Distinct HLA associations by stroke subtype in children with sickle cell anemia

Carolyn Hoppe, William Klitz, Janelle Noble, Lara Vigil, Elliott Vichinsky, and Lori Styles

Children with sickle cell anemia (SCA) carry a 200-fold increased risk for cerebral infarction. Stroke can be the result of small-vessel (SV) or large-vessel (LV) disease. However, it is unknown whether these subtypes result from the same pathophysiologic processes. Complete HLA genotyping was performed on 231 eligible children previously enrolled in the Cooperative Study of Sickle Cell Disease (CSSCD). Cerebral infarction on magnetic resonance imaging (MRI) was documented in 71 patients, and 160 patients had negative findings on MRI. Based on MRI/magnetic resonance angiography (MRA) findings, infarct size, and location, 36 patients were classified as having LV stroke and 35 as having SV stroke. When comparing the total MRI+ group with the MRI− group, HLA DPB1*0401 was associated with increased stroke risk (P = .01), whereas DPB1*1701 (P = .02) conferred protection from stroke. These DPB1 associations with stroke were attributed to the SV stroke group, in whom DPB1*0401 was associated with susceptibility (P = .003) and DPB1*1701 with protection from stroke (P = .06). In the LV stroke subgroup, HLA-A*0102 (P = .02) and -A*2612 (P = .007) conferred susceptibility, whereas -A*3301 (P = .04) protected from stroke. These results suggest that specific HLA alleles influence stroke risk and appear to contribute differently to SV and LV stroke subtypes. The distinct HLA associations with SV and LV stroke suggest that different pathologic processes may be involved in the development of stroke in children with SCA. If these results are confirmed in a larger study, HLA type may serve as a useful marker for the early identification of SCA patients at high risk for stroke.

The human leukocyte antigen (HLA) genes regulate inflammation. Specific HLA alleles have been identified as risk factors for vascular disease processes similar to stroke in SCA, including Moyamoya disease, idiopathic childhood stroke, aortic aneurysms, and cardiovascular disease. Data from in vitro studies have demonstrated the involvement of T cells and antigen-presenting cells in response to endothelial injury in SCA. Variation in HLA alleles may partially account for the immune-mediated events leading to stroke in susceptible persons with SCA.

We previously reported an association between HLA and stroke in a small local population of children with SCA. To determine whether unique HLA alleles contribute to a specific stroke phenotype based on LV versus SV disease, we extended our investigation of HLA and stroke risk to a prospective national cohort of children with SCA, the Cooperative Study of Sickle Cell Disease (CSSCD). The CSSCD has the strengths of random ascertainment, thorough and consistently collected clinical data, and relatively large sample sizes.

Patients, materials, and methods

Study patients and design

The CSSCD is a national, multicenter study designed to define the natural history of sickle cell disease (SCD) by following up more than 4000...
patients with SCD. Objectives, eligibility requirements, enrollment procedures, and data collection methods for the CSSCD have been reported. As part of this study, the CSSCD initiated a prospective infant cohort trial in 1978 into which almost 400 infants were enrolled at birth and were followed up prospectively to characterize their natural history. Children in this cohort underwent brain MRI at age 6 years and then every 2 years thereafter. These children have now been followed up for more than 13 years and are, on average, 13.1 ± 2.9 years of age. All children with adequate DNA and interpretable MRI results were included in this study. Children with SCA (homozygous hemoglobin [Hb] S) and MRI-documented cerebral infarction (asymptomatic or symptomatic) before age 15 years were included as case subjects. Children with MRI evidence of atrophy, cerebral hemorrhage, or other noninfarction abnormalities, unless accompanied by infarction, were excluded. MRI+ patients were subclassified as having LV or SV disease using an algorithm based on MRI/magnetic resonance angiography (MRA) findings, infarct size, and location (Figure 1). Using the definition of cerebrovascular disease previously described by the CSSCD, MRI-positive patients were also characterized by a history of clinical stroke. Children with SCA (homozygous HbS) who were older than 13 and had normal findings on MRI were included as control subjects. Only patients older than 13 were considered as controls to minimize the possibility of including in the control group a child in whom stroke would subsequently develop. Patient identities were masked, and patient samples were collected and stored with a study number previously assigned by the CSSCD. The present study was reviewed and approved by the Institutional Review Board of Children’s Hospital Oakland.

MRI scanning

The imaging systems and techniques used at each center for the brain MRI and the procedures for review of MRI images have been described. Usually, MRI was performed on a 1.5-T MRI scanner. A few centers used a 0.6- or a 1.0-T scanner. Criteria for acceptable images were established by the study neuroradiologists and included noncontrast spin-echo T1-weighted pulse sequence (short repetition time (TR), short echo time (TE), and T2-weighted axial spin-echo sequence (long TR, long TE) with intermediate (long TR, short TE) axial and coronal T2 images. Intermediate images also were obtained. All MRIs were reviewed centrally by 3 neuroradiologists who were blinded to patient clinical history and HLA status. Two neuroradiologists read each MRI scan independently and recorded interpretations on a study data sheet. The interpretations differed, consensus was reached in discussion with the third neuroradiologist. The scope of abnormalities defined by the review process was previously reported. This study included results from all MRI scans performed on eligible patients.

HLA typing

Samples from the CSSCD containing previously quantitated genomic DNA were first assessed to ensure adequate polymerase chain reaction (PCR) product for HLA typing. Complete HLA typing at class I (HLA-A, HLA-B, HLA-C) and class II (HLA-DRB1, DQB1, DPB1) loci was performed at an allelic level using well-described DNA-based methods. Briefly, DNA samples were amplified by PCR using locus-specific primers and were analyzed using immobilized sequence-specific oligonucleotide probe (SSOP) methods. Class I typing methodology modifications from published methods included amplification of exons 2 and 3 separately in a multiplex reaction, followed by hybridization to modified and additional probes for exons 2 and 3 on optimized immobilized probe strips (personal communications, Drs R. Apple and H. Erlich, Roche Molecular Systems, Inc, Nutley, NJ, September 2002). For allelic resolution, some sample types required group-specific amplifications followed by immobilized probe analysis to distinguish them. Computer software programs were used to identify types from the probe hybridization patterns. HLA alleles were classified according to nomenclature defined by the World Health Organization.

Statistical analysis

HLA allele frequency distributions at each of 6 loci were compared among disease status categories (MRI+ vs MRI−, SV vs MRI−, and LV vs MRI− groups) using tests of independence with the log-likelihood ratio (or G-statistic) for each of the 6 HLA loci examined. This approach uses a strategy often taken in population genetics, and it overcomes the multiple comparison problem inherent in testing for the presence or absence of an allele because the entire sample of all alleles at a locus is included in a single test. It results in an overall P value for a table and the individual deviation (contribution) of each allele, expressible as a G-statistic and as an odds ratio (OR). Rare alleles (here defined as those observed fewer than 3 times in the MRI+ and MRI− samples) were combined for global testing. A significantly deviant allele (uncorrected P < .05) is reported along with its OR. This testing strategy is conservative in that it assumes uniform likelihood of deviations and equal power for each allele. However, we expect only one or few HLA alleles to contribute to stroke predisposition. Moreover, allele frequencies vary widely. The numbers of homozygotes at each of the 6 HLA loci observed for each person ranged from 0 to 5. After binning of participants into 2 categories, one with 0 or 1 homozygote and the other with 2 to 6 homozygotes, the 2 × 3 (2 homozygosity classes and 3 disease status classes) were tested using the χ2 statistic.

Results

Brain MRI results

Of the 231 SCA patients included in this study, 160 (69%) had normal findings on brain MRI. Seventy-one (31%) patients demonstrated infarctive lesions on MRI; their mean age was 9.3 years (median, 9.0 years) at the time of first positive MRI results. Of these, 36 (51%) had MRI evidence of LV involvement (with or without SV involvement), and 35 (49%) had exclusively SV involvement. Clinical history of cerebrovascular accident (CVA) was documented in 26 (37%) of the 71 patients. Most (24 patients) CVAs were caused by LV disease, and only 2 were caused exclusively by SV disease.

HLA typing results

All 231 SCA patients were typed at high (allelic) resolution for the HLA loci A, B, C, DRB1, DQB1, and DPB1. To ensure that the CSSCD cohort reflects a representative sample of the African-American population, results from a recent study using DNA-based methods to type HLA-DRB1 and DQB1 on a series of 243 African-American control samples were used for comparison with the CSSCD sample. By contingency table testing, the 2
Table 1. Overall test of significance of allelic heterogeneity comparing SV, LV, and combined stroke groups (MRI+) against the MRI− group for each of the 6 HLA loci

<table>
<thead>
<tr>
<th>HLA locus</th>
<th>No. alleles tested</th>
<th>Log-likelihood ratio statistic (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28 (58)</td>
<td>41.1 (.005)</td>
</tr>
<tr>
<td>B</td>
<td>23 (47)</td>
<td>35.9 (.33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.3 (.56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.4 (.61)</td>
</tr>
<tr>
<td>C</td>
<td>19 (26)</td>
<td>16.5 (.62)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19.1 (.32)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.7 (.72)</td>
</tr>
<tr>
<td>DRB1</td>
<td>24 (37)</td>
<td>24.3 (.39)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.0 (.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.5 (.27)</td>
</tr>
<tr>
<td>DQB1</td>
<td>13 (17)</td>
<td>16.8 (.16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.9 (.20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.2 (.29)</td>
</tr>
<tr>
<td>DPB1</td>
<td>11 (22)</td>
<td>23.3 (.016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.4 (.03)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.8 (.39)</td>
</tr>
</tbody>
</table>

*Rare alleles, occurring fewer than 3 times in the entire sample, were binned into a combined group for testing.

African-American samples were indistinguishable (DRB1, P = .24; DQB1, P = .11).

Testing for overall significance among stroke categories at each of the 6 loci revealed differences in allele frequency distributions between the total MRI+ and MRI− groups at the HLA-A (P = .005) and DQB1 loci (P = .016) (Table 1).

The SV subgroup and the MRI− group also differed at DPB1 (P = .03). Nominally significant differences in HLA allele distributions were present at HLA-A in both subgroups (SV vs MRI−, P = .04; LV vs MRI−, P = .03). No significant overall differences in allele frequencies were found at HLA-B, C, DRB1, or DQB1. HLA loci with significant overall G-tests were then examined for allele-specific results comparing disease status categories (Table 2).

Allele frequencies for the MRI+ and MRI− groups were first compared to test for the effect of HLA alleles common to SV and LV stroke groups. Of the 6 loci, the HLA-DPB1 allele frequencies differed between the 2 groups (P = .03). Two alleles were responsible for the difference between the groups, DPB1*0401 was associated with an increased stroke risk (OR = 2.1; P = .01; 95% confidence interval [CI] = 1.2, 4.2), whereas DPB1*1701 (OR = 0.3; 95% CI = 0.13, 0.87) conferred protection from stroke. To detect HLA associations specific to a particular type of vessel disease, MRI+ patients were subclassified by LV versus SV involvement. When stratifying stroke patients by LV or SV disease, the DPB1 association with stroke was attributable to the LV stroke group, where DPB1*0401 was associated with susceptibility (OR = 3.0; P = .003; 95% CI = 1.4, 6.1) and DPB1*1701 was associated with protection (OR = .27; P = .06; 95% CI = 0, 1.1) from stroke. In addition, HLA-A*0205 was in apparent excess in the SV subgroup (OR = 7.1; P = .01; 95% CI = 1.4, ∞). In the LV stroke subgroup, HLA-A*0102 (OR = 4.7; P = .02; 95% CI = 1.3, 17.6) and HLA-A*3301 (OR = 1.1; P = .27; 95% CI = 0.3, 3.6) conferred susceptibility, whereas A*3101 (OR = 0.4; 95% CI = 0.0, 0.97) protected from stroke.

Overall HLA locus homozygosity was also assessed because it implies a reduced capacity to present foreign peptides to the immune system and it may also influence stroke risk. For the total sample of 231 CSSCD patients, homozygosity at any of the 6 HLA loci ranged from 0 to 6 loci. Because of the relatively small numbers in each category, 2 combined homozygosity categories were constructed with participants homozygous for 0 to 1 loci in one category and 2 to 6 loci in the other category. The resultant 2 × 3 table of proportions was significantly heterogeneous (Table 3). Although 12% of the MRI− group was homozygous at 2 or more loci, 29% of the LV stroke group displayed homozygosity at that level. The LV stroke group was intermediate at 19% (χ² = 6.57; P = .037).

**Discussion**

In searching for candidate genetic factors in SCA and stroke, particular focus on other similar diseases associated with vascular endothelial injury in the general population may provide additional clues. There is increasing recognition of the involvement of the immune system and the possible role of HLA genes in diseases, including atherosclerosis17,26 and aortic aneurysm, that are characterized by endothelial injury.15 Moyamoya disease, characterized by abnormal vascular collaterals around stenotic internal carotid arteries, is anatomically and histopathologically most similar to the intracerebral vasculopathy of SCA. Reports of a genetic basis for Moyamoya disease have shown an association with the class 2 HLA allele, DQB1*0502.13 Given the pathologically similar “moyamoya” changes seen in SCA patients with stroke, these reports suggest that perhaps other HLA alleles are associated with stroke in SCA.

In a previous study on a small local cohort of children with SCA, we documented that particular HLA phenotypes are associated with increased risk for cerebral infarction.19 Although our initial results suggested that specific HLA alleles contribute to stroke risk in SCA, analyses to localize the observed HLA effects to a particular stroke subtype were not possible in the relatively small population studied. Our present findings on a larger cohort of children participating in the CSSCD show unique HLA associations with stroke when comparing the MRI+ group with the MRI− group, with DPB1*0401 and DPB1*1701 conferring susceptibility to and protection from stroke, respectively. Subclassification of MRI-positive patients by LV versus SV involvement localized these observed HLA associations to the SV subtype of stroke, suggesting that unique antigen-dependent mechanisms contribute to the pathogenesis of different subtypes of stroke. Not surprisingly, the HLA associations found in this study differ from those found in our pilot institutional study19—the study populations are

---

**Table 2. HLA allelic associations with total MRI+, SV, and LV stroke groups**

<table>
<thead>
<tr>
<th>HLA locus</th>
<th>MRI+ vs MRI−</th>
<th>SV vs MRI−</th>
<th>LV vs MRI−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>P</td>
<td>95% CI</td>
</tr>
<tr>
<td>HLA-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*0102</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>*0205</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>*2612</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>*3301</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DPB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*0401</td>
<td>2.1</td>
<td>.01</td>
<td>1.2, 4.2</td>
</tr>
<tr>
<td>*1701</td>
<td>.30</td>
<td>.02</td>
<td>.13, .87</td>
</tr>
</tbody>
</table>

NS indicates not significant.
genetically and phenotypically distinct and, therefore, not comparable. The CSSCD, a prospective national cohort free of ascertainment and population biases and reflective of the genetic diversity among African-Americans, is a superior population in which to study the genetic determinants of stroke in SCA. Furthermore, the small sample in our pilot study precluded subgroup analyses based on LV or SV stroke.

Because stroke is a phenotypically heterogeneous condition, varying from large cortical artery distribution infarcts to mild lacunar infarcts, an assessment concerning the genetics of stroke requires an accurate classification and subgroup analysis of the type of stroke. The pattern of cerebral infarction on MRI suggests 2 separate pathogenetic mechanisms for stroke in SCA: proximal LV disease with inadequate cerebral perfusion (distal field insufficiency syndrome) and distal SV disease (sludging syndrome).30 Rheologic changes, increased endothelial adherence, intimal damage with smooth muscle hypertrophy, and thrombus formation are all factors that have been shown to contribute to the development of LV stroke,22 whereas SV lesions are thought to be related to peripheral vaso-occlusive events resulting in inadequate perfusion and subsequent infarction. Although previous studies to identify stroke risk factors have distinguished brain infarction from intracranial hemorrhage, further classification of ischemic stroke into subtypes based on presumed mechanism has not been performed. It remains unclear how the risk factor profiles for these 2 distinct manifestations of cerebrovascular disease differ. Determining which genes are responsible for severe phenotypic expression (eg, stroke from LV disease) versus those that confer milder effects (eg, lacunar infarction) may lead to a better understanding of the distinctive pathophysiologies of stroke. The association of DPB1*0401 and DPB1*1701 alleles with stroke from SV disease and of HLA A*0102, A*2612, and A*3301 alleles with LV stroke may reflect distinct genetic etiologies for stroke in SCA. Separation of ischemic stroke into subtypes based on presumed mechanisms may help clarify the contribution of HLA to stroke risk in SCA.

In addition to individual allele associations with stroke, our results show that homozygosity at HLA loci is associated with stroke risk. Homozygosity for HLA alleles may reduce the ability to mount an immunogenic response because of ineffective recognition and presentation of a diverse array of foreign antigens. Persons heterozygous at HLA loci are therefore able to present a greater variety of antigenic peptides than are homozygotes, resulting in a more productive immune response. In studies investigating the association of HLA and HIV disease, HLA homozygosity and reduced antigen disparity have been shown to increase the rate of HIV progression.31,32 We found a trend of increasing stroke risk with HLA locus homozygosity based on stroke subtype, with MRI—persons having the least degree of HLA homozygosity and those with LV and SV stroke subtypes having increasingly greater degrees of HLA homozygosity.

In conclusion, our results provide evidence for distinct HLA contributions to the development of LV and SV stroke subtypes in SCA. These results showing specific HLA effects based on stroke subtype emphasize the need for accurate phenotyping to elucidate the genetic basis for stroke in SCA. Given the extensive polymorphism at the HLA locus, even larger studies to confirm our results would be valuable. Although our findings demonstrate distinct HLA associations with particular stroke subtypes, mechanistic studies are still required to establish a causal role for HLA in the development of stroke in SCA. If confirmed, these findings could have a profound clinical impact on children with SCA because specific HLA “risk” alleles may help identify those at highest risk for stroke. Early identification of these high-risk patients would allow for preventive intervention, such as chronic transfusion or bone marrow transplantation, and for avoidance of these potentially toxic therapies in those who are at reduced risk.

References

21. Farber MD, Koshy M, Kinney TR. Cooperative


