criteria\textsuperscript{10} (Supplementary Table 1). Clinical characteristics of the patients and controls are displayed in Table 1. Informed consent was obtained from all patients, and the study protocol was approved by the local Ethics Committees of the participating centers.

**Table 1. Demographic Information of Participants**

<table>
<thead>
<tr>
<th></th>
<th><strong>Healthy Controls</strong></th>
<th><strong>CRPS Without Dystonia</strong></th>
<th><strong>CRPS With Dystonia</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>5,604</td>
<td>131</td>
<td>150</td>
</tr>
<tr>
<td>Percentage (N) of females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD) age</td>
<td>36.2 (8.7)</td>
<td>47.7 (12.6)</td>
<td>41.3 (12.4)</td>
</tr>
<tr>
<td>Mean (SD) age at onset, years</td>
<td>43.2 (10.8)</td>
<td>31.5 (12.0)</td>
<td></td>
</tr>
<tr>
<td>Median (IQR) disease duration, years</td>
<td>—</td>
<td>2.2 (.9–5.5)</td>
<td>8.7 (4.0–14)</td>
</tr>
</tbody>
</table>

Abbreviations: N, number; IQR, interquartile range.

**HLA Genotyping**

HLA DNA typing was performed for HLA-A (20 alleles), HLA-B (40 alleles), and HLA-C (14 alleles) using a commercially available reverse-line hybridization strip assay (RELI\textsuperscript{TM} SSO; Invitrogen, Washington, DC). Polymerase chain reaction (PCR) amplification and hybridizations were done according to manufacturer’s recommendations. HLA-DR (14 alleles) and HLA-DQ (7 alleles) were typed with a reversed approach of the PCR-sequence-specific oligonucleotide probe technique described elsewhere.\textsuperscript{31} The interpretation of the raw data was carried out with computer-assisted analysis software.\textsuperscript{11} Frequencies of HLA alleles in cases were compared with those of 5,604 randomly selected healthy Dutch blood donors who were previously genotyped (Table 1).

**Statistical Analysis**

This study had 2 aims. In the primary analysis, we tested whether the earlier described association between CRPS with dystonia and HLA-B\textsuperscript{62} and HLA-DQ\textsuperscript{8} alleles also exists in our CRPS sample. A multivariate logistic regression model with group (ie, patient or control) as dependent variable was used to test whether age and sex influenced the results. As a secondary, exploratory, analysis, we tested the possible association of other HLA alleles with CRPS. Genetic associations for HLA alleles were assessed by chi-square tests based on a 2×2 contingency table. P values were obtained with the two-sided Fisher’s exact test. Only for the secondary analyses, corrected P values (P\textsubscript{c}) were calculated to prevent type I errors due to multiple testing for multiple HLA alleles, as described before.\textsuperscript{8,23} P\textsubscript{c} values were calculated using the following formula: \(P_c = 1 - (1 - P)^p\), where \(p\) is the obtained \(P\) value and \(n\) the estimated number of HLA alleles present within the respective locus examined (ie, 20 alleles for HLA-A, 39 for HLA-B, 14 for HLA-C, 14 for HLA-DR, and 6 for HLA-DQ). A P or a P\textsubscript{c} less than .05 was considered a significant difference. Odds ratios (OR) with 95% confidence interval (CI) were calculated according to the Woolf-Haldane test.\textsuperscript{11}

**Re-analysis of HLA Associations in CRPS With Dystonia**

The results of our previous study of CRPS patients with dystonia were based on typing by serology and
compared with a smaller control panel (n = 2,440). Since that time, molecular HLA typing has become widely used in HLA laboratories, so to avoid methodological differences that may hamper comparisons between the 2 studies, we re-analyzed the allele frequencies of the CRPS sample with dystonia (n = 150) using the DNA control panel of 5,604 healthy Dutch blood donors.

**Results**

**Primary Analysis of HLA-DQ8 and HLA-B62 in CRPS**

As a primary analysis we tested the frequencies of HLA alleles DQ8 (molecularly typed as HLA-DQB1*03:02) and HLA-B62 (molecularly typed as HLA-B*15:01) in our set of 131 well-characterized CRPS patients who fulfilled the Budapest Research criteria and did not exhibit dystonia against 5,604 healthy Caucasian Dutch blood donors. Initial (uncorrected) analysis of the 2 target HLA alleles revealed a significant association with HLA-DQ8 (OR = 1.65 [95% CI 1.12–2.42], P = .014), whereas no association with HLA-B62 was found (OR = 1.22 [95% CI .78–1.92], P = .458) (Table 2). Logistic regression showed that HLA-DQ8 is independently associated with CRPS after adjusting for age and sex (Supplementary Table 2). To optimally compare the results with CRPS patients who also have dystonia, we re-analyzed the association by comparing the frequencies of HLA alleles with those of the same control panel. The 2 alleles showed significant association, similar to findings in the original study, with HLA-DQ8 having an OR = 1.85 (95% CI 1.30–2.63, P = .001) and HLA-B62 having an OR = 2.07 (95% CI 1.43–2.99, P < .001). Thus we provide genetic clues that CRPS without and CRPS with dystonia are genetically different, but overlapping, phenotypes.

**Secondary Analysis of HLA Alleles in CRPS Without Dystonia**

In a secondary analysis, we tested the remaining 93 HLA alleles of the HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ loci for association in our set of CRPS patients compared to the large cohort of Caucasian Dutch blood donor controls. The results of all tested alleles per locus are presented in Supplementary Tables 3 to 7.

**Discussion**

HLA alleles HLA-DQ8 and HLA-B62 showed genetic association in our previous study of CRPS patients who also developed dystonia. Here, we tested whether an association can also be found in a clinically homogeneous group of CRPS patients fulfilling the Budapest criteria but who did not suffer from dystonia. In our primary analysis, in which we tested only the HLA-DQ8 (molecularly typed as DQB1*03:02) and HLA-B62 (molecularly typed as HLA-B*15:01) alleles, an association was only found with HLA-DQ8. In our secondary, exploratory, analysis several associations were observed with other HLA alleles, but none survived multiple testing correction, likely due to the relatively small sample size in the case group. Still, we consider our findings of the secondary analysis noteworthy as future studies should investigate whether 1 or more of these associations are genuine. Regardless, this study and the study by de Rooij et al showed that the HLA system seems to be involved in CRPS, which would make the 6p21 region the first robustly replicated chromosomal region conferring some genetic risk to develop CRPS.

Our main genetic finding is that an association was found with HLA-DQ8 in both CRPS patients without dystonia (this study) and CRPS patients with dystonia. Thus, the evidence is accumulating that HLA-DQ8 predisposes to the development of CRPS. Although this is beyond the scope of this study, one could speculate on a possible biological pathway underlying CRPS. Within the HLA system of the major histocompatibility complex (MHC) region, molecules from HLA Class I bind to peptides derived from intracellular proteins and present these to CD8+ T-cells, whereas the HLA-peptide complex from Class II present extracellular peptides to CD4+ T-helper cells. Class III genes regulate complement components, tumor necrosis factor alpha, and other genes. The genotype HLA-DQ8, together with HLA-DQ2, has a strong association with, for instance, celiac disease, which in this case is characterized by an aberrant inflammatory response, in this case response of the small intestine to gluten. Also, in CRPS there are initial clinical features (ie, pain, swelling, redness, warmth) that suggest that aberrant inflammation is an important
mechanism in CRPS. In addition, several studies revealed that both nociceptive afferents and the skin’s immune system contribute to aberrant inflammation as observed in CRPS. In celiac disease the association with, for instance, HLA-DQ8 is explained by the role of HLA in binding gluten-peptides on antigen presenting cells. Gluten-specific CD4+ T-cells in the lamina propria of the small intestine respond to these peptides, which in turn enhances cytotoxicity of locally present lymphocytes against the intestinal epithelium. The subsequent development of the inflammatory process is also determined by a variety of factors involved in enhancing gluten peptides affinity to bind to gliadin T-cell epitopes and promoting downstream inflammatory effects. The proposed mechanism of celiac disease is that once a threshold of gluten on T-cells has been reached, a self-amplifying loop mediating a continuous inflammatory response is established. At present the exact nature of the inflammatory process in CRPS—in relation to HLA-DQ8, as well as other inflammatory responses—is unclear. Interestingly, though, studies on cytokines in artificially drawn blister fluid of the affected extremity of CRPS patients revealed that levels of interleukin-1 and tumor necrosis factor alpha remained elevated over a period of 3 years. Moreover, recently autoantibodies against muscarinic-2 receptor and β2 adrenergic receptor in CRPS were shown. It would seem possible that peptides derived from these 2 receptors, towards which autoantibodies were found, could possibly be presented in HLA-DQ8. This interesting possibility should be investigated in depth by additional studies. Despite important differences between celiac disease and CRPS, shared characteristics, including a role of HLA-DQ8, inflammation, and tissue injury in both disorders, seem to suggest that complex gene-environment interactions may serve as an interesting model for future research in CRPS. Given the relatively low OR observed in our studies, HLA-DQ8 plays a small role in the development of CRPS, and its presence is not required to develop the disease. This is not uncommon, because in most diseases there is a complex interplay between genetic and environmental factors.

Unlike with DQ8 that showed association in both CRPS with and CRPS without dystonia, we did not observe an association with HLA-B62 in patients who do not exhibit dystonia. This indicates that the 2 CRPS patient groups are genetically different. It is tempting to speculate why only in CRPS patients with dystonia is there an association with HLA-B62. One could postulate that there might be an interaction effect between Class I and Class II alleles in the development of dystonia. A combined role of HLA Class I and II in disease is uncommon but has been reported in, for example, diabetes mellitus type I. Additionally, MHC Class I molecules have been implicated in nonimmunological functions such as neural development and synaptic plasticity, and their presence was demonstrated presynaptically at the neuromuscular junction and in spinal motor neurons. Collectively, the limited data on MHC Class I molecules in neurons suggest that HLA Class I may play a role in biological pathways underpinning aberrant neuromuscular junctions. Axotomization of motoneurons in these mice led to synaptic reorganization that was strongly disrupted compared to wild-type mice. Collectively, the limited data on MHC Class I molecules in neurons suggest that HLA Class I may play a role in biological pathways underpinning aberrant neuromuscular junctions. A direct comparison of the 2 patient groups would need much larger numbers than 150 patients per group to take the various HLA alleles into account. This is due to the fact that HLA is an enormous polymorphic system, which is reflected by the more than 7,000 recognized different HLA alleles. By choosing a large control panel we are (almost) sure that all HLA alleles of the CRPS patients are covered in the control population. This is due to the fact that HLA is an enormous polymorphic system, which is reflected by the more than 7,000 recognized different HLA alleles. By choosing a large control panel we are (almost) sure that all HLA alleles of the CRPS patients are covered in the control population. Therefore, we consider the current approach valid and efficient. Moreover, we have been able to show robust results despite the relatively small sample size of the patient groups. The comparison of HLA-B62 frequencies between the control group and CRPS patients with dystonia is very significant, whereas the comparison between controls and CRPS patients without dystonia is not. Additionally, when inspecting the prevalence of HLA-B62 in CRPS patients without dystonia, the percentage of patients with HLA-B62 is almost equal to that of the control group.

### Table 3. Secondary Analysis—Odds Ratios and P Values Estimated for HLA Alleles Typed Among CRPS Cases and Control Subjects

<table>
<thead>
<tr>
<th>HLA Alleles</th>
<th>Serotype</th>
<th>% Cases</th>
<th>% Controls</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>Pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>B*13</td>
<td>B13</td>
<td>8</td>
<td>4</td>
<td>2.24</td>
<td>1.20–4.15</td>
<td>.024</td>
<td>.612</td>
</tr>
<tr>
<td>B*37</td>
<td>B37</td>
<td>7</td>
<td>3</td>
<td>2.32</td>
<td>1.18–4.56</td>
<td>.041</td>
<td>.805</td>
</tr>
<tr>
<td>C*04</td>
<td>Cw4</td>
<td>15</td>
<td>23</td>
<td>.58</td>
<td>.36–.94</td>
<td>.020</td>
<td>.251</td>
</tr>
<tr>
<td>C*06</td>
<td>Cw6</td>
<td>24</td>
<td>16</td>
<td>1.64</td>
<td>1.09–2.47</td>
<td>.023</td>
<td>.274</td>
</tr>
<tr>
<td>DRB1*04</td>
<td>DR4</td>
<td>37</td>
<td>28</td>
<td>1.51</td>
<td>1.05–2.16</td>
<td>.030</td>
<td>.348</td>
</tr>
<tr>
<td>DRB1*11</td>
<td>DR11</td>
<td>24</td>
<td>17</td>
<td>1.57</td>
<td>1.05–2.36</td>
<td>.043</td>
<td>.461</td>
</tr>
</tbody>
</table>

NOTE. The results of all tested alleles per locus are presented in Supplementary Tables 3 to 7.
patients with and without dystonia are genetically different or not. In conclusion, our data suggest that CRPS patients with and without dystonia may be genetically different and that the associations with HLA-DQ8 and HLA-B62 in CRPS with dystonia, and HLA-DQ8 only in CRPS without dystonia, could point to distinct pathophysiological mechanisms that might explain the perturbed regulation of inflammation and neuroplasticity in CRPS.

Acknowledgments

We thank the generous participation of CRPS patients and controls. We would like to thank Florencia Gosso for her contribution during the initial phase of the study.


