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Association of Natural Killer Cell Ligand Polymorphism, HLA-C Asn80Lys, with the Development of Anti-SSA/Ro Associated Congenital Heart Block

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Abstract

Objective—Fetal exposure to maternal anti-SSA/Ro antibodies is necessary but insufficient for the development of autoimmune congenital heart block (CHB), suggesting that other factors, such as fetal genetic predisposition, are important. Given the prior MHC and CHB risk association, here we test the hypothesis that a variant form of HLA-C Asn80Lys, which binds with high affinity to an inhibitory killer-cell immunoglobulin-like receptor (KIR) rendering an NK cell incapable of restricting inflammation, contributes to the development of CHB.

Methods—192 pedigrees (194 CHB, 91 unaffected siblings, 152 fathers, 167 mothers) and 1073 out-of-study controls were genotyped on the ImmunoChip single nucleotide polymorphism (SNP) microarray. Imputation was selected to identify associations at HLA-C Asn80Lys (Asn, C1; Lys, C2) and KIR. Tests for association used logistic regression. Matched analyses between affected and unaffected children employed McNemar's test and the pedigree disequilibrium test (PDT).

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Conflicts of Interest: None.

Results—Compared to the out-of-study controls of the same gender, the C2 allele was less frequent in the mothers (odds ratio (OR)=0.63, P=0.0014) and more frequent in the fathers (OR=1.40, P=0.0123), yielding a significant gender-by-C2 interaction (P=0.0002). The C2 allele was more frequent in the affected siblings than the unaffected siblings (OR=3.67, P=0.0025), which was consistent with the PDT (P=0.016); these results were observed in both genders and across the US and European cohorts. There was no difference in the inhibitory KIR genotype (AA KIRs) between the affected and unaffected children (P=0.55).

Conclusions—These data establish C2 as a novel genetic risk factor associated with CHB. This observation supports a model in which anti-SSA/Ro fetuses expressing C2 ligands may have impaired NK surveillance resulting in unchecked cardiac inflammation and scarring.

Introduction

Congenital heart block (CHB) is an autoimmune disease (1) characterized by the presence of maternal antibodies directed against components of the Ro/SSA–La/SSB ribonucleoprotein complex. Injury to the fetal heart most often occurs during gestational weeks 18 to 24 and is presumed to be dependent upon the transport of maternal IgG autoantibodies by neonatal Fc Receptor (FcRn). Maternal health status accompanying the production of anti-Ro/La autoantibodies is not a risk factor for this fetal disease. Many of the mothers are clinically asymptomatic at the time of CHB detection, and thus maternal autoantibody status is only tested after fetal injury is identified (1). Advanced conduction abnormalities are the signature phenotype of anti-SSA/Ro-associated cardiac injury. However, the disease spectrum can also extend to the myocardium and endocardium. 3° block is not reversible and is associated with significant mortality (17.5%) and morbidity (70% require pacing) (2). The case fatality rate approaches 50% when extranodal disease is present (2).

Maternal anti-SSA/Ro antibodies are identified in more than 85% of cases with CHB; however, their presence alone is not sufficient for CHB development. Only an estimated 2% of anti-SSA/Ro-positive mothers will have an affected CHB child, and furthermore the recurrence rate is approximately 18%, not 100%. Thus, although fetal exposure to maternal anti-Ro/SSA antibodies is necessary, it is not the sole driver of CHB progression. Identification of a potential fetal genetic contribution is challenging to disentangle from the maternal genome since there are strong associations between anti-SSA/Ro positivity and the extended DRB1*03:01 and HLA1-B8 haplotypes (3). Unsurprisingly, the first large-scale GWAS of 116 CHB cases versus healthy controls (4) identified an association of seventeen SNPs at 6p21 within the Human Leukocyte Antigen (HLA) region. In addition to reflecting the presumed contribution to the generation of maternal autoantibodies, this association is of interest given an interacting genetic effect between 6p21 and 19q13 (5). Specifically, HLA-C (6p21) acts as a ligand for the checkpoint receptor killer cell immunoglobulin-like receptors (KIR) encoded within 19q13 (5). A dimorphism at position 80 in HLA-C proteins creates two epitope subgroups, defined by their KIR interactions: C1 (Asn80) and C2 (Lys80) (5). The C1 designation includes HLA-C alleles: *01, *03, *07, *08, *12, *13, *14, *16:01, and *16:04; C2 includes: *02, *04, *05, *06, *15, *17, *18, and *16:02.

To date, no study has interrogated the interacting effect of KIR and HLA-C within anti-SSA/Ro-exposed affected and unaffected CHB siblings. To test the *a priori* hypothesis that a fetal genetic contribution to the pathogenesis of CHB might involve an inhibition of check point inhibitors, the current study focuses on the HLA-C Asn80Lys and *KIR* genes. Using U.S. and European based cohorts we specifically addressed whether the *C1/C2* dimorphism constitutes a susceptibility locus for the development of CHB.

Methods

Patients and Samples

Family data were collected from several sources: primarily, the United States Research Registry for Neonatal Lupus (RRNL) (138 CHB cases) with additional samples from collaborations in Italy (28 CHB cases) and France (28 CHB cases). There were 3 cases and 1 case of 1° CHB in the RRNL and French cohorts, respectively along with 1 case of 2° /NSR/1° CHB in the Italian cohort. Pedigrees were genotyped at HudsonAlpha, and samples passing quality control were used in analyses including: 194 CHB offspring, 152 fathers, 167 mothers, and 91 unaffected siblings. There were 90 independent (unrelated) sibling pairs of a CHB child and an unaffected child with the majority of these originating from the 139 complete trios (mother, father, and affected offspring); 13 of the pedigrees with paternal genotype data did not have maternal genotype data, and 28 of the pedigrees with maternal genotype data did not have paternal genotype data.

CHB was diagnosed if the following criteria were met: 1) the presence of a conduction defect (1°, 2° or 3° heart block) documented by electrocardiogram, echocardiogram and/or history of pacemaker; and 2) the presence of antibodies to the 52kD SSA/Ro, 60kD SSA/Ro, and/or 48kD SSB/La in the child and/or mother as determined by ELISA using recombinant proteins or by a commercial laboratory (90% of cases were tested in the research laboratory of JPB and RMC).

For case-control analyses, 1,073 healthy, out-of-study controls were obtained from the International Consortium on Systemic Lupus Erythematosus (SLEGEN), of which 635 were female. All control samples were of self-reported European ancestry.

Genotyping

All samples (cases and controls) were genotyped on the Illumina Infinium Immunochip array, as part of the larger Systemic Lupus Erythematosus Immunochip Consortium (>25,000 cases and controls from four ethnicities) (6). Illumina iScan machines were used in accordance with Illumina's protocols. The Oklahoma Medical Research Foundation (OMRF) performed genotyping and completed genotype calling from intensity data via OptiCall. SNPs that had call rates <95%, differential missingness between cases and controls, significant departure from Hardy-Weinberg equilibrium, or minor allele frequency <0.01 were excluded from analyses. Using the program ADMIXTURE, admixture estimates were computed on quality controlled genotype data, and genetic outliers were removed from subsequent analyses. Familial relationships were verified by using identity by descent

statistics in the software KING (7) and genotype data inconsistent with Mendelian inheritance were removed.

Imputation for Classical HLA alleles was completed using HIBAG (8). Only high quality SNPs were used as input and the European Ancestry reference panel (provided by HIBAG) was utilized. For HLA Class I alleles, HLA-C had the highest posterior probability (mean $\pm$ SD: 0.97 ± 0.08). For each allele, C1 or C2 status was designated based on amino acid at position 80, as originally identified by Winter et al (5): Lys80=C2; Asn80=C1 (5). For each person, C1 and C2 allele counts were based on best guess imputed HLA alleles. Best guess genotype data was completely consistent with Mendelian inheritance.

Imputation of KIR genes was completed using KIR*IMP V1.1.1 (<http://imp.mcri.edu.au>) (9). High-quality SNP genotypes were aligned with the reference sample and phased using SHAPEIT. The resulting phased haplotype output files from SHAPEIT were uploaded to the KIR*IMP V1.1.1 imputation server. Four Mendelian inconsistencies for the KIR A/B haplotypes were observed and removed.

Statistical Methods

Comparisons between unrelated cases and out-of-study controls were completed under a logistic regression model, which included admixture estimates as covariates. Admixture proportions were computed based on the Immunochip genotype data and the program Admixture as previously reported (4). A McNemar test was utilized to compare pedigree-matched pairs of affected and unaffected siblings.

Results

All analyzed pedigrees met the inclusion criteria of having a SSA/Ro-positive mother and data on an affected CHB offspring. The RRNL cohort of CHB was 54% female, and 98% had advanced heart block (2°/3° CHB). Similar data were reported for the Italian and French cohorts, except being enriched slightly for female CHB children.

In mothers, there was an enrichment of the C1 allele and a reduction of the C2 allele (mothers compared to female controls, OR=0.63; P=0.0014) (Table 1). In contrast, the C2 allele was observed at an increased frequency in the fathers compared to male controls (OR=1.40; P=0.0123). This gender-by-C2 interaction was statistically significant (P=0.0002). Affected children did not exhibit enrichment for C1 or C2 alleles in comparison to out-of-study controls (OR=1.14; P=0.2627). However, within the family data, affected and unaffected offspring exhibited dissimilar C1 and C2 frequencies.

Pedigree analyses exhibited differences in the HLA C1 and C2 frequencies between affected and unaffected offspring. Specifically, the affected offspring had comparable C2 frequencies to the fathers (affected offspring frequency=0.42; fathers frequency=0.45), and unaffected offspring C2 frequencies were comparable to the mothers (unaffected offspring frequency=0.31; mothers frequency=0.29). A formal assessment of C2 frequency differences in affected and unaffected offspring within a pedigree, which was computed using the McNemar test on matched-pedigree pairs of siblings (n=90 pairs), showed a significant

increase in the C2 allele in the affected offspring (OR=3.67; P=0.0025; Table 2). The results were comparable in the subset of CHB discordant sibling pairs where the mothers were homozygous for C1 (n=46 pairs, OR=2.75; P=0.07). When the paired-sibling analysis was stratified by geographic region (U.S., Europe), the results were still significant in each geographic stratum (U.S. OR=3.25; P=0.0290; Europe OR=4.50; P=0.0348). The replication and consistency of the effect sizes across the U.S. and European cohorts suggest the impact of the C1/C2 designation is representative of the CHB cohort and not an artifact of ancestral differences. The PDT, which incorporates both the differential parental transmission to affected offspring and the discordant siblings, also yielded significant results (P=0.016).

Given the interaction of C2 and KIRs, particularly within an AA (inhibitory) KIR haplotype, KIR genotypes were imputed by KIR*IMP. KIR AA haplotypes did not reveal enriched frequencies in either affected (AA=0.47) or unaffected children (AA=0.48) (P=0.5485). Similarly, no differences were identified between affected and unaffected siblings for specific KIR alleles (Figure 1). Individual KIR frequencies were consistent with published frequencies in European populations (10). The PDT was also not significant for the AA haplotype.

Discussion

In this study, the *HLA-C*-encoded supertypic epitope C2, which constitutes a high-affinity ligand for the inhibitory natural killer (NK) cell receptor KIR2DL1, was significantly enriched in CHB compared to unaffected siblings. The HLA-C2 genotype comparisons of mothers and fathers with the same gender out-of-study controls exhibited significant association in each gender but in opposite directions, yielding an intriguing gender-by-C2 interaction. The C1 and C2 frequencies reported in our control population are consistent with independent, healthy cohorts of European ancestry (11). For assessment of the inhibitory KIR genotype, more broadly referred to as AA KIRs, there were no differences when comparing affected and unaffected children.

The contribution of KIRs to NK cell phenotype varies depending on canonical and non-canonical NK states. For example, when considering the widely held role of NK cells, there is a strong dependence on the composition of KIRs, which are classically described in the context of MHC-1 recognition, whereby inhibitory KIRs engage self-MHC ligands to suppress NK cell activation to confer a low risk of autoimmunity. In the setting of viral infection, activating KIR receptors recognize ligands expressed on infected cells, which results in cytokine production and cellular cytotoxicity by NK cells. The integration of signals from activating and inhibitory KIR receptors and their ligands ultimately determine NK functionality.

Inhibitory KIRs contribute to a non-canonical role of NK cells. Mechanistically, the inhibitory KIR2DL1 binds to C2 with higher affinity than KIR2DL2/3 to C1 and KIR2DL2 to C2. The exceptional specificity and affinity of the KIR2DL1-C2 interaction might lead to stronger inhibition of NK cells, which could compromise the ability of NK cells to limit macrophage activation. For example, Bellora and coworkers report that NK cells serve an important role in limiting or restraining macrophages (12) and dendritic cells (13). Thus, the

C2 epitope participates as a checkpoint inhibitor ligand, which would be anticipated to diminish a physiological role by NK cells to forestall the consequences of inflammation to host tissues. Perhaps this explains the presence of macrophages and giant cells consistently observed in the autopsy tissue from CHB hearts (14) since C2 ligation conferred by the genetically vulnerable fetus would exhaust the restraining NK cells. These observations are compatible with a model in which C2 ligands impair NK-mediated surveillance in childhood acute lymphoblastic leukemia. In accord with our study, this model also does not include genetic differences in KIR (11).

Further evidence to support this hypothesis was published by Meisgen and colleagues who performed an association of targeted MHC molecules (HLA-A, -Cw and -DRB1 alleles), using low-resolution typing of 87 CHB families (15). HLA-Cw*05 variants (a C2 epitope) were significantly more frequently transmitted to affected offspring. However there was no significance when accounting for parent of origin. In further elaborations of the Meisgen study, because HLA-Cw*06 was enriched in unaffected and not affecteds, a hypothesis was advanced suggesting that HLA-Cw*05 serves as novel fetal HLA allele variant that confers susceptibility to CHB while Cw*06 emerges as a protective allele. Given that both alleles carry the C2 epitope, our current model and that of Meisgen are not in full alignment and require further study. Of note, both our study and that of Meisgen support a paternal contribution to the development of CHB.

These data require careful consideration and recognition of their inferential limits. Similar to most rare disease studies, relatively small sample sizes limit power. However, this does not negate the positive associations observed, including replication across U.S. and European cohorts. HLA Class I and KIRs assignments, which are based on imputation protocols, represent another limitation. However, the quality metrics of our assignments are within acceptable limits and practice.

In conclusion, there is an association of HLA-C Asn80Lys in the CHB children, which may represent novel susceptibility alleles. Given that the connection of autoantibodies and end organ injury is complex, the inclusion of a defective checkpoint inhibitor in the model may provide new biological insights.

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Figure 1.

KIR genotype frequencies in CHB and unaffected siblings. KIR2DL1 and KIR2DL3, which are derived from the same gene and their products recognize the same HLA ligands, are nearly monomorphic alleles. Both KIR2DS1 and KIR2DS2, which have been associated with autoimmune disorders, showed similar allelic frequency in both groups. Note CHB (filled bar) and controls (open bar).

Table 1

Comparison of HLA-C genotypes by C1/C2 designation. Association analyses with out-of-study controls used a logistic regression model with admixture estimates as covariates.

HLA-C	Family Data				Out-of-study, Healthy Controls	
	Fathers, n=152 (%)	Mothers, n=167 (%)	Affected children, n=194 (%)	Unaffected children, n=91 (%)	Female controls n=635 (%)	Male controls n=438 (%)
C1C1	48 (31.6)	87 (52.1)	62 (32.0)	46 (50.6)	259 (40.8)	177 (40.4)
C1C2	70 (46.1)	63 (37.7)	102 (52.6)	33 (36.3)	292 (46.0)	196 (44.8)
C2C2	34 (22.4)	17 (10.2)	30 (15.5)	12 (13.2)	84 (13.2)	65 (14.8)
Additive genetic model relative to C2*					P-value	OR (95% CI)
Mothers vs. female controls HLA-C2 genotype					0.0014	0.63 (0.45–0.89)
Fathers vs. male controls HLA-C2 genotype					0.0123	1.40 (1.06–1.81)

*. For gender interactions in parents relative to out-of study controls, P=0.0002

Table 2

Analysis of C2 status among paired siblings, affected versus unaffected. * **

	All pedigree pairs (n=90)	
Paired siblings	Unaffected with C2	
Affected with C2	No	Yes
No	24	6
Yes	22	38
McNemar P-value	P-value=0.0025	
	OR= 3.67	

* Each pedigree is represented by a single sibling pair.

** Pedigree disequilibrium test (PDT) for the C2 allele of HLA-C based on Asn/Lys dimorphism at position 80: P=0.016