Mapping Genetic Contributions to Cardiac Pathology Induced by Beta-Adrenergic Stimulation in Mice

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Background—Chronic stress-induced cardiac pathology exhibits both a wide range in severity and a high degree of heterogeneity in clinical manifestation in human patients. This variability is contributed to by complex genetic and environmental etiologies within the human population. Genetic approaches to elucidate the genetics underlying the acquired forms of cardiomyopathies, including genome-wide association studies, have been largely unsuccessful, resulting in limited knowledge as to the contribution of genetic variations for this important disease.

Methods and Results—Using the β-adrenergic agonist isoproterenol as a specific pathological stressor to circumvent the problem of etiologic heterogeneity, we performed a genome-wide association study for genes influencing cardiac hypertrophy and fibrosis in a large panel of inbred mice. Our analyses revealed 7 significant loci and 17 suggestive loci, containing an average of 14 genes, affecting cardiac hypertrophy, fibrosis, and surrogate traits relevant to heart failure. Several loci contained candidate genes which are known to contribute to Mendelian cardiomyopathies in humans or have established roles in cardiac pathology based on molecular or genetic studies in mouse models. In particular, we identify Abcc6 as a novel gene underlying a fibrosis locus by validating that an allele with a splice mutation of Abcc6 dramatically and rapidly promotes isoproterenol-induced cardiac fibrosis.

Conclusions—Genetic variants significantly contribute to the phenotypic heterogeneity of stress-induced cardiomyopathy. Systems genetics is an effective approach to identify genes and pathways underlying the specific pathological features of cardiomyopathies. Abcc6 is a previously unrecognized player in the development of stress-induced cardiac fibrosis.

Key Words: catecholamine ■ genome-wide association scan ■ genomics ■ heart failure ■ mouse

Heart failure (HF) is a common cause of death with a lifetime risk of ≥1 in 9 for both men and women in developed countries.1 Heart failure is a complicated syndrome, characterized by a large number of pathological changes, such as contractile dysfunction, cardiomyocyte hypertrophy, edema, and myocardial fibrosis.2–4 The onset and severity of these pathological manifestations are highly heterogeneous among HF patients, likely because of complex interactions between the genetic variants and the pathological stressors, including mechanical overload and humoral overstimulation. Indeed, several humoral factors, such as catecholamines and angiotensin II, are known to play key roles in triggering HF; however, the genetic variations underlying the pathological outcome in response to these stressors remain elusive. Dissecting the genetic contributions to specific pathological changes in the failing heart would provide important insights for the future development of personalized diagnoses and targeted therapies.

Clinical Perspective on p 49

In contrast to many other common disorders, genome-wide association studies (GWAS) of HF have had modest success in elucidating the genetics underlying this complex disease. Only 2 heart failure–related loci2 have reached accepted levels of genome-wide significance, despite meta-analyses of tens of thousands of patients.6,7 The challenge of performing GWAS in human HF is likely because of the complex nature of the disease, which can arise as a result of multiple underlying etiologies, such as myocardial infarction, hypertension, or metabolic disorders, each of which are complex traits with significant environmental confounders.1 Attempts to dissect the genetics of HF traits in rodents have been only modestly successful; although several loci for hypertrophy and fibrosis have been identified, the poor mapping resolution of traditional linkage analyses has complicated the identification of the underlying genes.8–11 The development of a method to perform high resolution, association-based mapping of complex

Received December 19, 2013; accepted October 28, 2014.

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Circ Cardiovasc Genet is available at http://circgenetics.ahajournals.org DOI: 10.1161/CIRCGENETICS.113.000732
traits in mice\textsuperscript{12} provided an opportunity to identify genetic factors contributing to common forms of HF under defined stress conditions.

In this study, we have conducted a comprehensive phenotypic characterization in a large panel of densely genotyped inbred mice from the hybrid mouse diversity panel (HMDP)\textsuperscript{12} following chronic treatment with a β-adrenergic agonist, isoproterenol (ISO). A wide spectrum of phenotypic changes was observed among the HMDP mice in ISO-induced cardiac hypertrophy, fibrosis, and peripheral edema. Using GWAS, we uncovered 7 significant loci and 17 suggestive loci, each containing an average of 14 genes. Several of these loci included genes with established causal roles in familial cardiomyopathies in humans or heart failure phenotypes in experimental models. In addition, we identified Abcc6 as a previously unrecognized regulator of ISO-induced cardiac fibrosis. Therefore, our study provides clear evidence that genetic variants have a significant contribution to the phenotypic heterogeneity of stress-induced cardiomyopathy.

Materials and Methods

Ethics Statement

All animal experiments were conducted following guidelines established and approved by the University of California, Los Angeles Institutional Animal Care and Use Committee. All surgery and echocardiography was performed under isoflurane anesthesia, and every effort was made to minimize suffering.

Online Database

All results and data can be accessed at http://systems.genetics.ucla.edu/data.

Mice and Isoproterenol Treatment

The mouse strains listed in Table 1 in the Data Supplement were obtained from The Jackson Laboratory and then bred in our colony. All mice have been previously genotyped at over 130000 locations. ISO (30 mg per kg body weight per day, Sigma) was administered for 21 days in 8- to 10-week-old female mice using ALZET osmotic mini-pumps, which were surgically implanted intraperitoneally. Abcc6 knockout (KO) and transgenic mice\textsuperscript{13,14} underwent the same protocol as described above, although both male and female mice were used in the analysis. No significant difference between genders was observed as a result of ISO treatment in these KO and transgenic animals.

Heart Weights

At day 21, mice were euthanized and body weight recorded. The heart was removed and weighed, then separated into its 4 component chambers, each of which was individually weighed as well. Each chamber of the heart was immediately frozen in liquid nitrogen for any future analysis and stored in a −80°C freezer. Lung and liver were removed and weighed. Additionally, the adrenal glands were removed, weighed, and frozen in liquid nitrogen.

Fibrosis and Calcification

A portion of the left ventricle (LV) was placed in formalin for 48 hours for preservation of ultrastructure. These samples were then washed with distilled water and sent to UCLA Department of Pathology and Laboratory Medicine for paraffin embedding and staining using Masson’s Trichrome and Alizarin Red for calcification. Sections were analyzed using a Nikon Eclipse, TE2000-U microscope and images captured of the entire cross-section of the heart. Fibrosis was quantified using the Nikon Imagine System Elements AR program by comparing the amount of tissue stained blue (for collagen) or red (for calcification) to the total tissue area. To confirm our results, we examined a subset of the strains using Sirius Red, another fibrosis-marking stain, and observed high concordance between our samples (R=0.75, data not shown). As expected,\textsuperscript{4,15} we observed strong correlations between cardiac fibrosis and total heart weight (P<1.0E-07). Our results compare favorably to prior quantifications of fibrosis in a limited number of strains.\textsuperscript{4,16}

Mice used for the Abcc6 validation experiments underwent an identical protocol to the one described above for Masson Trichrome and Alizarin Red staining, using 5 sections per heart for both Trichrome and Alizarin Red staining.

Echocardiography

Transsthoracic echocardiograms were performed using the Vevo 770 ultrasound system (VisualSonics, Inc., Toronto, ON, Canada). Inhaled isoflurane (1.25% during induction and 1% during maintenance) was administered to ensure adequate sedation in the mean time maintaining heart rate >450 beats per minute. A parasternal long-axis B-mode image was obtained. The maximal long-axis of the LV was positioned perpendicular to the ultrasound beam. A 90° rotation of the ultrasound probe at the papillary muscle level was performed to obtain a parasternal short-axis view of the LV. A M-mode image was captured to document LV dimensions. Then a semiapical long-axis view of the LV was obtained. The LV ejection time, E and A wave velocities were obtained from this view using pulse wave Doppler. Images were saved for analysis at a later time point using the Vevo 770 cardiac analysis package. In summary, a baseline echocardiogram was performed on all of the mice. Among control mice, a second echocardiogram was performed in 70 mouse strains at week 3. In ISO-treated mice, serial echocardiograms were performed at 1, 2, and 3 weeks. A single operator, who followed a standard operating protocol detailed above, performed all of the echocardiograms. Saved images were analyzed at a later time point by a single observer who was blinded to mouse strains.

Association Analysis

Unless otherwise noted, all analyses were performed using the R software environment. We performed the association testing of each single-nucleotide polymorphism (SNP) with a linear mixed model, which accounts for the population structure among the individuals.\textsuperscript{17} We estimated \( \mathbf{I}_n \times 1 \) vector of observed genotypes of the SNP (using additive coding of the SNPs), \( u \) denotes the identity-by-state kinship matrix estimated from all the genotypes, no transformation of the data was performed. Prior work\textsuperscript{17} determined our final significance threshold, 4.1E-7, by Bonferroni correction. Linkage disequilibrium (LD) was determined by calculating pairwise \( r^2 \) SNP correlations for each chromosome. Approximate LD boundaries were determined by visualizing \( r^2 \geq 0.8 \) correlations in MATLAB (MathWorks).

Locus Overlap with Other Studies

Gwas.gov was queried for all human GWAS loci for the terms heart failure or cardiac hypertrophy. All loci with \( P \) value <5E-7 were associated with clinical outcomes.\textsuperscript{4,19} These results were not included in our final significance threshold, 4.1E-7, by Bonferroni correction. Linkage disequilibrium (LD) was determined by calculating pairwise \( r^2 \) SNP correlations for each chromosome. Approximate LD boundaries were determined by visualizing \( r^2 \geq 0.8 \) correlations in MATLAB (MathWorks).
selected. The NCBI homology maps (http://www.ncbi.nlm.nih.gov/projects/homology/maps/) were used to find syntenic location of the 5 Mb region surrounding the peak SNP of the human HF locus. If a mapped syntenic region overlapped with the LD block of a suggestive locus from our study, it was considered a positive hit. A significance $P$ value was obtained by permutation testing, in which all suggestive loci were randomly placed across the genome and the number of overlaps measured a total of 100,000 times. Final significance was calculated as the number of permutations which surpassed the observed number of overlaps.

**Microarray and eQTL Analysis**

Following homogenization of LV tissue samples in QIAzol, RNA was extracted using the Qiagen miRNAasy extraction kit and verified as having a RIN>7 by Agilent Bioanalyzer. Two RNA samples were pooled for each strain/experimental condition, whenever possible, and arrayed on Illumina Mouse Reference 8 version 2.0 chips. Analysis was conducted using the Neqc algorithm included in the limma R package,18 and batch effects addressed through the use of COMBat.19 Expression quantitative trait loci (eQTLs) were then calculated for 13,155 expressed genes using EMMA, as described above. Significance thresholds were calculated as in Parks et al. Briefly, cis-eQTLs were calculated using a false discovery rate (FDR) of 5% for all SNPs that lay within 1 Mb of any probe (roughly 100 SNPs), using standard permutation analysis methods (total of 100 permutations of all data), previously used for cis-eQTL analysis.20–22 Our determined cutoff of 3.6E-3 further takes into account LD, which further reduces the effective number of SNPs in each 2 Mb window surrounding the peak SNP. We have previously observed that cis-eQTL at this level are highly conserved in the HMDP.24 Trans-eQTLs were calculated using the overall HMDP cutoff as determined in Kang et al and described above.27

**Results**

**Pathological Analysis of ISO-Induced Cardiomyopathy in HMDP Mice**

β-adrenergic stimulation is considered a common and critical driving force behind ongoing hypertrophy and progression to heart failure.23 We treated mice chronically with ISO, a synthetic nonselective β-adrenergic agonist.26,27 748 mice from 105 different strains of the HMDP were divided into control (average 2.2 per strain) and treated (average 4.1 per strain) cohorts (Table I in the Data Supplement). Treated mice were implanted with an Alzet micropump and given 30 mg/kg/d of ISO for 3 weeks, at which point all mice were euthanized. We characterized a variety of phenotypes to capture specific portions of the complex heart failure syndrome. For this report, cardiac hypertrophy and pulmonary and liver edema were assessed by measuring the weights of the 4 cardiac chambers, the lungs, and the liver. Cardiac fibrosis, a phenotype which is difficult to study in humans, was measured by histological quantification of fibrotic tissue area as a percentage of all tissue area in LV sections stained using Masson Trichrome. Functional analysis of the mouse hearts were performed using echocardiography. We observed statistically significant correlations between our calculated LV weights (LVW) from echocardiography and our measured LVW after harvesting ($R=0.82$, $P=5E-24$) as well as between LVW and LV internal dimension ($R=0.26$, $P=1.3E-11$). Further analysis of the functional data, including association analyses for each observed functional phenotype, are still under preparation and will be reported in a subsequent article.

As shown in Figure 1, we observed striking differences in cardiac hypertrophy, fibrosis, and degrees of pulmonary and hepatic edema among the strains. Our results are consistent with another report from a more limited strain survey.28 After treatment with ISO, mice were slightly heavier than their paired controls (23.5 g versus 21.83 g; $P=0.009$), likely because of edema. More significant changes in weight were observed for our traits of interest, such as total heart weight ($P=5.3E-24$) and lung weight (4.6E-12; Table II in the Data Supplement). Although it is likely that the ISO-treated organ weights are influenced by the slight increase in body weight, the magnitude of the results we observe in our study leads us to conclude that this influence is minimal when compared with the direct effects of ISO stimulation on the heart itself.

Of the 470 mice assigned to the treatment cohort, 139 (29.6%) died before the end of the protocol, most (127) within the first 48 hours of treatment, whereas none of the control cohort died (Figure I in the Data Supplement). There was no observable significant differences in baseline conditions between mice which survived the initial ISO challenge and mice which died after ISO challenge. Furthermore, we did not observe significant correlation between the strains which showed high mortality before end of protocol and any of our expressed genes before or after treatment (the most significant correlation was for the Riken gene 8430432M10Rik ($R^2=0.16$, $P=5.6E-5$), which fails to satisfy a Bonferroni-corrected threshold of 3E-6). Among the phenotypic traits (Bonferroni-corrected threshold of 4.8E-4), we observed that the post-ISO Mean Normalized Systolic Ejection Rate functional trait demonstrated significant correlation ($R^2=0.14$, $P=3E-4$) with prematurity mortality. This relationship will be explored in greater detail in our article focusing on echocardiographic functional traits. GWA on this premature death trait revealed a single locus (peak SNP rs29166005, $P=9E-6$), which contributes suggestively to this phenotype. These results suggest that the cause of our observed ISO-induced death is likely linked to underlying genetic effects or the interaction between genes and the ISO treatment.

**Genomewide Association**

Association analysis was performed using ~132,000 SNPs across the genome with the EMMA algorithm to correct for population structure. In addition to the absolute tissue weight measurements, analyses were performed on the ratios of each treated weight to its corresponding control weight as a measure of responsiveness to ISO treatment. Prior work with EMMA and the HMDP, using simulation and permutation, has suggested that an appropriate genome-wide significance threshold for a single trait is 4.1E-06.12 This is approximately equivalent to a Bonferroni correction.12 To correct for multiple comparisons, we have chosen the threshold of 4.1E-07 and a minimum minor allele frequency of 7.5% for our study. Given that the traits are correlated, this threshold (10-fold lower than the genome-wide significance level for a single trait) is conservative. Using these thresholds, we have identified 7 significant loci and 17 additional loci which matched the nominal significance threshold of 4.1E-06 (Tables 1 and 2; Table III in the Data Supplement). Although linkage analysis in mice typically exhibits a resolution of tens of Mb,59 the loci identified in this study averaged 1 to 2 Mb in size, based on LD, with the majority being <1 Mb.
The RV weight (RVW) and LVW variations mirrored each other closely, with associations being somewhat stronger for RVW (Figure 1B), although each locus identified in the RV was also detected at a lower level of significance in the LV (Figure II in the Data Supplement). In total, we observed 3 significant and 5 suggestive loci corresponding to treated RVW (Figure 2A), 1 significant and 1 suggestive locus for the ratio of treated to untreated RVW (Figure 2B), and a single

Table 1. Significant Heart Failure Trait Loci Identified in HMDP GWA

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Chr</th>
<th>Bp</th>
<th>P Value</th>
<th>LD</th>
<th>N</th>
<th>Gene</th>
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<tr>
<td>Hypertrophic loci</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>5</td>
<td>137934905</td>
<td>3.49E-10</td>
<td>137.93–138.15</td>
<td>11</td>
<td>Mospd3</td>
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<tr>
<td>RV</td>
<td>9</td>
<td>40202022</td>
<td>8.41E-08</td>
<td>39.77–40.52</td>
<td>15</td>
<td>Scn3b</td>
</tr>
<tr>
<td>RV</td>
<td>10</td>
<td>49818583</td>
<td>2.80E-07</td>
<td>48.19–54.24</td>
<td>22</td>
<td>Pln</td>
</tr>
<tr>
<td>RV ratio</td>
<td>9</td>
<td>80542295</td>
<td>2.94E-07</td>
<td>80.00–80.99</td>
<td>2</td>
<td>Myo6</td>
</tr>
<tr>
<td>Fluid retention loci</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>7</td>
<td>15251391</td>
<td>1.93E-07</td>
<td>15.13–18.75</td>
<td>57</td>
<td>Calm3</td>
</tr>
<tr>
<td>Lung</td>
<td>6</td>
<td>53975816</td>
<td>2.90E-07</td>
<td>53.88–55.57</td>
<td>17</td>
<td>Aqp1</td>
</tr>
<tr>
<td>Isoproterenol treated fibrosis loci</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrosis</td>
<td>X</td>
<td>10277028</td>
<td>4.10E-07</td>
<td>5–12.5</td>
<td>127</td>
<td>Srpx</td>
</tr>
</tbody>
</table>

The significance threshold is defined as P value <4.1E-07. RV, right ventricle; LV, left ventricle; and Lung represents isoproterenol-treated right ventricular, liver, and lung weights at week 3, respectively. Fibrosis represents isoproterenol-treated LV fibrosis at week 3. For each locus, the peak single-nucleotide polymorphism location, given by chromosome (Chr) and base pair position (Bp) in the NCBI-build-37 assembly, and association P value are reported, along with the number of genes (N) within the estimated LD block (LD) surrounding the peak single-nucleotide polymorphism and the top candidate gene (Gene). Bold entries represent genes which contain nonsynonymous mutations within the HMDP as reported by the Wellcome Trust Mouse Genome Project, whereas underlined entries possess significant cis-eQTLs. eQTLs indicates expression quantitative trait loci; HMDP, hybrid mouse diversity panel; LD, linkage disequilibrium; and RV, right ventricle.
suggestive locus for the ratio of right atrial weight. Similar to the heart weights, we observed marked variation of liver and lung weights after ISO treatment across the HMDP (Figure 1C and 1D). Lung weight in particular showed a robust increase with ISO treatment. We observed 1 significant and 4 suggestive loci corresponding to ISO-treated lung weights (Figure 2C) and 1 significant and 1 suggestive locus corresponding to ISO-treated liver weights (Figure 2D). Cardiac fibrosis also varied significantly in both baseline and treated mice, with the extent of fibrosis being much greater in treated mice (Figure 1E).

We observed a total of 3 suggestive loci for cardiac fibrosis in untreated animals (Figure 2E) and 1 significant and 4 suggestive loci in treated animals (Figure 2F).

eQTL Analysis for Candidate Genes From ISO-Treated HMDP Mice

To help identify candidate genes at the heart failure associated loci, we performed global expression analysis of LV heart tissue from 92 strains of ISO-treated mice. The loci controlling gene expression levels were mapped using EMMA and are referred to as eQTL. eQTLs were termed cis if the locus maps within 1 Mb of the gene and otherwise were termed trans. Overall, we observed 3093 cis eQTL (False Discovery Rate 5% = P < 3.6E-3, in line with previous measures in the HMDP). Additionally, the Wellcome Trust Mouse Genomes Project sequencing database, which has the full genomic sequence of 10 strains in our panel, was used to examine genomic variations, such as missense, nonsense, or splicing variations, in each locus. Together, these 2 approaches provided a powerful and systematic method for the identification of causal genes within each locus. All significant and suggestive loci as well as gene expression data are available at http://systems.genetics.ucla.edu/data.

Using eQTL analysis combined with GWAS, we identified a causal gene in one of the cardiac hypertrophy loci on chromosome 3 (Figure 3). The peak SNP (P = 1.9E-6) for the trait of treated-to-untreated right RVW ratio, lies between the second and third exons of Ppp3ca, encoding the alpha isozone of calcineurin A, which is also the only gene contained within the LD block surrounding the significantly associated SNPs. Calcineurin A is a known target of β-adrenergic signaling, with a well-described role in ISO-induced hypertrophy.

| Table 2. Suggestive Heart Failure Trait Loci Identified in HMDP GWA |
|------------------------|---|---|---|---|---|
| Phenotype | Chr | Bp | P Value | LD | N | Gene |
| Hypertrophic loci | | | | | | |
| RV | 1 | 134467906 | 5.75E-07 | 133.78–134.53 | 14 | … |
| RV | 5 | 23873494 | 1.23E-06 | 23.82–24.47 | 20 | Prkag2 |
| RV | 11 | 47181489 | 2.15E-06 | 46.18–49.3 | 41 | Sgc2 |
| RV Ratio | 3 | 136305887 | 7.83E-07 | 136.04–136.79 | 1 | Ppp3ca |
| RA Ratio | 7 | 142011844 | 1.41E-06 | 141.50–144.81 | 15 | Mymt |
| Fluid retention loci | | | | | | |
| Liver | 10 | 49468021 | 3.48E-06 | 48.19–54.24 | 22 | Pln |
| Lung | 5 | 111867706 | 1.28E-06 | 110.87–112.87 | 22 | Miat |
| Lung | 7 | 81814161 | 3.88E-06 | 79.8–82.2 | 6 | Slc3a1, Igapp1 |
| Lung | 14 | 14941056 | 3.34E-06 | 8.5–21.5 | 50 | Ppp3cb |
| Lung | 19 | 27061190 | 2.01E-06 | 26.68–27.43 | 1 | Vldl |
| Baseline fibrosis loci | | | | | | |
| Fibrosis | 2 | 139163425 | 2.51E-06 | 13.7–14.0 | 6 | Jag1 |
| Fibrosis | 4 | 84420058 | 2.20E-06 | 84–85 | 2 | Cntn |
| Fibrosis | 7 | 73365047 | 1.31E-06 | 72.3–74.3 | 7 | Tjp1 |
| Isoproterenol-treated fibrosis loci | | | | | | |
| Fibrosis | 7 | 52946331 | 7.11E-07 | 52.85–53.42 | 28 | Abcc6 |
| Fibrosis | 7 | 68593223 | 1.40E-06 | 60.5–69.5 | 18 | Snprn |
| Fibrosis | 7 | 73365047 | 1.40E-06 | 72.3–74.3 | 7 | Tjp1 |
| Fibrosis | 15 | 69907056 | 9.60E-07 | 68.4–71.4 | 3 | Col22a1 |

The suggestive threshold is defined as P value < 4.1E-06. RV, liver, and lung represents isoproterenol-treated right ventricular, liver, and lung weights at week 3, respectively. RV and RA ratio represents the ratio of isoproterenol-treated versus control right ventricle or right atrium weight at week 3. Fibrosis represents either control or isoproterenol-treated LV fibrosis at week 3. For each locus, the peak single-nucleotide polymorphism location, given by chromosome (Chr) and base pair position (Bp) in the NCBI-build-37 assembly, and association P value are reported, along with the number of genes (N) within the estimated LD block (LD) surrounding the peak single-nucleotide polymorphism and the top candidate gene (Gene). Bold entries represent genes which contain nonsynonymous mutations within the HMDP as reported by the Wellcome Trust Mouse Genome Project, whereas underlined entries possess significant cis-eQTLs. eQTLs indicates expression quantitative trait loci; HMDP, hybrid mouse diversity panel; LD, linkage disequilibrium; and RV, right ventricle.
Calcineurin A is the only gene in LD with the peak SNP and has a significant cis-eQTLs ($P=1.3E-3$) for the ratio of treated to control calcineurin A expression (Figure 3A and 3B). We also observed a modest correlation between the ratio of Ppp3ca expression and the ratio of heart weights in control and ISO-treated animals ($R=-0.18, P=0.01$). We further observed Ppp3cb, the beta isozyme of calcineurin A, in a locus on chromosome 14 ($P=3.3E-6$) for the trait treated lung weight. Ppp3cb has a strongly suggestive cis-eQTL ($P=4.7E-3$) as well as a minor allele with an insertion in a splice site in several strains of the HMDP. In addition to calcineurin A, we identified several other genes with well-established roles in cardiac physiology and pathology within other disease-associated loci. These include the key calcium cycling regulator phospholamban and structural protein Sgcd, as well as other genes, which have previously been implicated in cardiac hypertrophy, such as Prkag2, or cardiac malformation, such as Mospd3 (Tables 1 and 2).

The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to examine the genes located within LD of the peak SNPs of our study. We observed significant enrichment for calmodulin-related genes (benjamini-corrected $P=0.02$) and suggestive enrichment categories, such as calcium signaling (uncorrected $P=0.003$) and Epidermal Growth Factor signaling ($P=0.009$), both of which lie downstream of β-adrenergic signaling (Table VI in the Data Supplement). Examination of our top candidate genes within each locus reveals a strong bias toward genes known to be involved in catecholamine-stimulated cardiomyopathy, such as calcineurin, phospholamban, and calmodulin (Tables 1 and 2).

**Conservation of Cardiomyopathy Loci in Mice and Humans**

We explored whether the loci we identified overlap with human GWAS results by examining the top 12 previously identified significant and suggestive human loci. The human loci were mapped onto the mouse genome using the NCBI Homologene resource and compared with a set of loci identified for the weight traits based on a slightly relaxed stringency ($P<1E-05$, minor allele frequency >5%) from the HMDP study. We observed 6 out of 12 human loci, including one of the genome-wide significant loci near USP3, replicating in our study (Table IV in the Data Supplement). We determined that this overlap is highly significant ($P=3.5E-4$)

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**Figure 2.** Manhattan plots of heart failure (HF) traits. A, Treated right ventricular weights; B, ratio of treated to untreated right ventricular weights; C, treated lung weights; D, treated liver weights; E, baseline cardiac fibrosis; and F, treated cardiac fibrosis. The red line indicates the threshold for suggestive association (4.2E-6) between a single-nucleotide polymorphism and a phenotype, whereas the blue line indicates the threshold for significant association (4.2E-7). Proposed candidate genes are indicated by gene symbols above peaks. QQ plots for each phenotype may be found in Figure VIII in the Data Supplement.
by permutation analysis. Our result supports the concept that genetic influences in \( \beta \)-adrenergic signaling significantly contribute to polygenic human HF. Furthermore, 16 loci for HF-related traits have previously been identified via linkage studies in mice for HF-related traits. We further observed that 7 of these 16 QTLs overlap with weight loci identified in this study \((P=1.2E-3)\) by permutation analysis \((\text{Table V in the Data Supplement})\), despite the fact that some of the linkage studies used different hypertrophy-inducing stressors, such as a calsequestrin transgene,\(^8\) which likely influence distinct HF pathways.

Validation of \textit{Abcc6} as Causal Gene for ISO-Induced Cardiac Fibrosis

We have identified a locus contributing to ISO-induced fibrosis on chromosome 7 \((\text{Figure 4, } P=7.1E-7)\). One of the 28 genes within the LD block, \textit{Abcc6}, has a splice site variation,\(^8\) resulting in a premature stop codon that is found in 19 of the strains we analyzed for cardiac fibrosis \((\text{KK/HJ, C3H/HeJ, DBA/2J, 11 BxD, 2 BxH, 3 CxB})\) in the HMDP. In untreated animals, we did not observe any significant difference \((P=0.25)\) between the degrees of fibrosis present in the left ventricle among HMDP strains divided based on \textit{Abcc6} genotype. In contrast, we observed a marked increase in cardiac fibrosis in the mice containing the \textit{Abcc6} splice mutation allele in response to ISO treatment \((P=1E-4; \text{Figure 4B})\).

\textit{Abcc6} deficiency is the cause of pseudoxanthoma elasticum, a disorder characterized by progressive tissue calcification,\(^{13,39}\) and a deficiency of \textit{Abcc6} has previously been linked to calcification phenotypes in aged mice by our laboratory.\(^{38}\) However, the fibrosis phenotype observed in these studies is clearly distinct from that of calcification. In fact, we did not observe a significant association with heart calcification in our study under basal \((P=0.8)\) or ISO treatment condition \((P=0.12)\) in mice with different \textit{Abcc6} genotypes, although we did observe some outlier strains, such as KK/HJ, that had markedly increased calcification after ISO stimulation \((\text{Figure 4C})\). Therefore, \textit{Abcc6} is a likely candidate gene contributing to ISO-induced fibrosis in heart.

To validate the role of \textit{Abcc6} in cardiac fibrosis, we studied a previously described \textit{Abcc6} KO mouse carrying a targeted mutation in a C57BL/6J strain background \((\text{KO})\), as well as the wildtype C57BL/6J \((\text{Control})\) mice.\(^{13}\) We previously reported that the \textit{Abcc6} KO mice exhibited increased cardiac calcification beginning from 6 months of age.\(^{14}\) At 3 months of age, neither the wildtype nor the KO mice exhibited significant differences in calcification as judged by Alizarin Red staining or cardiac fibrosis based on Masson Trichrome staining in the absence of ISO treatment \((\text{Figure 4D})\). Consistent with our observations of the entire panel, the ISO treatment significantly increased fibrosis levels in the \textit{Abcc6} KO animals without significantly increasing calcification levels in these
mice. This result suggests that the age-associated calcification phenotype observed in Abcc6 KO is distinct from the ISO-induced cardiac fibrosis phenotype.

To further establish Abcc6 as a causal gene for ISO-induced cardiac fibrosis, we studied transgenic mice carrying the Abcc6 wildtype locus from a C57BL/6J bacterial artificial chromosome on the background of the fibrosis-susceptible C3H/HeJ strain. Strain C3H/HeJ mice lack functional Abcc6 as a result of a splice variation. In the absence of ISO, neither C3H/HeJ mice nor C3H/HeJ mice carrying the Abcc6 bacterial artificial chromosome-transgene exhibited significant calcification or fibrosis in the heart, whereas after ISO treatment, the C3H/HeJ mice but not the Abcc6-bacterial artificial chromosome transgenic mice exhibited substantial fibrosis and calcification. TG, n=8; KO, n=6 (*P<0.05).

**Figure 4.** Abcc6 plays a role in the regulation of cardiac fibrosis after isoproterenol (ISO) stimulation. A, The locus on chromosome 7 which contains Abcc6 spans ∼800 kb and contains 28 genes within LD. B, Calcification in post-ISO treated hearts is increased in mice lacking Abcc6. C, Expression of Abcc6 in a mouse that lacks the gene is sufficient to rescue the mouse from the ISO-induced fibrosis and calcification. D, Knockout of Abcc6 in a mouse is sufficient to cause ISO-induced fibrosis, but does not cause a significant increase in calcification. WT, n=4; KO, n=3. E, The expression of Abcc6 as a transgene is sufficient to significantly reduce both calcification and fibrosis. TG, n=8; KO, n=6 (*P<0.05).

is unknown, but DAVID analysis of genes significantly correlated with Abcc6 expression in heart showed highly significant enrichment for mitochondrial genes (Table VII in the Data Supplement). Systemic factors are clearly involved in the calcification phenotype of Abcc6 deficiency, but it is noteworthy that, based on our expression profiling data, Abcc6 is clearly expressed in heart. The Abcc6 locus is suggestively associated (P<0.01) with several hypertrophic phenotypes in our study, namely whole heart weight (P=4E-3), right atrial weight (P=5E-3), and LVW (P=7E-3). Transcript coexpression network analysis was performed on Abcc6, but the module into which Abcc6 fell was not significantly enriched for any particular Gene Ontology term using DAVID (data not shown).

**Discussion**

We have used a strategy involving GWAS in a large panel of inbred mice to perform fine mapping of loci contributing to specific pathological features of cardiomyopathy after treatment with ISO. We have combined this strategy with global gene expression analysis in the heart to help identify...
candidate genes. A significant number of genetic loci revealed from this study are replicated in human GWAS analysis, supporting a conserved genetic network contributing to human heart failure. Several loci contain genes known to be involved in cardiomyopathy based on previous biochemical or genetic studies, supporting the validity of this approach to uncover important mechanisms and pathways related to the onset of heart failure. Finally, we validated Abcc6, a candidate GWAS hit, as a novel player in stress-induced cardiac fibrosis. These findings should complement human studies to identify genes and pathways contributing to this common and poorly understood disorder.

Systems genetics is a potent approach to reveal genes and pathways underlying the specific pathological features of cardiomyopathies, of which GWAS represents only one potential avenue for exploration. Using the resources presented here, it should be possible to perform additional analyses, including the generation of transcript coexpression networks for the identification of groups of genes involved in maladapted cardiac remodeling. The genetic information and the phenotypic spectra established by this study should provide a valuable resource for future heart failure studies.

Acknowledgments

We thank the excellent technical assistance from Ms. Mary Tuteryan, Ms. Haiying Pu, and Ms. Melanie Rosales.

Sources of Funding

This work was supported by National Institute of Health (NIH) grants HL110667 and HL28481. C.D. Rau was supported by NIH training grant T23HL97666 and J. Wang was supported by NIH training grant HL007895.

Disclosures

None.

References


Heart failure (HF) is characterized by insufficient pump action to maintain adequate blood flow, and common forms can result from myocardial infarction, hypertension, valvular disease, and viral infection. It constitutes the major cause of hospitalization in the United States with a lifetime risk of \( \approx 1 \) in 5. Genetic approaches to understand the common forms of HF, including genome-wide association studies have been largely unsuccessful, likely because of the extremely heterogeneous pathogenesis of HF. Using a defined pathological stress, chronic stimulation by isoproterenol, to circumvent the problem of heterogeneity, we performed genome-wide association studies for genes influencing cardiac hypertrophy and fibrosis in a large panel of inbred mice. We identified 24 significant or suggestive loci, several of which contain known HF-related genes, such as phospholamban and calcineurin A. We also validated \( Abcc6 \) as a causal gene at one of the fibrosis loci. Our results have the potential to help identify novel pathways leading to human HF and to develop novel therapies.
Mapping Genetic Contributions to Cardiac Pathology Induced by Beta-Adrenergic Stimulation in Mice
Christoph D. Rau, Jessica Wang, Rozeta Avetisyan, Milagros C. Romay, Lisa Martin, Shuxun Ren, Yibin Wang and Aldons J. Lusis

Circ Cardiovasc Genet. 2015;8:40-49; originally published online December 5, 2014; doi: 10.1161/CIRCGENETICS.113.000732
Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1942-325X. Online ISSN: 1942-3268

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**Figure S1.** *Isoproterenol-Induced Lethality.* The HMDP strains varied in the percent survival in response to ISO. In some strains, survival was 100% even when large numbers (>10) of mice were studied while, in other strains, 100% of mice died, usually within the first week.
**Figure S2.** Left Ventricular Loci Mirror Right Ventricular Loci at Lower Significances. Left ventricular GWAS and Right ventricular GWAS are displayed. Yellow bars indicate the location of each significant or suggestive right ventricular locus. Each significant or suggestive right ventricular locus reported in our study is mirrored in the left ventricular data at a lower significance level.
Figure S3. Distribution of Total Heart Weights Before and After Isoproterenol Treatment in the HMDP. Data is organized based on the untreated heart weight of the strain and displays mean +/- standard deviation.

Figure S4. Distribution of Right Ventricle Weights Before and After Isoproterenol Treatment in the HMDP. Data is organized based on the untreated heart weight of the strain and displays mean +/- standard deviation.
Figure S5. Distribution of Lung Weights Before and After Isoproterenol Treatment in the HMDP. Data is organized based on the untreated heart weight of the strain and displays mean +/- standard deviation.

Figure S6. Distribution of Liver Weights Before and After Isoproterenol Treatment in the HMDP. Data is organized based on the untreated heart weight of the strain and displays mean +/- standard deviation.
**Figure S7.** Distribution of Cardiac Fibrosis Before and After Isoproterenol Treatment in the HMDP. Data is organized based on the amount of fibrosis observed after isoproterenol treatment and displays mean +/- standard deviation.
Figure S8. QQ plots for each phenotype studied
### Table S1. List of all strains used in the study

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Table S2. Average values for each weight trait of interest before and after ISO stimulation.
All weights are in grams. P-value calculated using Student's T-test.

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Table S3. Expanded details on each locus

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Tm2d3  
Fibrosis |
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Mir30b  
Mir30d  
Zfat  
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AU022751  
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Table S4. Significant overlap of HMDP and human GWA loci for heart failure traits. Human loci were considered to overlap with mouse loci if they fell within 5 MB of a mouse locus peak. Overall, six of the twelve currently reported loci in human are matched in the HMDP study. DCM represents dilated cardiomyopathy, LVM represents Left ventricular, HF represents heart failure, and Death represents mortality among HF patients. Syntenic region represents the mouse region that is syntenic to the loci reported in the human studies. The genomic locations are notated by chromosome and bp position in Mb. RV represents right ventricular weight, TH represents total heart weight, RV* and RA* represents the difference in Right Ventricle and Right Atrial weight between isoproterenol-treated and control animals at week 3, respectively.

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<th>Study</th>
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<th>Peak SNP</th>
<th>P-value</th>
<th>Syntenic Region</th>
<th>Trait</th>
<th>P-value</th>
<th>SNP Location</th>
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Table S5. Significant overlap of HF GWAS loci in HMDP with QTL from previous mouse linkage analyses. Overall, 7 of the 16 reported QTLs relating to heart failure in mice are duplicated in the study. HR represents heart rate, HW represents heart weight, DCM represents dilated cardiomyopathy, and HF represents heart failure. LV represents the LV weight in controls, TH' represents isoproterenol-treated total heart weight at week 3, RA represents right atrial weight in controls, and RA* represents the difference in RA between isoproterenol-treated and control animals at week 3.

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Table S6. DAVID enrichment of all genes contained within a reported locus
No. represents the number of genes in each term category. Enrichment represents fold enrichment by DAVID. Adj. p-value represents the Benjamini-Hochberg adjusted p-value.

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<td>257</td>
<td>9.61</td>
<td>0.0069</td>
<td>0.81</td>
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Table S7. DAVID enrichment of all genes significantly correlated with Abcc6 expression in ISO treated mouse heart. No. represents the number of genes in each term category. Enrichment represents fold enrichment by DAVID. Adj. p-value represents the Benjamini-Hochberg adjusted p-value.

<table>
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<tr>
<th>Term</th>
<th>Count</th>
<th>%</th>
<th>No.</th>
<th>Enrichment</th>
<th>Adj. p-value</th>
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