Genetic Modifier Loci Affecting Survival and Cardiac Function in Murine Dilated Cardiomyopathy

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Background—Understanding the role for genetic factors in human heart failure is difficult because environmental factors cannot be standardized and genetic variation is great. One approach to identify genes that modify disease outcome is to use mouse models that show strong genetic variation of the disease phenotype.

Methods and Results—In this study, we used transgenic mice that develop severe dilated cardiomyopathy due to the cardiac-specific overexpression of calsequestrin. Transgenic mice showed marked strain-specific variation of cardiac function and survival, independent of transgene expression. A reciprocal backcross strategy was employed using two inbred strains showing distinct differences in survival and cardiac function. To map the genes that modified the heart failure phenotype, progeny from the 2 reciprocal backcrosses were used in a genome-wide scan for linkage. We identified two loci significantly linked to survival with a maximum likelihood ratio statistic of 36.2 (LOD score \( \approx 7.8 \)) on chromosome 2 and of 26.5 (LOD score \( \approx 5.7 \)) on chromosome 3. The chromosome 3 locus was also significantly linked to cardiac function with a maximum likelihood ratio statistic of 42.9 (LOD score \( \approx 9.3 \)). Because only a single strong modifier locus was found in each backcross, we applied a haplotype analysis to map crossovers and successfully narrowed the critical intervals for each locus.

Conclusion—Using a sensitized mouse model, we identified major modifier loci that affect the genetically complex disease of heart failure. This approach should allow the rapid identification of candidate genes involved in disease susceptibility in human populations and new insights into the pathogenesis of heart failure. (Circulation. 2002;105:1824-1829.)

Key Words: heart failure ■ genes ■ linkage mapping ■ transgenic mice ■ genetics

The pathogenesis of heart failure is complex, polygenic, and multifactorial. Considerable variation in clinical presentation and outcome indicates that unidentified genetic factors play a significant role in “modifying” the heart failure phenotype. Unfortunately, modifier genes have been difficult to identify in human populations because environmental factors cannot be standardized and the genetic variability is large. Although genetic mapping leading to gene identification for human disease traits has been widely successful for Mendelian disease, complex diseases have generally been recalcitrant to this approach,\(^1,2\) relying more on a candidate gene strategy.\(^3,4\)

Although rodent models have been successfully used to map disease susceptibility loci for complex disease, these studies have often resulted in the identification of multiple loci of weak to moderate effect, making it difficult to identify the genes involved.\(^5\) In order to circumvent these difficulties, we sought to simplify the genetic contribution to an otherwise complex phenotype by using a mouse model of heart failure as a “disease-sensitized” strain. The mouse model was generated by the cardiac overexpression of the sarcoplasmic reticulum Ca\(^{2+}\)-binding protein calsequestrin (CSQ),\(^6,7\) which sequesters the contractile-dependent pool of Ca\(^{2+}\).\(^8\) CSQ transgenic mice recapitulate many of the phenotypic features of human dilated cardiomyopathy, including cardiomegaly, depressed contractile function, abnormal \( \beta \)-adrenergic receptor signaling, and premature death.\(^6,7,9\) However, the effects of the CSQ transgene are highly strain-specific, showing wide variability in phenotype when crossed into different inbred mouse strains. The goal of this study was to use a genetically sensitized mouse model to map modifier genes that affect the heart failure phenotype.

Methods

Experimental Animals

All mice were handled according to approved protocol and animal welfare regulations by the Institutional Review Board at Duke University Medical Center. DBA CSQ mice were described previously.\(^5,8\) DNA preparation and genotyping for the CSQ transgene were performed by standard procedures. The F1 hybrid breeders were treated with the \( \beta \)-blocker metoprolol (350 mg/kg of body weight per day) from day 7 to prolong their survival in order to generate the backcrossed progeny.\(^7\) Wild-type mice of the C57BL/6 (B6) and DBA strains (Jackson Laboratory, Bar Harbor, Me) were used for backcrossing.
Echocardiography
Transthoracic M-mode echocardiography was performed on all mice at the age of 4 weeks in the conscious state using a HDI 5000 CV echocardiograph machine with a 10.5-MHz frequency probe (ATL Ultrasound).

Western Blotting
Whole hearts from wild-type DBA/B6, DBA CSQ, F1 (B6×DBA) CSQ, N2 (F1×B6) CSQ, and N2 (F1×DBA) CSQ mice were homogenized in lysis buffer, and 15 μg of cytosolic extracts were used for immunoblotting with a monoclonal CSQ antibody (kindly provided by Dr Larry Jones, Krannert Institute, Indiana University, Indianapolis).

Genome-Wide Scan
Seventy progeny from the N2 (F1×B6) and 56 progeny from the N2 (F1×DBA), chosen because their phenotypic values were greater than 0.5 standard deviations from the mean, were screened using 100 microsatellite markers (Research Genetics) spaced at approximately 15-cM intervals through the 19 mouse autosomes (http://www-genome.wi.mit.edu). An additional 39 progeny from the N2 (F1×B6) and 21 progeny from the N2 (F1×DBA) chosen at random were included in the analysis to follow-up on regions with evidence of a QTL. DNA was visualized by one of the following protocols: fluorescent labeling using an ABI Prism 377 sequencer (Applied Biosystems), ethidium bromide staining on agarose gels, or radioactive labeling on acrylamide gels. Data were collected with GeneScan 2.1 software (Applied Biosystems).

Statistical Analysis
MapManager QTX was used to perform all the statistical functions used in QTL mapping, including the estimation of the phenotypic variability due to each locus identified. In order to establish linkage thresholds, permutation tests were performed on our data at 1-cM intervals for 10 000 permutations. Suggestive (P = 0.05), significant (P < 0.05), and highly significant (P < 0.001) thresholds were established on the guidelines suggested by Lander and Kruglyak. Single locus association tests were performed between each marker and the phenotype to identify regions of interest in our initial genome scan. Chromosomes containing markers that reached or exceeded the established genome-wide significant linkage threshold (P < 0.05) were analyzed further using simple interval mapping at 1-cM intervals. The logarithm of the odds (LOD) score equivalent was calculated by dividing the likelihood ratio statistic (LRS) value by 4.6. Genetic distances were based on those reported in the Mouse Genome Database (MGD) (http://www.informatics.jax.org/).

Haplotype Analysis
We selected animals that had a recombinant haplotype in our regions of interest (chromosome 2 between markers D2Mit91 and D2Mit526 for the F1 backcross to B6 and chromosome 3 between markers D3Mit42 and D3Mit16 for the F1 backcross to DBA). Animals that were included in the haplotype analysis had a phenotypic value for survival beyond a 0.5 standard deviation from the mean (survival for the N2 (F1×B6) mice was 137.6±46.7 and the N2 (F1×DBA) mice was 33.5±5.3 days).

Results
To determine whether there was strain dependence to the cardiomyopathic phenotype, CSQ mice inbred on a DBA/2J (DBA) background were crossed with wild-type C57BL/6 (B6) mice to produce F1 (DBA/B6) progeny. To monitor the severity of the cardiomyopathy, two phenotypic traits (survival and cardiac function) were measured in the parental CSQ (DBA) mice and F1 generation. The average survival of the F1 CSQ progeny was significantly reduced to 52.4±9.0 days compared with 156.9±48.6 days in the parental CSQ mice (P < 0.0001) (Figure 1A). Echocardiographic assessment of the cardiac function showed greater dysfunction in 4-week-old F1 CSQ mice compared with the parental CSQ (DBA) mice (Figures 1B and 1C), indicating substantial acceleration of the heart failure phenotype with increased LVEDD and reduced fractional shortening compared with the parental CSQ (DBA) mice; n = 43 for CSQ (DBA) and n = 47 for the F1 (DBA/B6) CSQ; *P < 0.0001 vs parental CSQ (DBA).

Figure 1. Phenotype in the DBA CSQ and the F1 CSQ by echocardiography. A, Days survival showed remarkable decrease in the F1 (DBA/B6) CSQ mice compared with the parental CSQ (DBA) mice. Lifespans of wild-type (WT) DBA and B6 mice are approximately 600 days and 800 days, respectively (http://www.informatics.jax.org/external/festing/mouse/STRAINS.shtml); n = 44 for CSQ (DBA) and n = 58 for the F1 (DBA/B6) CSQ. B and C, F1 (DBA/B6) CSQ mice show acceleration of the heart failure phenotype with increased LVEDD and reduced fractional shortening compared with the parental CSQ (DBA) mice; n = 43 for CSQ (DBA) and n = 47 for the F1 (DBA/B6) CSQ; *P < 0.0001 vs parental CSQ (DBA).
Although we were unable to obtain echocardiographic data on 33 out of 94 (39.4%) N2 (F1\times B6) CSQ progeny because they died before their 4-week echocardiogram, echocardiograms on the remaining 61 N2 (F1\times B6) CSQ progeny showed a significant increase in left ventricular (LV) dimension and reduction in cardiac function (measured by FS) compared with the DBA CSQ and the F1 CSQ mice (Figures 2B and 2C). These data indicate that the heart failure phenotype was accelerated in the N2 progeny of the backcross to B6 compared with the F1 CSQ mice. When the phenotype was accelerated in the N2 progeny of the backcross, the initial genome screen included 70 progeny to detect QTLs linked to survival in the N2 progeny. For the N2 (F1\times B6) backcross, the initial genome screen included 70 progeny to detect QTLs linked to survival. We identified one marker on chromosome 2 that showed linkage to survival with a LRS 31.7 (P<0.001) (Figure 3A). For interval mapping of this locus at 1-cM intervals, 39 more N2 progeny were genotyped with additional markers providing a denser map of the interval with a maximum LRS of 36.2 (equivalent to a LOD score of 7.8) (Figure 3B). We demonstrate the existence of Hfm1 (for heart failure modifier 1) on chromosome 2 and estimate that it accounts for approximately 29% of the variance in survival in this cross. Because 39% of the N2 (F1\times B6) progeny died before their 4-week echocardiogram, we could not screen for QTLs affecting cardiac function in this backcross. To show a relationship between phenotype and genotype, survival was plotted against genotype at the chromosome 2 locus that showed the highest LRS (marker D2Mit327) (Figure 3C). As shown, a heterozygous genotype (DBA/B6) in the Hfm1 region is more often associated with increased survival than a homozygous (B6/B6) genotype. These data support our hypothesis that the DBA genetic strain is contributing a dominant protective allele to the phenotype.

To determine whether the difference in the phenotypic expression of the N2 progeny might be due to differences in expression level of the CSQ transgene, we performed Western blotting analysis on heart extracts from the parental DBA CSQ, the F1, and the N2 progeny. Importantly, the level of CSQ protein expression was equal in all progeny tested, indicating that the variation in phenotype of the CSQ mouse cannot be accounted for by variation in the level of CSQ protein in the heart (Figure 2D).

To map the genes that modify the heart failure phenotype, progeny from the 2 reciprocal backcrosses were used in a genome-wide scan for linkage. One hundred microsatellite markers were chosen with an average spacing of 15 cM to detect quantitative trait loci (QTLs) linked to LVEDD and survival in the N2 progeny. For the N2 (F1\times B6) backcross, the initial genome screen included 70 progeny to detect QTLs linked to survival. We identified one marker on chromosome 3 that showed linkage to survival with a LRS 31.7 (P<0.001) (Figure 5A). Linkage to this locus was confirmed with a LRS of 26.5 (equivalent to a LOD score of 5.7) (Figure 4B). We demonstrate the existence of Hfm2 on chromosome 3 and show that this modifier locus accounts for approximately 28% of the variance in survival in this backcross. There was also one marker mapping near Hfm2 that showed linkage to LVEDD with a peak LRS of 33.4 (P<0.001) (Figure 5A). Linkage to this region was also confirmed by interval mapping with a maximum LRS of 42.9 (equivalent to a LOD score of 9.3) and accounts for approximately 42% of the variance in LVEDD in this backcross (Figure 5B). Both traits (survival and LVEDD) appear to be linked to the same QTL (Hfm2) on chromosome 3, which is supported by the multipoint linkage analysis for both regions. We focused on the marker D3Mit86, which showed one of the highest LRS for both phenotypes, to determine whether there was an association between LVEDD and survival at that specific locus (Figure
Importantly, a heterozygous genotype (DBA/B6) in the Hfm2 region is more often associated with a larger ventricle and shortened survival than a homozygous (DBA/DBA) genotype.

Because only one significant QTL was identified in each of the backcrosses, we used a haplotype analysis to further narrow the 2 candidate intervals. We identified 15 N2 progeny from the F1 backcross to DBA with both a recombinant haplotype for Hfm2 and a survival value greater than 0.5 standard deviation from the mean (Figure 6A). Ten mice were grouped into an early death category, and genotyping revealed that these animals were heterozygous (B6/DBA) in the region of the QTL. Five mice were grouped into a late death category and showed a homozygous DBA genotype for the QTL. The minimal candidate interval is defined by 11 independent crossovers (animal Nos. 25, 91, 12, 411, 501, and 590 for the proximal limit; animal Nos. 153, 64, 123, 72, and 316 for the distal limit), which narrow the Hfm2 candidate interval to a 1.1-cM region between markers D3Mit320 and D3Mit260.

The same approach was used to refine the Hfm1 interval; we identified 14 N2 progeny from our F1 backcross to B6 that have both a recombinant haplotype in the QTL region and a survival value greater than 0.5 standard deviation from the mean (Figure 6B). Six of these animals were grouped into an early death category, and genotyping revealed that these mice were homozygous for B6 within the region of the QTL. Eight animals were grouped into a late death category and showed a heterozygous (B6/DBA) genotype for the QTL. The minimal candidate interval is defined by 6 independent crossovers (animal Nos. 31, 237, 1025, and 470 for the proximal limit; animal Nos. 478 and 1350 for the distal limit), which narrow the Hfm1 interval to 5.2 cM between markers D2Mit325 and D2Mit418. Interestingly, 1 animal (No. 1181) appears discordant, with a survival just above the upper limit of mean ± 1 SD exclusion.

Discussion

Using a reciprocal-backcross strategy to separate the effects of dominant modifiers on this sensitized genetic background, we identified a single strong modifier locus influencing cardiomyopathy and outcome for each cross. Because both mapped modifiers act as dominant alleles, but in reciprocal orientations (in one instance B6 over DBA, in the other DBA
over B6), they will not appear together in the reciprocal backcrosses; thus allowing us to separate the genetic effects for more precise mapping of each locus. Furthermore, this strategy allowed us to treat each locus as a "Mendelian" allele and, therefore, apply a haplotype analysis to map crossovers and rapidly narrow the critical interval. Although a haplotype analysis strategy is usually reserved for disorders of Mendelian inheritance, the presence of a single very strong QTL in each of the backcrosses provided the opportunity to use this approach for the mapping of modifiers in a complex genetic disease.

The phenotype-genotype relationship (Figures 3C and 5C) supports our hypothesis that the B6 genetic strain is contributing a dominant susceptibility allele to the cardiomyopathic phenotype in this model and is consistent with the clinical data showing the relationship between cardiac size and outcome (survival). However, our estimates of the percent-age of genetic contribution to the phenotypes defined by Hfm1 and Hfm2 are overestimates, because we excluded animals clustered around the mean values for each. Regardless, these loci collectively do not account for 100% of the phenotypic variance in LVEDD and survival. We note that there is reproducible variation in the phenotype even in genetically identical F1 animals (Figure 2), suggesting that experimental error and stochastic events contribute to the variance. Furthermore, because the measured traits are continuous, we assume that the interaction of multiple modifiers with weaker effects may be at work but each may fall below the threshold for detection. Nonetheless, when we hold constant the Hfm1 and Hfm2 loci in the respective backcross mapping analyses, we find that only a single locus on chromosome 9 reaches the $P=0.05$ level, in only the N2 progeny from the DBA backcross.

Figure 5. Mapping QTLs linked to cardiac chamber size determined by LV end-diastolic dimension in N2 (F1×DBA) CSQ. A, Single marker association tests for a whole genome scan with each bar representing a single microsatellite marker for 56 animals. B, Interval mapping of the chromosome 3 locus showing highly significant linkage to cardiac dimension with a maximum LRS of 42.9 ($n=74$ due to marker dropout in 3 animals). C, Scatter plot showing the phenotype-genotype interaction between survival and LV end-diastolic dimension at the marker D3Mit86 in the N2 (F1×DBA) progeny. This marker, unlike some used for haplotype analysis, was genotyped in all progeny of the backcross.

Figure 6. Refining the candidate intervals by haplotype analysis for the modifier loci. A, Hfm2, solid bars represent regions where the mouse is heterozygous for B6 and DBA alleles, and the open bars represent regions where the mouse is homozygous for the DBA allele. B, Hfm1, solid bars represent regions where the mouse is homozygous for the B6 allele, and the open bars represent regions where the mouse is heterozygous for DBA and B6 alleles. The change in the bar symbol from open to solid represents the widest marker position at which the indicated genotype is identified. For both loci, the hatched lines designate the borders of the current candidate interval.
Because only one major modifier locus was identified in each of the backcrosses, we were able to apply a haplotype analysis to map individual crossovers and further narrow the Hfml and Hfml2 intervals. The large number of independent crossovers that lie within the critical interval confirms that the modifiers are acting in a dominant manner and validates the experimental approach of using haplotype analysis. In the N2 (F1×B6) backcross, the haplotype of one animal (No. 1181) was found to be discordant. This is not surprising given the severity of the phenotype in this backcross and the clustering of survival around the mean. Indeed, animal No. 1181 died within 2 days of the upper threshold for exclusion and underscores the need for multiple independent crossover events to narrow the Hfml interval.

Within the Hfml region, a large number of genes map, including the gene titin, where it has recently been shown that mutations can cause autosomal-dominant dilated cardiomyopathy. Future studies will determine whether DNA sequence alterations in titin are responsible for the observed differences in the heart failure phenotype. Importantly, for both the Hfml and Hfml2 intervals, additional recombinant events within these regions will further refine the loci and lead to the identification of potential candidate genes.

Population studies have long suggested that common diseases (such as heart failure) have a strong genetic component. Although progress has been made in identifying disease loci causing inheritable forms of cardiomyopathy, such as that caused by mutations in cardiac actin, it is apparent that modifying genes define susceptibility to progressive cardiac dysfunction and sudden death in the heart failure population and are likely to be particularly important in mediating outcome. However, detecting which genes are relevant is difficult in human studies because environmental factors cannot be standardized and because human individuals differ in many genes in addition to the genes of interest. In this regard, the mouse provides a solution to both these difficulties and provides the opportunity to dissect the genetic factors involved in the disease process. Although not all mouse modifier loci will lead to the identification of corresponding loci in humans, we demonstrate here how the use of a sensitized mouse strain bred into different genetic backgrounds can significantly enhance the likelihood of successful mapping of modifier QTLs. In this regard, modifier genes have been successfully mapped in other mouse models of human disease, such as the secretory phospholipase gene Pla2g2a, identified as a modifier of adenomatous polyps, and a mouse gene that modifies cystic fibrosis. Whether modifier genes identified using this approach will be applicable to human heart failure will ultimately be determined through association studies in patient populations. Nonetheless, mapping of QTLs in mice may provide novel mechanistic insights into the pathogenesis and progression of heart failure.

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