Published in final edited form as:

Arthritis Rheum. 2012 December ; 64(12): 4060–4065. doi:10.1002/art.34663.

Enrichment of Associations in Genes with Fibrosis, Apoptosis, and Innate Immunity Functions with Cardiac Manifestation of Neonatal Lupus

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Abstract

Objective—The proposed pathogenesis of the cardiac manifestations of neonatal lupus (cardiac-NL) involves maternal autoantibodies to the ribonucleoproteins SSA/Ro and SSB/La enhanced by as yet unknown factors likely to involve the dysregulation of both inflammatory and fibrotic fetal responses. This study was designed to improve the power to detect specific associations in genes with candidate biological functions.

Methods—Using data from our genome-wide association study (GWAS) in 116 cardiac-NL Caucasian children and 3,351 Caucasian controls, we tested for enrichment of SNP associations in genes with candidate biological functions related to fibrosis, immune, apoptosis, T cell function, cell infiltration, innate immune cell function, interferon, Toll like receptors and calcium channels. After linkage disequilibrium pruning and exclusion of the extended HLA region, a total of 15,103 SNPs in 3,068 genes remained.

Results—A highly significant enrichment of P-values was observed in genes related to fibrosis ($P=2.27 \times 10^{-9}$), apoptosis ($P=7.67 \times 10^{-7}$), innate immune cell ($P=2.53 \times 10^{-6}$), immune ($P=5.01 \times 10^{-4}$), T cell ($P=2.23 \times 10^{-4}$), and interferon functions ($P=1.64 \times 10^{-3}$). The most significant non-HLA associations included the sialyltransferase *ST8SIA2* (rs1487982, $P=3.38 \times 10^{-5}$, OR [95% CI]=2.20 [1.52–3.19]), the integrin *ITGA1* (rs2432143, $P=4.54 \times 10^{-5}$, OR [95% CI]=2.31 [1.54–3.45]), and the complement regulator *CSMD1* (rs7002001, $P=6.33 \times 10^{-5}$, OR [95% CI]=2.41 [1.57–3.72]).

Conclusion—This study identified novel candidate genes associated with cardiac-NL and highlights the value of this cohort in advancing our knowledge regarding the genetic etiology of this syndrome. Identification of causal alleles is expected to provide critical insight into the molecular mechanisms responsible for linking maternal autoantibodies to cardiac scarring in these fetuses/neonates.

Neonatal lupus (NL) is a syndrome whose clinical manifestations include transient cutaneous rash, hematologic and hepatic laboratory abnormalities, and irreversible cardiac damage. The cardiac manifestations of NL (cardiac-NL), which include atrioventricular conduction defects (heart block) and life-threatening cardiomyopathy, are associated with substantial mortality and morbidity (1). While maternal antibodies to components of the SSA/Ro-SSB/La ribonucleoprotein complex are necessary for the development of disease, the rarity of advanced injury at 2–5% suggests that fetal factors are highly contributory (2) to a complex pathogenic cascade which links autoantibody to scar. A fetal genetic contribution to the development of cardiac-NL is supported by a high sibling risk ratio ($\lambda_s = 10\text{--}3000$), recurrence rate of approximately 18%, concordance rates in monozygotic twins of 33% (reviewed in 3), as well as the results from our published genome-wide association study (GWAS) in cardiac-NL (4).

While the precise pathogenic cascade is yet to be fully defined, one scenario posits that injury is initiated by increased apoptosis of the cardiocytes which exposes Ro/SSA antigens and associated ssRNA generating an immune complex phagocytosed by macrophages, which sustain both an inflammatory response and a fibrotic response, resulting in the scarring of the heart (5). This hypothesis is supported by the results from our GWAS (4), where several genome-wide significant variants were identified in the MHC region, as well as at 21q22.3, near the *erythroblast transformation-specific transcription factor ERG*, which is a regulator of embryonic development, cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis.

Despite the significant associations identified in the GWAS (4), we hypothesize that there may be a global enrichment of specific pathways and genetic variation potentially overlooked if only the most significant statistical associations are considered. A judicious and efficient way to identify genetic variation predisposing to complex diseases is the application of a candidate gene framework that incorporates prior biological knowledge. Unlike agnostic GWAS, this approach narrows the hypothesis space to provide a more focused and powerful examination of the data. Indeed, several studies have shown that an essentially Bayesian approach of selecting candidate genes serves to increase the reliability and likelihood of finding genes that are truly associated with disease (6, 7).

In accord with these considerations, we tested for a potential enrichment of significant associations in genes with candidate biological functions by mining the cardiac-NL GWAS. Given their potential contribution to inflammation, fibrosis, and conduction abnormalities in the heart, we selected genes with functions contributing to apoptosis, calcium electrogenesis, immune function, and fibrosis. Figure 1 illustrates our approach for the identification of cardiac-NL risk loci. Several associations within genes in candidate pathways relevant to the pathogenesis of cardiac-NL were identified supporting the potential of this approach for gene discovery.

Materials and Methods

The Ingenuity Pathway Analysis (www.ingenuity.com) was used to catalogue a list of all human genes with known genomic positions and with immune (n=1,993 genes), fibrotic (n=327 genes), apoptosis (2,283 genes), T cell (980 genes), cell infiltration (311 genes), innate immune cell (1,381 genes), interferon (102 genes), Toll like receptors (TLRs) (10 genes) and calcium channel (13 genes) functions. Genes with bone functions (n=568 genes) served as a control. Ingenuity has a comprehensive database that contains biological findings and annotations manually curated from the literature and several databases, including thorough gene function information.

All SNPs within a 10 kb interval of each gene from our published GWAS (4) were considered in this analysis. Briefly, our GWAS analyzed 346,110 SNPs genotyped on the Illumina HumanCNV370 array in 116 cardiac-NL Caucasian children and 3,351 Caucasian controls for association with cardiac-NL. Only SNPs that met the following quality control (QC) criteria were considered: 1) no differential missingness between cases and controls ($P > 0.05$), 2) overall missing genotype data $<10\%$, 3) no significant departures from Hardy-Weinberg equilibrium expectations ($P > 0.0001$ for cases and $P > 0.01$ for controls), and 4) minor allele frequency (MAF) >0.02 in the control samples. Tests for association were adjusted for potential confounding effects of population structure by including principal components derived from the GWAS as covariates in logistic regression models. The primary inference for this study was based on the additive genetic model, unless the lack-of-fit to an additive model was statistically significant ($P < 0.05$), in which case the minimum P value from the dominant, additive, or recessive models was reported. The observed inflation factor for the original GWAS was 1.026.

The list of SNPs in our GWAS that met statistical QC and mapped within 10 kb up- and downstream of each gene were pruned to contain only those SNPs in no linkage disequilibrium (LD: $r^2 > 0.4$). For each pathway, a Z-test for proportions was computed to test for a potential statistical difference between the number of observed and expected significant associations (at $P < 0.001$) using the pruned SNPs that met quality control criteria. To further avoid biases due to LD, the extended HLA region was also excluded.

Finally, the most significant genes in each of the biological functions that showed an enrichment of associations were identified. The top five most significant non-HLA SNPs ($P < 5 \times 10^{-4}$) in each function were selected and their regions analyzed using all SNPs that met QC. Regions in which the most significant association was not corroborated by neighboring SNPs in LD were flagged as likely false positives.

Results

The Ingenuity Pathway Analysis database was used to compile a list of all molecules with candidate biological functions. A total of 1,993 human genes with immune functions and known genomic positions were identified, 327 genes with fibrotic functions, 2,283 with apoptosis, 980 with T cell, 1,381 with innate immune cell, 311 with cell infiltration, 102 with interferon, 10 with Toll like receptor (TLR), 13 with calcium channel, and 568 genes with bone functions (to serve as control).

A total of 36,434 SNPs that met QC were identified in 3,121 genes. 417 of these SNPs mapped to 45 genes within the extended HLA region. Once this list was pruned to eliminate redundant SNPs in linkage disequilibrium (LD), 15,103 SNPs in 3,068 genes remained outside the extended HLA region. Table 1 shows the results of the tests for a potential statistical difference between the number of observed and expected significant associations (at $P < 0.001$) in these genes.

As shown in Table 1 (first row), 8,100 pruned SNPs in genes were identified with immune functions that met quality control criteria. We would expect 8 of these SNPs with a cardiac-NL association P -value < 0.001 . However, we observed 18 SNPs not in LD that exhibited an association P -value < 0.001 (over twice the number of expected SNPs). This 8:18 expected:observed ratio is statistically significant ($P = 5.01 \times 10^{-4}$), suggesting an enrichment of associations in genes with immune functions.

We observed a significant enrichment of P -values in genes with immune ($P = 5.01 \times 10^{-4}$), T cell ($P = 2.23 \times 10^{-4}$), and interferon functions ($P = 1.64 \times 10^{-3}$), and highly significant

enrichment of P-values in genes with fibrosis ($P=2.27\times 10^{-9}$), apoptosis ($P=7.67\times 10^{-7}$) and innate immune cell ($P=2.53\times 10^{-6}$) functions.

For most of the biological functions, inclusion of the extended HLA region did not significantly alter these results. The most striking difference was the enrichment of SNP associations within immune function genes ($P<1\times 10^{-9}$ with the HLA), which is expected given the abundance of immune function genes in this region. A stronger enrichment of associations within genes with apoptosis ($P=7.35\times 10^{-9}$) and innate immune cell functions ($P=3.06\times 10^{-7}$) was also observed when the HLA region was included. For the rest of the biological functions the enrichment of significant associations remained similar, emphasizing a modest role of HLA variation in these pathways.

Next the genes driving the enrichment of associations in fibrosis, immune, apoptosis, T cell, innate immune cell, and interferon functions were identified. As expected, the most significant SNPs mapped to the HLA region. The most significant SNP in this region located in the *natural cytotoxicity triggering receptor 3* gene (NCR3, rs2857595, $P=1.96\times 10^{-9}$, OR[95% CI]=2.37[1.79–3.14], which was reported in the GWAS (4). Outside of the HLA, the most significant associations were observed in genes with immune and apoptosis functions (Table 2). In this study, the most significant non-HLA signal was observed in the ST8SIA2 gene, which in the GWAS ranked 14th when the results were ordered by region.

As shown in Table 2, immune function genes included the *integrin alpha 1* (ITGA1, rs2432143, $P=4.54\times 10^{-5}$), the protein phosphatase *CUB and Sushi multiple domains 1* (CSMD1, rs7002001, $P=6.33\times 10^{-5}$), and the *opioid receptor mu 1* genes (OPRM1, rs2272381, $P=7.34\times 10^{-5}$). Apoptosis genes included the sialyltransferase *ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2* (ST8SIA2, rs1487982, $P=3.38\times 10^{-5}$) and the aforementioned ITGA1 and OPRM1 genes.

The most significant non-HLA associations in genes with T cell functions included the *c-src tyrosine kinase* (CSK, rs1378942, $P=9.95\times 10^{-5}$) and *phospholipase C beta 3* genes (PLCB3, rs2244621, $P=1.24\times 10^{-4}$) (Table 2). Innate immune cell function genes included the aforementioned ITGA1, CSK and PLCB3. Among the genes with interferon functions, only two regions had SNPs that met QC criteria and $P<5\times 10^{-4}$, the *adenosine A1 receptor* (ADORA1, rs3753473, $P=2.05\times 10^{-4}$) and *toll-like receptor 4* genes (TLR4, rs1554973, $P=4.46\times 10^{-4}$). Finally, non-HLA fibrosis function genes included the *cytochrome P450, family 2, subfamily A* (CYP1A2, rs2472299, $P=8.47\times 10^{-5}$) and the aforementioned ADORA1 and TLR4 genes (Table 2).

To reduce the potential of false positives the regions of association in the most significant genes with biological functions were examined (Table 2). With the exception of GLIS2, EPHA2 and ATF3, all of the associations were corroborated by evidence of association from neighboring SNPs not included in the original analysis and that are in linkage disequilibrium with the associated SNP. In the majority of cases all signals appear to be driven by variants within the candidate genes. The exceptions include PLCB3, ADORA1, and the CYP1A2-CSK genes, which are in regions of strong LD ($r^2>0.8$). In this situation, the possibility that variation in neighboring genes is responsible for these associations can not be excluded. For all the other genes, their involvement in physiological functions relevant to cardiac-NL supports their association with this syndrome.

Discussion

The goal of this study was to identify genetic variation that leads to dysregulation of genes with biological functions involved in the pathogenesis of cardiac-NL. This candidate gene approach based on prior knowledge was selected because it serves to increase the power,

reliability and likelihood of finding genes that are associated with disease (6, 7). This approach is illustrated in Figure 1.

A highly significant enrichment of associations was observed in fibrosis-and immune-related genes, suggesting that genetic variations of these pathways are likely involved in predisposing to cardiac-NL. The specific immune-related functions that appear to have driven the enrichment were apoptosis, innate immune cell, T cell and interferon functions. Exclusion of genes in the extended HLA region did not alter these results, demonstrating that the results were not driven just by HLA variation in these pathways. Genes with TLR, cell infiltration, and calcium channel functions did not show an enrichment of associations.

Several genes with potential involvement in cardiac injury were uncovered. Outside of the HLA, the strongest signal mapped to the *ST8SIA2* gene, also known as STX, a member of the glycosyltransferase family 29. This polysialyltransferase catalyzes the transfer of sialic acid to N-linked oligosaccharides and glycoproteins. It is expressed in the neonatal atrium and its expression was shown to be sufficient to modulate cardiomyocyte excitability (8). Aberrant glycosylation has been shown to modulate cardiac sodium ion channel activity and electrical signaling (8), which supports this molecule's potential role in the etiology of cardiac-NL.

ITGA1, also known as VLA1, encodes the alpha 1 subunit of integrin receptors. It heterodimerizes with the beta 1 subunit to form a cell-surface receptor for collagen and laminin. Integrins, together with multiple cytoskeletal proteins, join the sarcomeric contractile apparatus to the extracellular matrix across the plasma membrane and elicit intracellular signaling pathways, promoting the cardiomyocyte hypertrophy program, e.g., MAPK pathway, including ERK1/2, JNK, and p38 MAPK, which are important for mediating hypertrophic cardiac growth (9). Indeed, integrin $\alpha 1\beta 1$ is required for down-regulation of the profibrotic EGF receptor signaling (10), of collagen IV synthesis (11), and of oxidative stress-mediated damage (12). Down-regulation of integrin $\alpha 1\beta 1$ expression was observed in fibroblasts isolated from patients with systemic sclerosis (13). Furthermore, integrin $\alpha 1$ -null mice develop exacerbated glomerulosclerosis following injury (11), are more susceptible to fibrosis following injury (12).

Another significant gene, *CSMD1*, encodes a soluble protein that can block the classical complement activation pathway. *CSMD1* is highly expressed in the central nervous system and epithelial tissues, where it may play important roles in controlling complement activation and inflammation (14). It is also expressed in areas of regenerative growth, and it might be developmentally regulated (14). Given that *CSMD1* may be a fetal membrane-bound complement regulator, it may provide a mechanism of fetomaternal tolerance during development by protecting the embryo from spontaneous complement activation (15).

Although the objective of this study was to perform a comprehensive analysis of all genes with several immune, calcium channel and fibrosis functions, several limitations are acknowledged. Clearly, associations could be missed due to the genomic coverage of the genotyping array or the *a priori* selection of specific genes reported in the literature to have the aforementioned functions. Nevertheless, the associations herein reported are supported by the fact that these genes are involved in the proposed pathogenesis of cardiac-NL, which involves a dysregulation of both inflammatory and fibrotic responses (5). We also note that, despite the observed enrichment of associations in genes with several candidate biological functions in the affected child, it is possible that such enrichment reflects an inherited maternal component. Application of this same approach to SLE cases might facilitate the distinction of fetal from maternal effects. However, since many mothers do not have SLE, and likewise many SLE patients do not have anti-Ro antibodies, the only way to definitively

disentangle a fetal enrichment from the maternal one is to genotype and perform the same analysis in the cardiac-NL mothers which is planned in a future study. Follow-up studies are needed to confirm these results and to delineate the molecular mechanisms by which these variants predispose to cardiac-NL.

In summary, this is the first pathway-based analysis in children with cardiac-NL. Through this gene function paradigm, a highly significant enrichment of associations in genes with immune and fibrosis functions, as well as novel loci associated with cardiac-NL was observed. Taken together, these new data expand the list of genes that show association with cardiac-NL and emphasize the potential genetic contribution to dysregulated immune and fibrosis pathways. Identifying the fetal genes predisposing to cardiac scar and understanding how these genetic factors might contribute to pathogenesis should ultimately lead to important opportunities for the delineation of risk profiles and development of therapeutic targets to prevent and possibly reverse cardiac injury in these children.

Acknowledgments

Supported by the NIH (National Institute of Arthritis and Musculoskeletal and Skin Diseases) R01-AR-42455, AR-42271 and N01-AR-042220, the American Heart Association Grant Number 0655938T, and Wake Forest School of Medicine Center for Public Health Genomics.

We gratefully acknowledge the International Consortium on Systemic Lupus Erythematosus Genetics (SLEGEN) and Drs. John B. Harley, Kathy L. Moser, Lindsey A. Criswell, Marta E. Alarcon-Riquelme, Chaim O. Jacob, Robert P. Kimberly, Betty P. Tsao, and Timothy J. Vyse for the use of the SLEGEN control data.

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Figure 1.

Approach for the identification of genes predisposing to cardiac manifestations of neonatal lupus (cardiac-NL). An approach that focuses the hypothesis space based on biological knowledge provides a more powerful and efficient way to identify disease-causing variation. The identification of an enrichment of associations in genes believed to be relevant to the etiopathology of cardiac-NL uncovered novel associations in genes with such functions.

Table 1

P-value enrichment in genes with candidate biological functions.

	n	Expected	Observed	Pr(exp) (%)	Pr(obs) (%)	P-Value
Fibrosis	1556	1.6	9	0.10	0.58	2.27E-09
Apoptosis	10211	10.2	26	0.10	0.25	7.67E-07
Innate immunity	5736	5.7	17	0.10	0.30	2.53E-06
T cell	3804	3.8	11	0.10	0.29	2.23E-04
Immune	8100	8.1	18	0.10	0.22	5.01E-04
Interferon	294	0.3	2	0.10	0.68	1.64E-03
Calcium channels	251	0.3	1	0.10	0.40	1.35E-01
Control	2891	2.9	5	0.10	0.17	2.15E-01
Cell infiltration	1321	1.3	2	0.10	0.15	5.54E-01
TLRs	26	0.0	0	0.10	0.00	8.72E-01

The SNP list was pruned and the extended HLA region excluded. n – number of SNPs, exp – number of expected SNPs with $P < 0.001$, obs – number of observed SNPs with $P < 0.001$, Pr(exp) and Pr(obs) – probability of expected and observed SNPs, respectively.

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Table 2
Location of top five most significant non-HLA SNPs ($P < 5 \times 10^{-4}$) within each biological function.

SNP	Gene ¹	Chr	Position (Mb)	MA	MAF		P-value ²	OR [95% CI]	Biological functions
					Cases	Controls			
rs11260745	EPHA2 [*]	1	16.339	C	0.11	0.06	2.51E-04	2.27 [1.46–3.53]	Fib, Tcell
rs7543038	PBX1	1	162.990	T	0.44	0.37	2.52E-04 ^r	2.23 [1.45–3.43]	Tcell
rs3753473	ADORA1	1	201.398	A	0.13	0.07	2.05E-04 ^d	2.27 [1.47–3.51]	Fib, Apop, Innate, IFN
rs11811628	ATF3 [*]	1	210.823	A	0.07	0.03	2.15E-04 ^d	2.84 [1.63–4.93]	Fib, Apop, Innate
rs2432143	ITGA1	5	52.167	G	0.16	0.09	4.54E-05 ^d	2.31 [1.54–3.45]	Innm, Apop, Innate
rs1196175	GJA1	6	121.808	A	0.15	0.26	3.08E-04 ^d	0.47 [0.31–0.71]	Tcell
rs2272381	OPRM1	6	154.584	G	0.03	0.14	7.34E-05 ^d	0.19 [0.08–0.43]	Innm, Innate, Apop
rs4392700	PARK2	6	162.644	C	0.24	0.15	1.28E-04	1.84 [1.35–2.51]	Apop
rs7002001	CSMD1	8	4.564	T	0.11	0.05	6.33E-05	2.41 [1.57–3.72]	Innm
rs1554973	TLR4	9	119.521	G	0.35	0.25	4.46E-04	1.65 [1.25–2.18]	IFN, Fib
rs2073577	GFI1B	9	134.852	G	0.43	0.36	2.43E-04 ^r	2.26 [1.46–3.48]	Tcell
rs2244621	PLCB3	11	63.783	T	0.04	0.15	1.24E-04 ^d	0.22 [0.10–0.48]	Tcell, Innate
rs2472299	CYP1A2	15	72.820	A	0.39	0.27	8.47E-05	1.72 [1.31–2.25]	Fib, Innm
rs1378942	CSK	15	72.864	G	0.47	0.35	9.95E-05	1.68 [1.29–2.19]	Innm, Tcell, Innate, Apop
rs1487982	ST8SIA2	15	90.796	C	0.26	0.17	3.37E-05 ^d	2.20 [1.52–3.19]	Apop
rs611704	GLIS2 [*]	16	4.335	A	0.08	0.03	9.76E-05 ^d	2.78 [1.66–4.65]	Fib
rs762960	TSPO	22	41.881	T	0.20	0.32	9.60E-05	0.52 [0.38–0.72]	Apop

Chr - chromosome; MA: minor allele; MAF: minor allele frequency; OR - odds ratio; CI: confidence interval;

¹ Associations denoted with the

^{*} are not supported by the association of neighboring SNPs;

² The superscript after the P-value denotes the genetic model if other than additive:

^r recessive,

^d dominant;

Fib: fibrosis; Innm: immune; Tcell: T cell; Apop - apoptosis; Innate: innate immune cell; IFN -interferon.