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Identification of a Common Gene Expression Signature in Dilated Cardiomyopathy Across Independent Microarray Studies

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OBJECTIVES This study was designed to identify a common gene expression signature in dilated cardiomyopathy (DCM) across different microarray studies.

BACKGROUND Dilated cardiomyopathy is a common cause of heart failure in Western countries. Although gene expression arrays have emerged as a powerful tool for delineating complex disease patterns, differences in platform technology, tissue heterogeneity, and small sample sizes obscure the underlying pathophysiologic events and hamper a comprehensive interpretation of different microarray studies in heart failure.

METHODS We accounted for tissue heterogeneity and technical aspects by performing 2 genome-wide expression studies based on cDNA and short-oligonucleotide microarray platforms which comprised independent septal and left ventricular tissue samples from nonfailing (NF) (n = 20) and DCM (n = 20) hearts.

RESULTS Concordant results emerged for major gene ontology classes between cDNA and oligonucleotide microarrays. Notably, immune response processes displayed the most pronounced down-regulation on both microarray types, linking this functional gene class to the pathogenesis of end-stage DCM. Furthermore, a robust set of 27 genes was identified that classified DCM and NF samples with >90% accuracy in a total of 108 myocardial samples from our cDNA and oligonucleotide microarray studies as well as 2 publicly available datasets.

CONCLUSIONS For the first time, independent microarray datasets pointed to significant involvement of immune response processes in end-stage DCM. Moreover, based on 4 independent microarray datasets, we present a robust gene expression signature of DCM, encouraging future prospective studies for the implementation of disease biomarkers in the management of patients with heart failure. (J Am Coll Cardiol 2006;48:1610–7) © 2006 by the American College of Cardiology Foundation

Dilated cardiomyopathy (DCM) is characterized by dilatation and impaired contraction of 1 or both ventricles in the absence of significant coronary artery disease. The incidence of DCM has been estimated to be 5 to 8 cases per 100,000 individuals, with a prevalence of 36 per 100,000 (1). Thus, DCM is a leading cause of heart failure and cardiac transplantation in Western countries (2). The high morbidity and mortality associated with DCM underscore the need for a better understanding of the underlying molecular events leading to heart failure in DCM. So far, the natriuretic peptides are the best characterized markers for diagnosing and managing heart failure. However, there is significant heterogeneity in expression levels of these molecular disease markers that is not explained by left ventricular function alone (3–5). Transcriptional signature analysis is a powerful technique capable of identifying new molecular targets which could ultimately become important for diagnosis and therapy of heart failure. Yet, differences in platform technologies, experimental design, and the biological heterogeneity associated with the use of human tissue samples form obstacles to the successful comparison and integration of results obtained by different microarray studies in heart failure. In addition, substantial regional variation in gene expression exists in mammalian myocardium including atrium, ventricle and septum or left and right side of the heart (6–9). These differences make it difficult to determine which transcripts are related to DCM as opposed to other sources of biological variability.

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The aim of the present study was 2-fold: First, we wanted to identify common genes and shared biological processes based on Gene Ontology (GO) (10) across 2 independent microarray studies in human DCM to minimize overinterpretation of either dataset. Biological variability was accounted for by examining 20 DCM and 20 nonfailing (NF) myocardial samples from different regions of human

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Abbreviations and Acronyms

DCM = dilated cardiomyopathy
GO = Gene Ontology
ICM = ischemic cardiomyopathy
NF = nonfailing

myocardium (septum and left ventricle of different transmural origin). Our second goal aimed at establishing a robust set of genes of DCM and NF hearts for the 2 present and 2 additional, publicly available, microarray datasets. As a result, we present a set of 27 genes which classified 108 NF and DCM samples in 4 independent microarray datasets with >90% accuracy.

METHODS

Study design. We performed 2 microarray studies with a total of 40 patient samples. The cDNA microarray study (dataset A) was based on 28 septal myocardial samples obtained from 13 DCM hearts at the time of transplantation and 15 NF donor hearts that were not transplanted because of palpable coronary calcifications. The latter patient group was not known to have any history of overt cardiovascular disease. For our oligonucleotide microarray study (dataset B), 12 independent subendocardial left ventricular samples were collected from 7 DCM patients and 5 NF donors. Detailed patient characteristics are listed in supplemental Table 1 (see Appendix). All transplanted patients gave written informed consent. The investigation was approved by the Institutional Review Board.

After excision, all tissue specimens were frozen in liquid nitrogen and stored at −80°C. RNA isolation, sample preparation, labeling, and hybridization to RZPD Unigene 3.1 cDNA (37.5 K) and to Affymetrix U133A (22.2 K) arrays were carried out as described previously (6,11,12). The original data files for datasets A and B were deposited in the Gene Expression Omnibus database (GEO) (13) and are accessible through GEO series accession numbers GSE3585 and GSE3586.

For the classification and the verification of the classifier gene set, we included 2 additional studies, for which data were publicly available. Dataset C consists of 6 NF, 21 DCM, and 10 ischemic cardiomyopathy (ICM) samples hybridized to Affymetrix HG-U133A arrays (14). Normalized gene expression data were downloaded from GEO (GSE1869). Dataset D is available online through a program for genomic application funded by the National Heart, Lung, and Blood Institute and consists of 14 NF, 27 DCM, and 32 ICM samples hybridized to Affymetrix HG-U133 2.0-plus arrays (15). In summary, we used 68 DCM and 40 NF samples from 4 independent studies for classification.

Data extraction and statistical analysis. Preprocessing and most of the statistical analysis were done using R (16) and Bioconductor (17). After quality control, all cDNA microarray data were normalized using arrayMagic (18) and "VSN" (19). Normalized data were filtered with respect to signal intensity. Quality of the HG-U133A arrays was assured by controlling for dynamic range, perfect match saturation, pixel noise, grid misalignment, and signal-to-noise ratio. Microarray data of all samples in microarray study B were normalized in common using robust multi-array average (RMA) (20) implemented in Bioconductor's "affy" package. Probe sets with "absent" calls in more than 50% of tissue samples in either group (NF and DCM) were excluded.

To determine differentially expressed genes, 2-class unpaired significance analysis of microarrays (SAM) (21) was applied in both studies. Differences in gene expression were regarded as statistically relevant if a false discovery rate (FDR) of $q < 0.05$ and a fold-change of ≥ 1.2 were achieved.

Mapping of transcripts between cDNA clones and Affymetrix probe sets was achieved by means of the MatchMiner software tool (22). Functional annotation of differentially expressed genes was based on hierarchical system of GO domains "cellular component," "biological process," and "molecular function." Over-representation of specific GO classes in a gene set was statistically analyzed by "FatiGO" (23).

Expression values of selected transcripts were validated by quantitative real-time polymerase chain reaction (PCR) (ABI Prism 7900HT Sequence Detection System; Applied Biosystems, Weiterstadt, Germany). A detailed list of genes examined by RT-PCR including protocols used for RT-PCR is given in the supplemental Materials and Methods section (see Appendix).

Identification of a gene expression signature for DCM. Prediction analysis for microarrays (PAM) (24) was used for classification. The ability to correctly classify the status of DCM and NF samples was assessed by complete cross-validation implemented in the Bioconductor package "MCRestimate" (25). First, the samples in every study were randomly divided into equally sized subsets. In each following step, 1 subset was left aside and the classifier (filtering and PAM) was built on the remaining samples (training set). The status (NF vs. DCM) of the left-out samples was predicted and compared with the clinically diagnosed status. Optimization of the PAM parameter and of the number of genes remaining after variance filtering was achieved through a second cross-validation within each training set. To estimate the variability of the cross-validation result based on different sample compositions of the training set, the procedure was repeated 50 times. A sample was called "misclassified" if it was incorrectly classified in more than half of all cross-validations.

RESULTS

Common changes of biological processes in DCM. To identify differentially expressed genes, we analyzed our 2 experimental studies, which included septal myocardial

tissue samples from 13 DCM and 15 NF hearts (dataset A) and left ventricular samples from 7 DCM and 5 NF hearts (dataset B) by SAM analysis. In dataset A, 1,353 transcripts were up-regulated and 384 were down-regulated in DCM. In dataset B, 399 transcripts were up-regulated and 75 transcripts were down-regulated in DCM (supplemental Table 2, see Appendix). In both studies, up-regulation was about 4 to 5 times more common than down-regulation, indicating a net transcriptional activation in heart failure. Overall, 76 transcripts were found to be consistently deregulated in both studies, representing ~16% overlap at the single gene level between both microarray studies (supplemental Table 3). These transcripts included known marker genes of heart failure, such as pro-brain natriuretic peptide (pro-BNP) (26), and chemokine (C-C motif) ligand 2 (CCL2) (27), but also many genes previously not associated with cardiomyopathies. Validation of microarray expression values was done by using quantitative real-time PCR. As a result, we found a strong correlation for the expression ratios between arrays and quantitative PCR for 11 of 12 differentially expressed genes analyzed in dataset A (supplemental Table 4, see Appendix).

To gain a comprehensive insight into the biological processes associated with DCM, we related differentially expressed genes to their respective GO classes. Thereby, we were able to identify specific biological processes which were consistently enriched in up- or down-regulated transcripts of both studies (supplemental Table 5, see Appendix). For example, both studies showed a marked up-regulation of transcripts involved in protein biosynthesis in DCM. However, it is interesting to note that this functional GO class comprises qualitatively different genes in the 2 studies. Whereas the cDNA microarray study (dataset A) detected many elongation factors, ribosomal transcripts were more frequently recognized in the Affymetrix study (dataset B) (supplemental Table 6, see Appendix).

The biological processes "immune" and "inflammatory response" displayed the most significant changes in the group of down-regulated genes in DCM. These functional gene classes included components of the complement system (C1QB, C1QR1, C1R, C3), chemokines (CCL2, CCL11, CCL18), interferon-induced genes (IFI27, IFI30, IFITM1, IFITM3, STAT3), calgranulins (S100A8, S100A9), and leukocyte antigens (CD14, CD53, CD163), suggesting a profound deregulation of the immune system in DCM (Fig. 1). In accordance with down-regulation of immune response genes in DCM, the functional class of "chemokine activity" displayed the most prominent down-regulation specified by level 6 of GO category "molecular function."

Consistent with prominent structural remodeling in end-stage DCM, many deregulated transcripts were related to extracellular matrix composition and turnover. Notably, up-regulation of collagen transcripts (COL5A1, COL8A1) and the procollagen C-endopeptidase enhancer 2 (PCOLCE2) which binds to type I procollagen and poten-

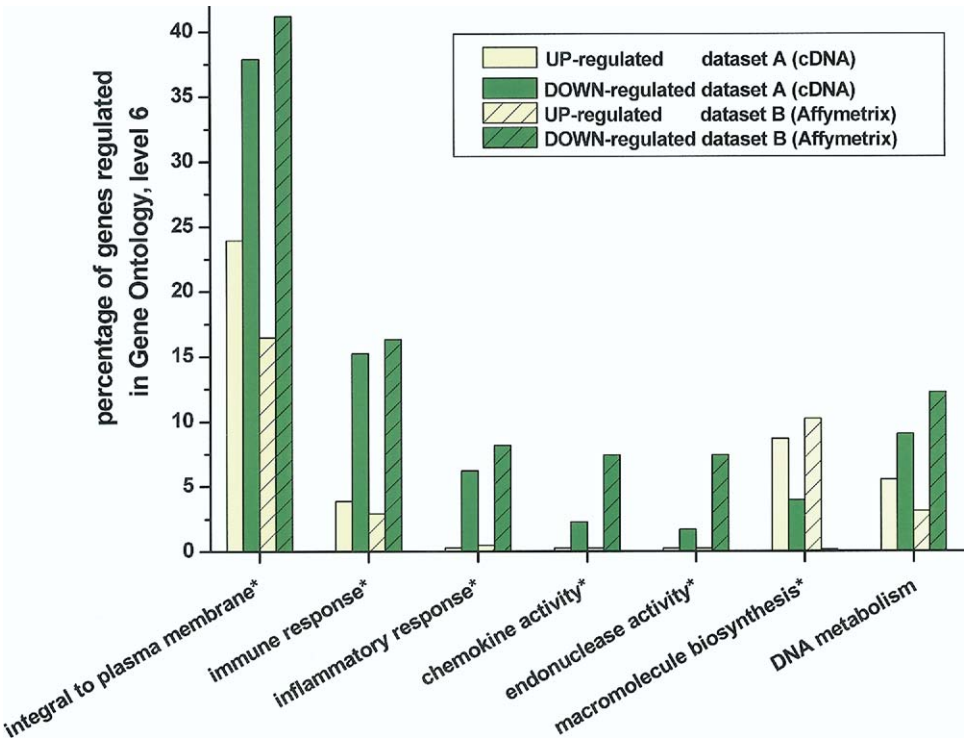

Figure 1. Functional analysis based on Gene Ontology for selected gene classes comparing up-regulated genes in DCM **(yellow bars)** and down-regulated genes **(green bars)** in dilated cardiomyopathy (DCM) of datasets A **(open bars)** and B **(striped bars)**. Differences in gene classes marked by an asterisk were statistically significant (Fisher exact test; $p < 0.05$) according to "FatiGO" (23). Results for cellular component, biological process, and molecular function are based on supplemental Table 5 (see Appendix).

tiates its cleavage by procollagen C-proteinases, was observed. In addition, extracellular matrix protein 2 (a member of the small leucine rich proteoglycans (SLRP), important for collagen fibrillogenesis), asporin, and most other members of the SLRP family were found to be up-regulated as well (decorin, lumican, biglycan, fibromodulin, osteoglycin, and osteomodulin), highlighting their importance in extracellular remodeling. Furthermore, we noted prominent up-regulation of genes coding for Z-disc components, including caldesmon 1, sarcospan, sarcoglycan epsilon, utrophin, spectrin, titin, vinculin, sarcoglycan D and G, alpha-actinin, LIM-domain binding 3, and alpha-2-capping protein. The Z-disc is thought to act as a sensor, linking biomechanical forces to the activation of stress pathways (28). In this sense, we were able to corroborate the desensitization of beta-adrenergic signaling by gene expression analysis with down-regulation of adrenergic, beta-1-receptor in end-stage DCM (supplemental Table 2, see Appendix). Profound changes in signal transduction were also reflected in the down-regulation of the GO class "integral to plasma membrane" specified by level 6 of GO category "cellular component." With regard to the deregulation of important signaling pathways, we found transcriptional repression of the oncostatin M receptor (OSMR), anti-apoptotic gene BCL2L1, and signal transducer STAT3, which are involved in the protection of the myocardium from heart failure via the JAK-STAT pathway. Ultimately, the balance between pro- and anti-apoptotic programs may determine if relevant loss of myocytes occurs. In line with this notion, we noted up-regulation of anti- (FGF1, DSIPI) and pro-apoptotic transcripts (CCL2, BCLAF1, FOXO3A).

Identification of a gene expression signature for DCM. The second goal of our analysis was to identify a specific set of transcripts that could reliably classify DCM and NF samples. We approached this issue by performing the PAM classification method in 4 independent microarray studies. Very low misclassification rates were found in our 2 studies (datasets A and B) and in dataset D for the classification of NF vs. DCM samples (Fig. 2). One out of 12 samples was misclassified in dataset B. Likewise, datasets A and D showed similar results, with 1 out of 28 and 3 out of 41 misclassified samples, respectively. In contrast, the classification algorithm did not show any predictive power in dataset C. This was unexpected, because the expression levels of established molecular cardiomyopathy markers, including pro-BNA or pro-ANP, suggested a clear separation into NF and failing ventricular samples (Fig. 3).

The smallest number of probe sets used for classification was found in dataset B, with a median of 5 probe sets comprising 31 different probe sets and a median absolute deviation of 2.9. Therefore, we evaluated the ability of this set of 31 probe sets coding for 27 genes (Table 1) to correctly classify DCM and NF samples of the remaining 3

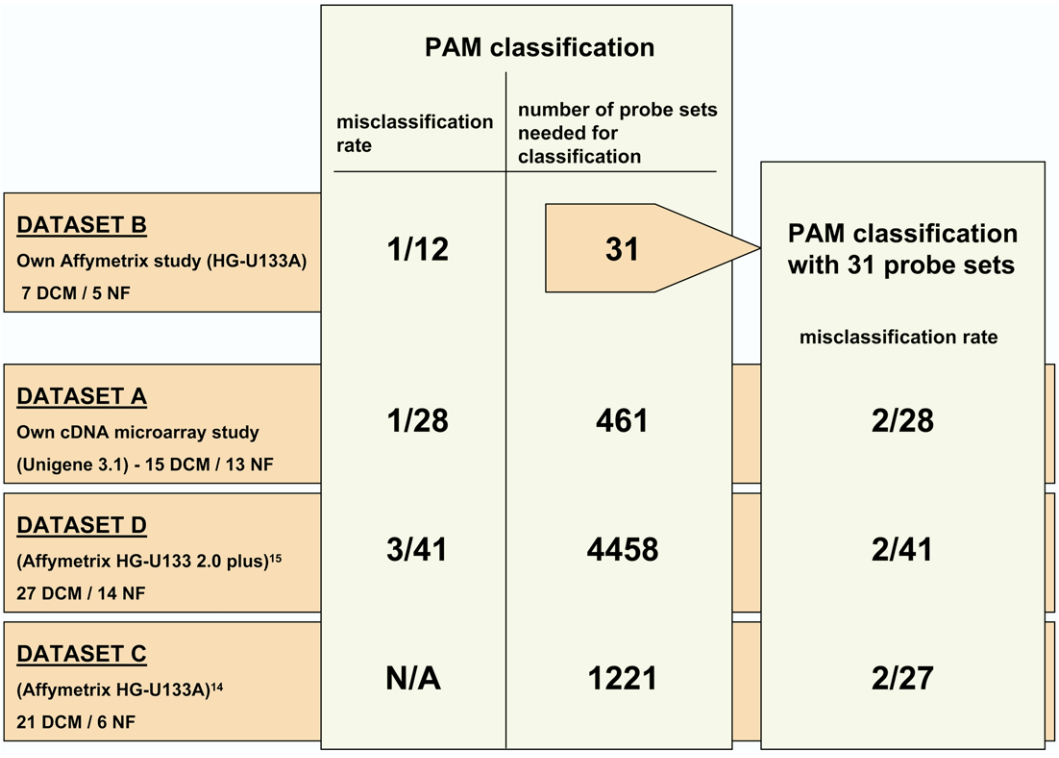

Figure 2. Prediction analysis for microarrays (PAM) classification. In the first step, PAM classification was applied to all 4 datasets separately. Very low misclassification rates were found in datasets A, B, and D for the classification of nonfailing (NF) versus dilated cardiomyopathy (DCM) samples, whereas the classification algorithm did not show any power in dataset C. In the second step, we repeated the procedure with the smallest gene signature obtained from dataset B (31 probe sets which correspond to 27 transcripts), now achieving more than 90% accuracy for classifying DCM and NF samples across all studies, including dataset C.

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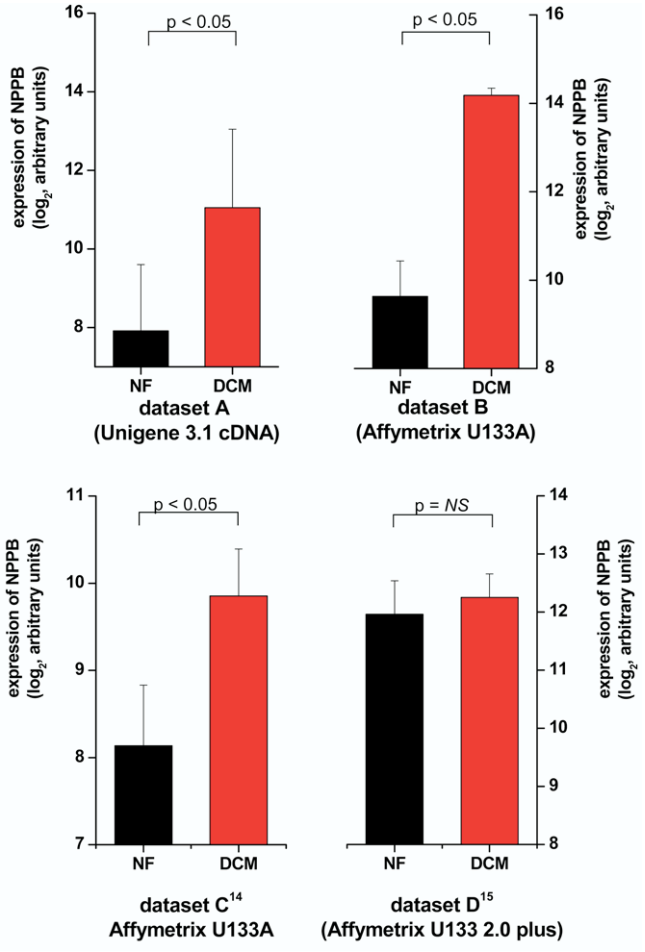

Figure 3. Mean expression ± SEM of pro-brain natriuretic peptide (NPPB) in nonfailing (NF) **(black bars)** and dilated cardiomyopathy (DCM) samples **(red bars)** in datasets A to D. Statistical comparison was carried out by Student *t* test.

studies. First, we reduced datasets C and D to the 31 probe sets that were used for classification in dataset B. Next, we used PAM without filtering on the reduced set for classification and estimated the prediction power by performing a complete classification procedure. For dataset A, we first determined the presence of 17 out of 27 genes on the cDNA microarray and proceeded with the classification as previously described. In summary, we found that a small set of 27 genes was sufficient to classify DCM and NF hearts across all 4 independent studies with more than 90% accuracy (Fig. 2). Remarkably, this comprehensive gene set was able to classify DCM and NF samples in dataset C, for which the PAM method had initially failed.

The 27-gene signature included known marker genes of heart failure like pro-BNP, pro-atrial natriuretic peptide (pro-ANP), corin (which converts pro-ANP to biologically active ANP), transcripts encoding for sarcomer structure proteins (MYH6, MYH10), apoptotic processes (CCL2, PHLDA1, SNCA), cell growth (FRZB, SFRP4, SPOCK, CTGF), and cell cycle control (G0S2, ETV5, RARRES1). Notably, the selection of individual known marker genes such as pro-BNP alone was not sufficient to classify the DCM cases because of the heterogeneous gene expression across all 108 myocardial samples (Fig. 3).

Because several previously described genes for heart failure were part of the classifier gene set, its validity might well hold for heart failure in general, irrespective of etiology. We tested this hypothesis by PAM analysis for ICM and NF samples included in datasets C (ICM: n = 10; NF: n = 6) and D (ICM: n = 32; NF: n = 14) and found that this gene set classified more than 90% of these samples correctly as well (data not shown).

DISCUSSION

Different etiologies and duration of dilated cardiomyopathy, differences in age, gender, and medications, as well as individual course of the disease contribute to the variability of gene expression data. In addition, it is very difficult to obtain true "non-failing" human ventricular tissue, because donor hearts may have been exposed to varying degrees of hypoxia or hemodynamic stress which are known to be potent inducers of chemokine and BNP gene expression (29). In the present work, we considered these variables by integrating independent microarray studies from a large number of failing and non-failing hearts to identify common transcripts and biological processes involved in the pathogenesis of DCM and to define a robust, common denominator for DCM and NF hearts. Based on the smallest gene set for classification from dataset B, the PAM method classified all 4 human heart failure datasets with low misclassification rates. Notably, despite the large variation of gene expression values for single genes, the classifier as a whole is highly valuable to distinguish DCM and NF samples. This supports the usefulness of this molecular approach for future diagnostic applications. In addition, the classificator gene set based on DCM and NF hearts also achieved a similarly high accuracy of classification in ICM as in DCM samples, suggesting that this gene set could be representative of molecular changes of heart failure in general.

Of note, the classificator based on dataset B performed as well in datasets A and D as if one used classificators generated from these 2 datasets alone. What is more, the gene signature from dataset B was also able to accurately discriminate NF and DCM samples in dataset C. As noted by the authors of dataset C, differences in gene expression were greater between left ventricular assist-device (LVAD) and non-LVAD hearts in the DCM group than between DCM and NF samples (14). This peculiarity might impede the PAM approach for identifying a useful classifier between DCM and NF within this dataset itself.

The classifier gene signature can be grouped into different functional sets with respect to the pathogenesis of DCM. Up-regulation of the cardiomyopathy markers pro-ANP and pro-BNP is well established in heart failure (26) and mediated by neurohormonal dysregulation (30). Activation

Table 1. Classifier Gene Set in DCM

Gene Name	Mean of NF (Dataset B) ± SD [Arbitrary Units]	Mean of DCM (Dataset B) ± SD [Arbitrary Units]	Linear Fold-Change (Dataset B)	Ranking Dataset B	Ranking Dataset A	Ranking Dataset C	Ranking Dataset D
Adipocyte enhancer binding protein 1	349 ± 122	911 ± 415	2.61	16	12	21	17
Alanine-glyoxylate aminotransferase 2-like 1	205 ± 129	82 ± 27	0.4	19	—	8	13
Asporin	1,305 ± 614	3,142 ± 1,134	2.41	17	16	3	5
Activating transcription factor 3	580 ± 376	271 ± 83	0.47	18	—	28	12
Chemokine (C-C motif) ligand 2	2,315 ± 1,039	761 ± 197	0.33	9	2	11	1
Complement factor H-related 3	321 ± 128	1,099 ± 373	3.42	5	—	12	11
Corin	932 ± 567	281 ± 255	0.3	25	—	26	26
Connective tissue growth factor	567 ± 270	2,006 ± 737	3.54	7	5	24	28
Ets variant gene 5	310 ± 57	561 ± 70	1.81	31	—	25	30
Ficolin 3	1,040 ± 587	465 ± 306	0.45	27	15	5	8
Frizzled-related protein	230 ± 72	819 ± 449	3.56	12; 21	14	16; 30	7; 9
Putative lymphocyte G0/G1 switch gene	1,584 ± 593	711 ± 210	0.45	30	—	27	21
Inhibitor of DNA binding 4	149 ± 62	474 ± 198	3.17	8; 28	—	9; 29	19; 23
Kelch-like 3	802 ± 103	1,722 ± 444	2.15	15	13	20	18
Myosin, heavy polypeptide 6, cardiac, alpha	6,357 ± 2,812	2,933 ± 2,703	0.46	24	—	1	2
Myosin, heavy polypeptide 10, non-muscle	1,653 ± 148	3,126 ± 797	1.89	29	4	13	22
Natriuretic peptide precursor A	6,216 ± 3,194	26,824 ± 3,506	4.32	3	9	17	14
Natriuretic peptide precursor B	1,343 ± 1,354	19,173 ± 4,557	14.29	1	10	19	27
Ornithine decarboxylase 1	888 ± 239	2,001 ± 358	2.27	11	1	7	31
Procollagen C-endopeptidase enhancer 2	549 ± 98	1,081 ± 394	1.96	23	3	6	16
Pleckstrin homology-like domain, family A, member 1	365 ± 124	1,425 ± 490	3.90	2; 4	8	2; 4	20; 24
Retinoic acid receptor responder 1	738 ± 603	119 ± 48	0.16	6; 13	17	15; 18	3; 10
S100 calcium binding protein A8	1,084 ± 487	266 ± 142	0.25	14	—	31	6
Secreted frizzled-related protein 4	178 ± 32	474 ± 252	2.67	22	11	23	4
Synuclein, alpha	420 ± 104	843 ± 223	2.01	26	—	14	15
Sparc/osteonectin proteoglycan	904 ± 359	2,268 ± 679	2.51	10	7	22	29
Zinc finger and BTB domain containing 16	785 ± 472	1,430 ± 373	1.82	20	6	10	25

Twenty-seven transcripts classifying dilated cardiomyopathy (DCM) and nonfailing (NF) samples (generated by prediction analysis for microarrays [PAM] classification from dataset B and listed in alphabetical order). In addition to expression values for DCM and NF samples of dataset B, the ranking of the single genes for classification of datasets A to D based on PAM parameters is given. When transcripts were represented by 2 probe sets, the ranking of both is indicated. Genes indicated with a dash in dataset A were not present in the cDNA array dataset.

of pro-fibrotic stress hormone pathways leads to prominent structural remodeling in DCM, exemplified by deregulation of genes coding for sarcomer structure and extracellular matrix proteins such as myosin 6 and 10, asporin, PCOLCE2, kelch-like 3 (KLHL3), and adipocyte enhancer binding protein 1 (AEBP1). In addition to pro-BNP, further transcripts of this set of classifier genes, including the transcription factor ZBTB16 (31), the connective tissue growth factor CTGF (32), and the chemokine CCL2 (33), characterize important targets of the renin-angiotensin system in failing myocardium, because they all have been shown to be regulated by angiotensin-II.

Other transcripts of this classifier gene set belong to apoptotic (PHLDA1, SNCA, CCL2) and cell growth (FRZB, SFRP4, SPOCK, CTGF) processes. Of note, the 2 genes coding for frizzled-related protein (FRZB) and secreted frizzled-related protein 4 (SFRP4) are members of the Wnt signaling pathway, implicated in wound healing and regeneration of heart failure (34,35). Especially, the expression of the Wnt antagonist SFRP4 is associated with myocyte apoptosis in overload-induced heart failure (34).

Furthermore, the genes coding for the complement factor H-related 3 (CFHL3), ficolin 3 (FCN3), chemokine ligand 2 (CCL2), and calgranulin A (S100A8) are related to stress and immune response. Remarkably, the GO classes "immune response" and "inflammatory response" showed the most significant changes of all biological processes in our 2 datasets, A and B. Given that DCM has a very heterogeneous etiology, it is plausible that a subgroup of DCM represents post-infectious autoimmune disease, especially in individuals with genetic susceptibility. However, independent experimental models of cardiomyopathy suggest that cardiac remodeling itself is able to trigger immune response (36,37). Chemokine ligand 2 is a prominent member of the broader functional group of immune and inflammatory processes and was found to be down-regulated in both datasets A and B. This chemokine, capable of interacting with tumor necrosis factor alpha and interleukin-6–related pathways, has been localized to the cardiomyocyte compartment by immunohistochemistry (27). It promotes attraction and invasion of activated leukocytes into the failing myocardium but is also involved in shaping the extracellular matrix by modulating the activity of matrix metalloproteinases and collagen turnover (38) as well as cell proliferation and induction of apoptosis (27). Down-regulation of CCL2 transcripts in end-stage heart failure may therefore represent an adaptive mechanism to promote cell survival. Of note, additional chemokines like CCL11 and CCL18 also were found to be down-regulated in Affymetrix and Unigene arrays, respectively.

Given the limited availability of human myocardial samples, future studies will need to correlate gene expression patterns in myocardium to tissue specimens more readily available from individual patients and suitable as myocardial surrogates (possibly peripheral blood leukocytes). In addition to the known cardiomyopathy markers pro-ANP and pro-BNP, we identified further genes involved in the natriuretic system and immune response processes which may be promising candidates for disease biomarkers. In this respect, the identification of a robust molecular signature of DCM across different microarray platforms and independent studies enables careful "hypothesis driven" validation studies of biomarkers of heart failure and testing their clinical utility.

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APPENDIX

For an extended version of the Materials and Methods and supplemental tables, please see the online version of this article.