Transcriptomic Biomarkers for the Accurate Diagnosis of Myocarditis

Bettina Heidecker, MD; Michelle M. Kittleson, MD, PhD; Edward K. Kasper, MD; Ilan S. Wittstein, MD; Hunter C. Champion, MD, PhD; Stuart D. Russell, MD; Ralph H. Hruban, MD; E. Rene Rodriguez, MD; Kenneth L. Baughman, MD†; Joshua M. Hare, MD

Background—Lymphocytic myocarditis is a clinically important condition that is difficult to diagnose and distinguish. We hypothesized that the transcriptome obtained from an endomyocardial biopsy would yield clinically relevant and accurate molecular signatures.

Methods and Results—Microarray analysis was performed on samples from patients with histologically proven lymphocytic myocarditis (n=16) and idiopathic dilated cardiomyopathy (n=32) to develop accurate diagnostic transcriptome-based biomarkers using multiple classification algorithms. We identified 9878 differentially expressed genes in lymphocytic myocarditis versus idiopathic dilated cardiomyopathy (fold change >1.2; false discovery rate <5%) from which a transcriptome-based biomarker containing 62 genes was identified that distinguished myocarditis with 100% sensitivity (95% confidence interval, 46 to 100) and 100% specificity (95% confidence interval, 66 to 100) and was generalizable to a broad range of secondary cardiomyopathies associated with inflammation (n=27), ischemic cardiomyopathy (n=8), and the normal heart (n=11). Multiple classification algorithms and quantitative real-time reverse-transcription polymerase chain reaction analysis further reduced this subset to a highly robust molecular signature of 13 genes, which still performed with 100% accuracy.

Conclusions—Together, these findings demonstrate that transcriptomic biomarkers from a single endomyocardial biopsy can improve the clinical detection of patients with inflammatory diseases of the heart. This approach advances the clinical management and treatment of cardiac disorders with highly variable outcome. **(*Circulation*. 2011;123:1174-1184.)**

Key Words: biomarker ■ gene expression ■ gene microarray ■ heart failure ■ myocarditis

The myocardites are inflammatory diseases of the heart that have variable clinical presentations and are caused by a range of underlying inflammatory variants.[1,2] Of new-onset heart failure, 10% to 30% may be caused by cardiac inflammation, and viral infection,[3,4] systemic or local inflammatory diseases, and genetic predisposition represent inciting factors.[5–7] Myocarditis can be difficult to diagnose, requiring multiple endomyocardial biopsies (EMBs).[8–11] Even with multiple biopsies, consensus among pathologists has been difficult to attain.[12] Inaccurate or uncertain diagnosis is of major concern because emerging therapies specifically targeting inflammatory or viral heart disease have the potential to reverse the disease process.[11,13–15] In a previous decision analysis investigating the value of EMBs in improving clinical outcome with specific therapy, histological inaccuracy was a major limiting factor for treatment efficacy.[11]

Clinical Perspective on p 1184

Current attempts to improve diagnostic accuracy include screening for viral RNA in EMBs,[16,17] serum antiheart autoantibodies,[16] and use of magnetic resonance imaging.[18,19] Transcriptomics has emerged as a highly valuable tool for complex pathological diagnosis. Examples include delineation of childhood tumors,[20] determination of organ rejection,[21,22] and delineation between ischemic and nonischemic heart disease.[23] On the basis of recent findings indicating that a single EMB contains sufficient RNA to perform a microarray without amplification,[24,25] we sought to test the hypothesis that the transcriptome could be used to create biomarkers that add diagnostic accuracy to clinical, pathological, and imaging modalities currently used to diagnose myocarditis.

Received May 21, 2010; accepted January 13, 2011.

From the University of Miami, Miller School of Medicine, Miami, FL (B.H., J.M.H.); Johns Hopkins Hospital, Baltimore, MD (E.K.K., I.S.W., S.D.R., R.H.H.); University of Pittsburgh, PA (H.C.C.); Cleveland Clinic Foundation, Cleveland, OH (E.R.R.); Department of Medicine, Division of Cardiology, University of California, Los Angeles (M.M.K.); and Brigham and Women's Hospital, Boston, MA (K.L.B.).

†Deceased.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.110.002857/DC1.

Correspondence to Joshua M. Hare, MD, Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, Biomedical Research Bldg, Room 824, PO Box 016960 (R-125), Miami, FL 33101. E-mail jhare@med.miami.edu

© 2011 American Heart Association, Inc.

Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.110.002857**

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

Methods

Study Population

We performed transcriptomic analysis of EMBs in matched cohorts of patients with idiopathic cardiomyopathy (IDCM; n=32) and myocarditis (n=16) selected from a biorepository containing samples from patients with new-onset heart failure (n=350). The baseline conditions between groups were compared using Student's *t* test or Fisher exact test as appropriate and were found to have no differences (Table 1). Similarity of baseline conditions was tested with the Student *t* test and Fisher exact test. There was no difference between the 2 groups. Four to 6 biopsy specimens were obtained from each patient and examined by an experienced cardiac pathologist. Myocarditis was defined according to Dallas criteria,[26,27] whereas IDCM was a diagnosis of exclusion.[9] If the diagnosis was equivocal on the basis of standard histology, special stains were performed such as immunofluorescence for IgG, IgM, IgA, C1q, C3d, C4d, and fibrinogen and stains for acid fast bacilli, fungi, elastosis, glycogen, or iron accumulation.

One biopsy sample from each patient, obtained independently from the histological samples, was flash-frozen and stored in liquid nitrogen for microarray analysis. A total of 115 biopsy samples were included for microarray analysis in this study: 81 samples were newly processed and 34 samples from a previous study were included for validation.[23] Forty-eight samples were selected for our first transcriptomic study, including samples from patients with myocarditis (n=16)[26,27] and IDCM (n=32) selected in a case-control fashion. In addition, samples from 6 patients with myocarditis and divergent baseline criteria were used for independent validation of the transcriptome-based biomarkers (TBBs). Furthermore, we tested the ability of the biomarker to detect active myocardial inflammation in patients with secondary cardiomyopathies associated with myocarditis (n=27). This group included patients with stress-induced cardiomyopathy (Takotsubo) (n=4), sarcoidosis (n=9), peripartum cardiomyopathy (n=6), arrhythmogenic right ventricular dysplasia (n=3), giant-cell myocarditis (n=3), and systemic lupus erythematosus (n=2). Finally, we tested the transcriptomic biomarker for myocarditis in samples from a previous study,[23] which included samples from patients with normal hearts (n=11), ischemic cardiomyopathy (n=8), and IDCM (n=15), and analyzed them with a prototype microarray, the Affymetrix U133A Gene Chip. By using this approach, we evaluated the generalizability of the molecular signature to various heart conditions, tested its performance in hearts free of disease, and evaluated its intraplatform reproducibility.

Transcriptomic Analysis

Total RNA was extracted and hybridized as previously described.[24,25] Microarray data were normalized with robust multiarray average[28] and analyzed with significance analysis of microarrays[29] to identify differentially expressed genes in patients with myocarditis (n=16) compared with IDCM (n=32). The resulting gene list was further processed with MetaCore pathway analysis from GeneGo Inc (St. Joseph, MI). To determine the minimum number of differentially expressed genes required for the detection of patients with myocarditis, we used prediction analysis of microarrays.[20] The nearest shrunken centroid classifier was developed from a training set (n=33) consisting of two thirds of the data and applied to an independent test set (n=15) containing one third of the data.[20]

After developing the TBB with a case-control design, we tested its performance in unmatched samples (n=6) with higher ejection fractions (65±4.7%) to evaluate generalizability.

To test whether previously established classification algorithms can further reduce the number of genes necessary for accurate prediction, we applied misclassification-penalized posteriors classification (MiPP), a novel classification software package.[22] We subsequently applied the following classification rules implemented in the MiPP package: supervector machine (svm) with radial basis function (rbf), svm with lineal function as kernel (lin), quadratic discriminant analysis (qda), lineal discriminant analysis (lda), and a combination of lda, qda, and svm-rbf. Models were based on 5-fold cross-validation in a training set (two thirds of data) and subsequent validation in an independent test set (one third of data).

To evaluate whether distinct models are generated from additional random splits, we performed 50 random divisions to develop individual classification models, which were then validated in 200 independent splits. In addition, we performed principal components analysis (PCA) to illustrate how well patients with myocarditis can be separated from patients with IDCM on the basis of the original 62-gene molecular signature and to test whether genes that we identified by MiPP analysis to be the most robust classifiers would also be discovered to be important when PCA was applied. PCA depicts highly robust classifiers with vectors having their end points far from the center.

Table 1. Baseline Conditions of Patients With Idiopathic Dilated Cardiomyopathy and Lymphocytic Myocarditis

	Idiopathic Dilated Cardiomyopathy (n=32)	Myocarditis (n=16)
Age, y	48±3	45±6
Male, n (%)	11 (38)	11 (69)
NYHA class, n (%)		
I	9 (28)	4 (25)
II	10 (31)	3 (19)
III	13 (59)	8 (50)
IV	3 (9)	1 (6)
LVEF, %	26±2	33±4
LVIDD, cm	5±0.3	5±0.2
PAP, mm Hg		
Systolic	38±3	37±3
Diastolic	18±2	15±2
PCWP, mm Hg	15±2	12±2
Systolic BP, mm Hg	128±5	119±5
Diastolic BP, mm Hg	76±2	70±4
Medications, n (%)		
B-antagonist	20 (62)	9 (56)
ACE inhibitor	20 (62)	14 (88)
Aldosterone antagonist	4 (13)	1 (6)
Diuretic	14 (64)	13 (81)
Intravenous inotropic therapy	NA	NA

NYHA indicates New York Heart Association; LVEF, left ventricular ejection fraction; LVIDD, left ventricular internal dimension, diastole; PAP, pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; BP, blood pressure; and ACE, angiotensin-converting enzyme. Values are mean±SEM when appropriate.

Validation of Microarrays With Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

Validation with real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed in a randomly selected subset of patients (IDCM, n=10; myocarditis, n=10) with replication in triplicate. First-strand cDNA was synthesized from 100 ng total RNA and amplified with the MessageAmp II Amplification Kit. Importantly, this amplification step was performed only on validation samples, after the original biomarker was developed from pure total RNA that did not undergo any amplification, to eliminate any possibility of amplification bias that may affect the resulting molecular signature. TaqMan probes were designed for a subset of 13 candidate genes from microarray analysis: *CD14*, *FCER1G*, *TLR1*, *TLR2*, *TLR7*, *ITGB2*, *SIGLEC* 1, *ADCY7*, *MEGF9*, *PTPLAD1*, *SWAP70*, *MSI1*, and *LCE1E*, as well as the housekeeping gene *18S RNA*. Finally, the results from RT-PCR were illustrated as a heat map

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

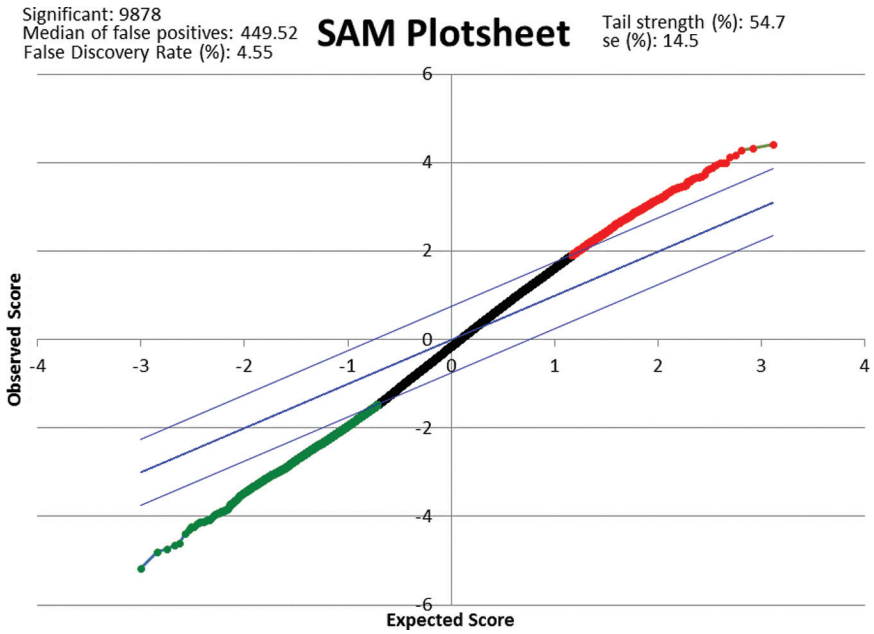

Figure 1. Significance analysis of microarrays (SAM) plot of differentially expressed genes in lymphocytic myocarditis vs idiopathic dilated cardiomyopathy (IDCM). There were 9878 genes differentially expressed in myocarditis (n=16) vs IDCM (n=32; q<5%; fold change >1.2), of which 2313 were overexpressed (red) and 7565 were downregulated (green).

created with unsupervised hierarchical clustering based on euclidean distance.

For detailed methods, see the online-only Data Supplement.

Results

Table 1 depicts baseline clinical variables of patients of the selected case-control population with IDCM and Dallas criteria[26,27]–defined lymphocytic myocarditis.

Phenotype-Specific Differences in Gene Expression

To identify differential gene expression between patients with IDCM (n=32) and those with lymphocytic myocarditis (n=16), we used oligonucleotide microarrays to analyze RNA obtained from EMBs from affected patients at first presentation with new-onset heart failure. We identified 9878 differentially expressed genes (q<5%; fold change [FC] >1.2) in patients with IDCM compared with myocarditis (Figure 1). Transcripts with FC >2 (141 overexpressed and 16 downregulated transcripts) are provided as Tables I and II in the online-only Data Supplement. Pathway analysis with GeneGo MetaCore revealed overexpression of 8 networks in myocarditis compared with IDCM (Table III in the online-only Data Supplement).

Molecular Signature to Distinguish Myocarditis From Noninflammatory Cardiomyopathy

We applied prediction analysis of microarrays in a training set containing two thirds of the data (IDCM, n=22; myocarditis, n=11) and evaluated its accuracy in an independent test set containing one-third of the data (IDCM, n=10; myocarditis, n=5). The developed transcriptomic diagnostic biomarker consisted of a minimal set of 62 transcripts (Table 2). When the molecular signature was tested in matched independent samples (n=15), it performed with 100% accuracy (sensitivity, 100%; 95% confidence interval [CI], 46 to 100; specificity, 100%; 95% CI, 66 to 100; positive predictive value, 100%; 95% CI, 46 to 100; negative predictive value, 100%; 95% CI, 66 to 100; Figure 2). All samples were predicted correctly regardless of degree of inflammation (borderline or active myocarditis).

We next tested the transcriptomic biomarker in an additional set of independent samples derived from patients with myocarditis (n=6), who presented with higher ejection fractions (65±4.7%) compared with the case-control samples. In this group, the molecular signature still identified 83% of patients with myocarditis correctly (sensitivity, 91%; 95% CI, 57 to 100; specificity, 100%; 95% CI, 66 to 100; positive predictive value, 100%; 95% CI, 66 to 100; negative predictive value, 91%; 95% CI, 57 to 100; data not shown).

Performance of Predictive Algorithm in Secondary Cardiomyopathy/Myocarditis

To evaluate generalizability in an additional relevant population, we applied the transcriptomic biomarker to biopsies from patients with secondary cardiomyopathies associated with myocarditis (stress induced cardiomyopathy, n=4; sarcoidosis, n=9; peripartum cardiomyopathy, n=6; arrhythmogenic right ventricular dysplasia, n=3; giant-cell myocarditis, n=3; and systemic lupus erythematosus, n=2). In this setting, the biomarker distinguished myocarditis with an accuracy similar to that of idiopathic myocarditis (sensitivity, 100%; 95% CI, 46 to 100; specificity, 95%; 95% CI, 75 to 100; positive predictive value, 83%; 95% CI, 36 to 99; negative predictive value, 100%; 95% CI, 80 to 100; Figure 3). Among this set of secondary cardiomyopathies, 5 biopsies were found to contain significant inflammatory changes based on immunohistochemistry, of which 1 was from a patient with stress-induced cardiomyopathy (sample STR2), 1 was from a patient with systemic lupus erythematosus (sample SLE1), and 3 were from patients with giant-cell myocarditis. Indeed, all samples were correctly identified as inflammatory cardiomyopathy, whereas in the remaining samples, the molecular signature successfully ruled out inflammatory disease with very high accuracy. Only 1 patient with sarcoidosis (sample SARC1) was misclassified.

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

Table 2. Transcriptomic Diagnostic Biomarker for Detection of Patients With Myocarditis: 62 Genes

Probe Set ID	Gene Symbol	Gene Title	Gene Ontology Biological Process Term
1552302_at	*FLJ77644, TMEM106A*	Similar to transmembrane protein 106A, transmembrane protein 106A	NA
1552310_at	*C15orf40*	Chromosome 15 open reading frame 40	NA
1553212_at	*KRT78*	Keratin 78	NA
1555349_a_at	*ITGB2*	Integrin, β-2 (complement component 3 receptor 3 and 4 subunit)	Apoptosis, inflammatory response, leukocyte adhesion
1555878_at	*RPS24*	Ribosomal protein S24	Translation
1556033_at	NA	NA	NA
1556507_at	NA	NA	NA
1558605_at	NA	NA	NA
1559224_at	*LCE1E*	Late cornified envelope 1E	Keratinization
1562785_at	*HERC6*	Hect domain and RLD 6	Protein modification process
1565662_at	NA	NA	Maintenance of gastrointestinal epithelium
1565830_at	NA	NA	NA
202375_at	*SEC24D*	SEC24-related gene family, member D (*S cerevisiae*)	Transport, intracellular protein transport
202445_s_at	*NOTCH2*	Notch homolog 2 (*Drosophila*)	Cell fate determination
203741_s_at	*ADCY7*	Adenylate cyclase 7	cAMP biosynthetic process, signal transduction
204222_s_at	*GLIPR1*	GLI pathogenesis-related 1	NA
206052_s_at	*SLBP*	Stem-loop binding protein	mRNA processing, histone mRNA 3′-end processing
206333_at	*MSI1*	Musashi homolog 1 (*Drosophila*)	Nervous system development
206770_s_at	*SLC35A3*	Solute carrier family 35 [UDP-*N*-acetylglucosamine (UDP-GlcNAc) transporter], member A3	UDP-*N*-acetylglucosamine metabolic process, transport
209307_at	*SWAP70*	SWAP-70 protein	Somatic cell DNA recombination, isotype switching
211089_s_at	*NEK3*	NIMA (never in mitosis gene a)-related kinase 3	Protein amino acid phosphorylation, mitosis
211341_at	*LOC100131317, POU4F1*	Similar to hCG1781072, POU class 4 homeobox 1	Transcription, regulation of transcription, DNA dependent, regulation of transcription from RNA polymerase II promoter
212511_at	*PICALM*	Phosphatidylinositol binding clathrin assembly protein	Protein complex assembly, endocytosis, receptor-mediated endocytosis
212830_at	*MEGF9*	Multiple EGF-like domains 9	NA
212999_x_at	*hCG_1998957, HLA-DQB1/2, HLA-DRB1/2/3/4/5*	Major histocompatibility complex, class II, DR [bet]a 1/2/3/4/5; similar to major histocompatibility complex, class II, DQ beta 1	Antigen processing and presentation of peptide or polysaccharide antigen via major histocompatibility complex class II
213501_at	*ACOX1*	Acyl-coenzyme A oxidase 1, palmitoyl	Generation of precursor metabolites and energy, lipid metabolic process
213831_at	*HLA-DQA1*	Major histocompatibility complex, class II, DQ α 1	Antigen processing and presentation of peptide or polysaccharide antigen via major histocompatibility complex class II
217054_at	NA	NA	NA
217182_at	*MUC5AC*	Mucin 5AC, oligomeric mucus/gel forming	Cell adhesion, digestion, fibril organization and biogenesis
217322_x_at	NA	NA	NA
217777_s_at	*PTPLAD1*	Protein tyrosine phosphatase-like A domain containing 1	I-κB kinase/NF-κB cascade
218803_at	*CHFR*	Checkpoint with forkhead and ring finger domains	Protein polyubiquitination, mitotic cell cycle, ubiquitin-dependent protein catabolic process
219425_at	*SULT4A1*	Sulfotransferase family 4A, member 1	Lipid metabolic process, steroid metabolic process
221663_x_at	*HRH3*	Histamine receptor H3	Signal transduction, G-protein–coupled receptor protein signaling pathway, neurotransmitter secretion
223077_at	*TMOD3*	Tropomodulin 3 (ubiquitous)	NA
224327_s_at	*DGAT2*	Diacylglycerol O-acyltransferase homolog 2 (mouse)	Glycerol metabolic process, lipid metabolic process, lipid biosynthetic process, triacylglycerol biosynthetic process
224996_at	NA	NA	NA
225579_at	*PQLC3*	PQ loop repeat containing 3	NA
226240_at	*MGC21874*	Transcriptional adaptor 2 (ADA2 homolog, yeast)-β	Transcription, regulation of transcription, DNA dependent

(*Continued*)

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

Table 2. Continued

Probe Set ID	Gene Symbol	Gene Title	Gene Ontology Biological Process Term
227280_s_at	*CCNYL1*	Cyclin Y-like 1	NA
227618_at	NA	NA	NA
227983_at	*RILPL2*	Rab interacting lysosomal protein-like 2	NA
228980_at	*RFFL*	Ring finger and FYVE-like domain containing 1	Intracellular protein transport, apoptosis
229191_at	*TBCD*	Tubulin folding cofactor D	Protein folding, β-tubulin folding
230836_at	*ST8SIA4*	ST8 α-*N*-acetyl-neuraminide α-2,8-sialyltransferase 4	Protein modification process, protein amino acid glycosylation, nervous system development
231599_x_at	*DPF1*	D4, zinc and double PHD fingers family 1	Transcription, regulation of transcription, DNA dependent, induction of apoptosis
234495_at	*KLK15*	Kallikrein-related peptidase 15	Proteolysis
234986_at	NA	NA	NA
234987_at	NA	NA	NA
236232_at	*STX4*	Syntaxin 4	Transport, neurotransmitter transport, intracellular protein transport
236404_at	NA	NA	NA
236698_at	NA	NA	NA
238327_at	*LOC440836*	Similar to MGC52679 protein	Cell growth
238445_x_at	*MGAT5B*	Mannosyl (α-1,6-)-glycoprotein β-1,6-*N*-acetyl-glucosaminyltransferase, isozyme B	NA
239463_at	NA	NA	NA
242383_at	NA	NA	NA
242563_at	NA	NA	NA
243819_at	NA	NA	NA
244841_at	*SEC24A*	SEC24-related gene family, member A (*S cerevisiae*)	Transport, intracellular protein transport, ER to Golgi vesicle-mediated transport
32069_at	*N4BP1*	NEDD4 binding protein 1	NA
44673_at	*SIGLEC1*	Sialic acid binding Ig-like lectin 1, sialoadhesin	Inflammatory response, cell adhesion
53720_at	*C19orf66*	Chromosome 19 open reading frame 66	NA

NA indicates not applicable.

In addition, we evaluated the biomarker performance in patients from a previous data set (n=34)[23] containing samples with ischemic cardiomyopathy (n=8), IDCM (n=15), and normal heart (n=11). All samples were correctly classified.

Additional Novel Classification Strategies

To obtain a parsimonious molecular signature, we first applied multiple established classification algorithms using the MiPP package in R that includes lda, qda, svm-rbf, and svm-lin. When applied to the 62-gene signature, these algorithms identified a highly diagnostic set of 3 transcripts (mean error, 0.167 in independent validation sets; n=18). Table 3 contains the mean error for each established set of genes developed by individual rules or combinations of rules.

We continued our analysis by testing whether a different random split of data would reveal distinct models. Splitting of data into a training (two thirds) and test (one third) set and selecting a model for a given split were repeated 50 times.

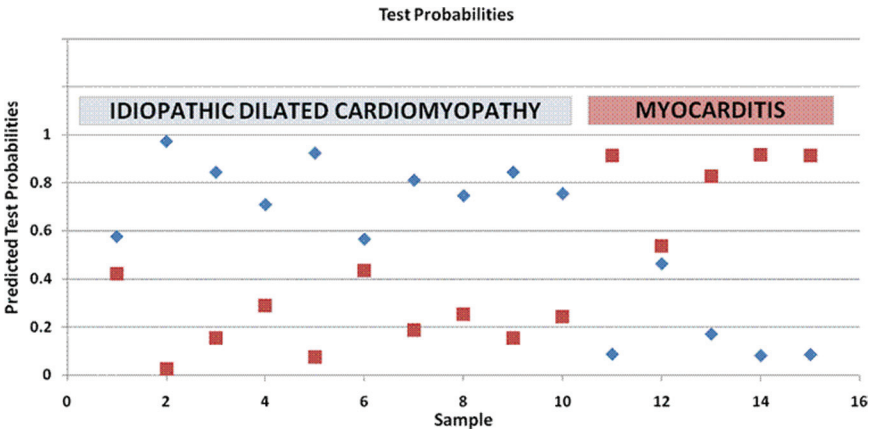

Figure 2. Validation of a 62-gene molecular signature in an independent test set (idiopathic dilated cardiomyopathy, n=10; myocarditis, n=5) using prediction analysis of microarrays (PAM). Samples identified as noninflammatory cardiomyopathy are illustrated in blue; samples identified as myocarditis are illustrated in red. The *y* ordinate illustrates the predicted test probability values obtained from PAM analysis; the *x* ordinate lists the number of samples. Although samples were assigned to different classes with varying probability values, the classification accuracy of the transcriptomic biomarker was 100%.

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

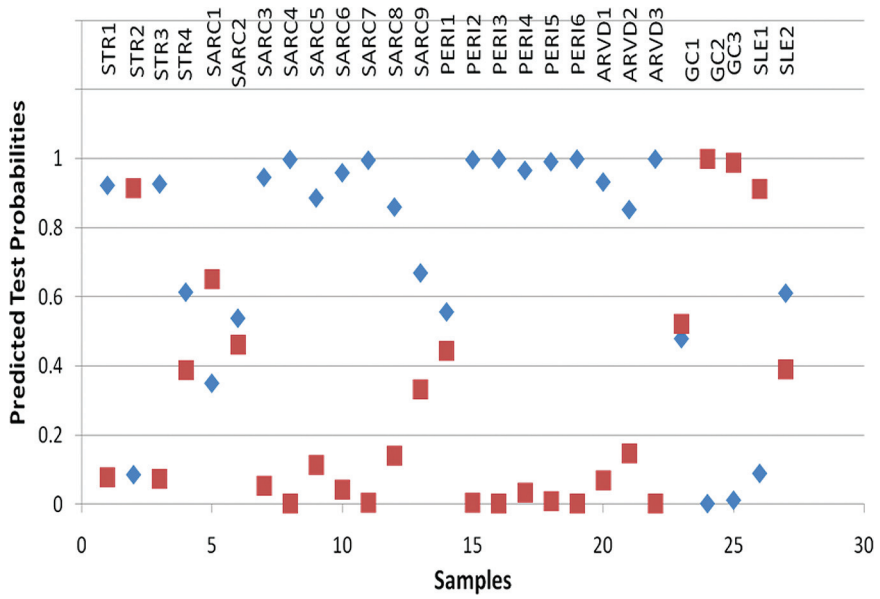

Figure 3. Prediction analysis of microarrays applying the developed molecular signature for inflammatory cardiomyopathy in patients with secondary cardiomyopathy (n=27). Samples identified as noninflammatory cardiomyopathy are depicted in blue, while samples identified as inflammatory cardiomyopathy are depicted in red. The transcriptomic biomarker performed with 100% sensitivity and 95% specificity in identifying inflammation in patients with stress-induced cardiomyopathy (STR; n=4), sarcoidosis (SARC; n=9), peripartum cardiomyopathy (PERI; n=6), arrhythmogenic right ventricular dysplasia (ARVD; n=3), giant-cell myocarditis (GC; n=3), and systemic lupus erythematosus (SLE; n=2). One patient with STR (sample STR2) and another with SLE (sample SLE1) were identified as inflammatory cardiomyopathy. Indeed, when results from immunohistochemistry were revised, those 2 samples contained significant lymphocytic infiltrates. One sample from the group with sarcoidosis (sample SARC1) was misclassified as inflammatory cardiomyopathy, but the report from histopathology revealed no signs of inflammation. All samples from patients with giant cell myocarditis were correctly identified.

KRT78, *MSI1*, *POU4F1*, and *LCE1* and the transcript 1556507_at resulted as top classifiers (mean error, 0.086 after validation in 200 independent splits; Table 4). As an additional measure for performance of a given gene model, we evaluated mean standardized MiPP, a parameter that approximates 1 with increasing accuracy. When the top 5 gene models (Table 4) were validated in 200 independent random splits, mean standardized MiPP ranged from 0.776 to 0.791 (Table 4). Because those models were built from 50 initial random splits, it is likely that identical gene clusters are identified in subsequent splits, as occurred in our analysis (Table 4; splits 17 and 45). PCA is a valuable tool to illustrate the importance of individual genes for classification of their corresponding phenotype. In agreement with results from our MiPP analysis, the transcripts 1556507_at, *KRT78*, *LCE1E*, *MSI1*, and *POU4F1* were identified as highly important, with vectors having their end points distant from the center (Figure 4A). Additional highly robust transcripts were *ITGB2*, *HERC6*, *ADCY7*, *NEK3*, and *MEGF9*, as well as the ESTs 1558605_at and 1565662_at (data not shown).

In addition, PCA clustered patients with similar expression patterns as 1 principal component. As shown in Figure 4B, samples from patients with myocarditis noticeably separated from samples from patients with IDCM.

Table 3. Most Predictive Gene Signatures Identified by Misclassification-Penalized Posteriors Classification in a Data Set of Patients With Myocarditis (n=16) Versus Idiopathic Dilated Cardiomyopathy in Training (n=32)

Gene Signatures	Selection Method	Prediction Rule	Class Comparison	Mean ER in Training Set	Mean ER in Validation Set
MSI1, 1556507_at	MiPP	svm-rbf	2	0	0.167
KRT78	MiPP	svm-lin	2	0.033	0.167
KRT78, 1556507_at	MiPP	qda	2	0	0.167
KRT78, 1556507_at	MiPP	lda	2	0	0.167
1556507_at	MiPP	lda, qda, svm-rbf	2	0	0.167

ER indicates error; MiPP, misclassification-penalized posteriors classification; svm, supervector machine; rbf, radial basis function; lin, lineal function as kernel; qda, quadratic discriminant analysis; and lda, lineal discriminant analysis. Validation was performed in independent test sets (n=18).

Validation With Quantitative Real-Time RT-PCR

To obtain technical validation of the results from microarray analysis, we performed real-time RT-PCR on a subset of 13 genes (Table 5). Genes were selected from the resulting gene lists of our bioinformatic approach on the basis of biological plausibility and robustness as classifiers for lymphocytic myocarditis. Biological plausibility was defined according to pathway analysis, which identified those genes as being significantly involved in inflammation and remodeling.

The FC of most genes measured by quantitative real-time RT-PCR strongly correlated with data obtained from microarray analysis, except for *MSI1*, for which real-time RT-PCR data revealed much stronger downregulation in patients with myocarditis versus lymphocytic cardiomyopathy compared with microarray data. The genes with the highest FC accord-

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

Table 4. Models Obtained From 50 Random Splits Into Training and Test Sets

Split	Gene1	Gene2	Gene3	Gene4	Gene5	Gene6	Mean ER	Mean sMiPP	5% ER	50% ER	95% ER
17	*KRT78*	*1556507_at*	NA	NA	NA	NA	0.078	0.789	0.188	0.063	0
45	*KRT78*	*1556507_at*	NA	NA	NA	NA	0.078	0.789	0.188	0.063	0
44	*MSI1*	*POU4F1*	*1556507_at*	NA	NA	NA	0.09	0.776	0.188	0.063	0
43	*MSI1*	*POU4F1*	*1556507_at*	*LCE1E*	NA	NA	0.091	0.789	0.188	0.063	0
41	*LCE1E*	*POU4F1*	*MSI1*	NA	NA	NA	0.092	0.791	0.188	0.063	0

Genes obtained from 50 random splits were further validated in 200 independent random splits. Results from the top 5 gene clusters with the lowest mean error (ER) are given. Mean sMipp is an additional parameter for performance and converges toward 1 as accuracy of the model increases. sMiPP indicates standardized MiPP.

ing to RT-PCR were *CD14* (FC=6.8), *FCER1G* (FC=5), *TLR1* (FC=4.2), *TLR2* (FC=5.9), *SIGLEC1* (FC=4.3), and *ADCY7* (FC=4.2; Table 5). However, among the 5 candidate genes from MiPP analysis, *KRT78* and *POU4F1* could not be confirmed with real-time RT-PCR. Because *KRT78* appeared highly robust as a classifier based on microarray results, we used 2 different primer pairs to detect either the 3′ or the 5′ end of the gene sequence. However, neither of them was able to detect KRT78 in any of the samples. When we used total RNA from immortalized keratinocytes as positive control, we received a signal from each primer pair. To exclude the possibility of cross-hybridization that may have occurred on the microarray assay, we performed a batch search in the National Center for Biotechnology Infronation database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the target sequence that was used on the Affymetrix chip. However, there was no significant sequence homology with any gene other than *KRT78*. Despite this minimal incoherence between microarray analysis and the more specific real-time RT-PCR, we minimized the diagnostic biomarker to a very small set of 13 genes that performed highly robustly with both methods (100% sensitivity, 100% specificity; Figure 5). Finally, we confirmed overrepresentation of HLA-DQ1+ patients in myocarditis (60%), whereas only 20% of patients with IDCM were positive for DQ1 (data not shown) by real-time RT-PCR.

When applied to a subset of myocarditis patients with higher ejection fractions, the 13-gene signature performed with a sensitivity of 75% (95% CI, 36 to 96), specificity of 100% (95% CI, 52 to 100), positive predictive value of 100% (95% CI, 52 to 100), and negative predictive value of 75% (95% CI, 36 to 96).

Discussion

Distinction of inflammatory compared with noninflammatory cardiomyopathies by standard histology represents a major diagnostic challenge.[9,27,30] Moreover, delineating between different inflammatory cardiomyopathies with highly variable clinical courses is an even more challenging task.[3,31] Given the emerging value of transcriptomics to add greatly to the accuracy of complex diagnoses,[23,32,33] we sought to apply this technology to the problem of diagnostic inaccuracy in myocarditis. Here, we report our success with this approach.

Inflammatory disorders of the heart are notoriously difficult to diagnose because of the patchy nature of the inflammation.[11] In addition, a wide variety of underlying inflammatory conditions with highly variable clinical outcomes can affect the heart.[2] Here, we used the transcriptome obtained from a single EMB to develop a biomarker that enhances diagnostic accuracy for lymphocytic myocarditis. Our findings are in agreement with previous transcriptomic approaches in heart disease.[23–25,33,34] Specifically, Ruppert et al[35] reported a set of 42 genes that are different between inflammatory versus noninflammatory cardiomyopathy. Their findings suggested that the transcriptomes of various subtypes of cardiomyopathy differ significantly from each other and that these differences may be used as a diagnostic biomarker, as shown successfully here. Consistent with the data from Ruppert and colleagues,[35] we found significant activation of the Toll-like receptor signaling pathway in inflammatory cardiomyopathy. In particular, genes such as *TLR1*, *TLR2*, *TL7*, and *CD14* were overexpressed in patients with myocarditis compared with IDCM.[36] Furthermore, in agreement with their findings, we found more overexpressed than downregulated genes in inflammatory compared with noninflammatory cardiomyopathy. Entirely novel in our study was the identification of the smallest set of genes required to identify inflammatory cardiomyopathy from a single EMB and validation of the developed molecular signature in multiple independent sets of samples consisting of various types of cardiomyopathy and normal heart.

We have previously used TBBs to distinguish between idiopathic and ischemic cardiomyopathy[23] and to predict long-term prognosis in new-onset dilated cardiomyopathy.[24] Margulies and colleagues[37] discovered a biomarker that predicts recovery from heart failure, and Deng and coworkers[34] developed a molecular signature that detects early cardiac transplant rejection that has now entered the clinic.[21] Our discoveries reported here are clinically relevant because high diagnostic sensitivity in cardiomyopathy facilitates the appropriate use of new myocarditis-specific therapies.[2,3,12–15,38–42] Early and accurate diagnosis of this condition is essential to avoid excessive myocardial damage resulting from failure to apply therapies. New candidate therapies for myocarditis include antiinflammatory cytokines,[42] antiviral agents, and immunoabsorption.[2,3,12–15,38–42] In this regard, interferon B therapy has been safely applied in humans, leading to increased left ventricular function and elimination of viral infection.[13] Immunoglobulin administration[41] in acute myocarditis and application of calcium channel blockers[42] are potential approaches with promising preliminary data that require further evaluation. Although immunosuppressive therapy in inflammatory cardiomyopathy is highly controversial,[12,14,15,40,43]

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

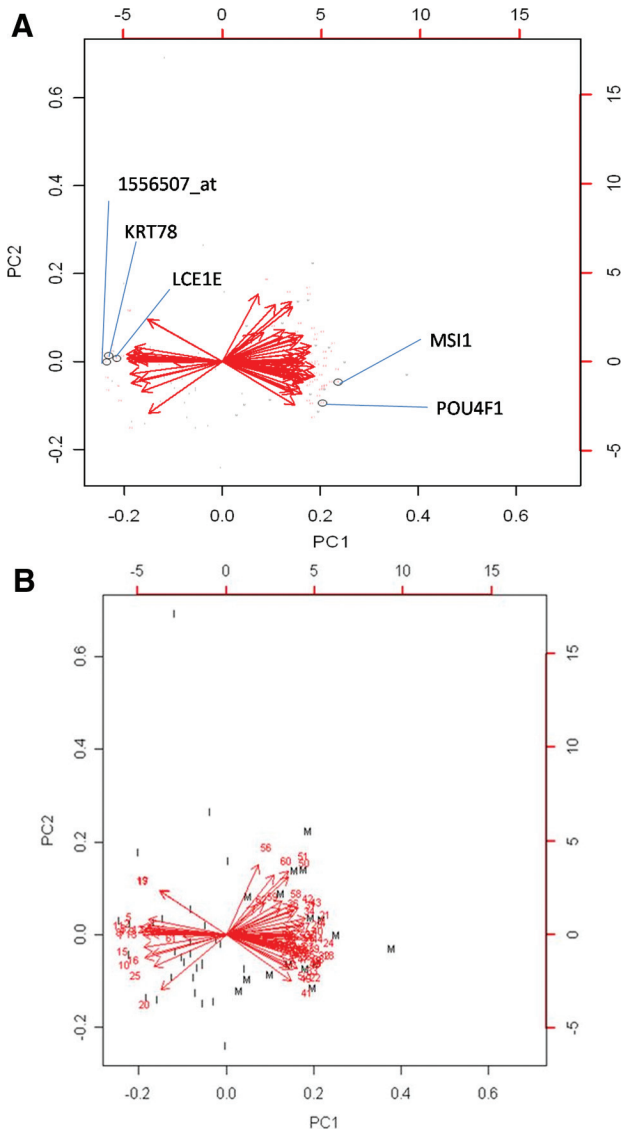

Figure 4. Principal components analysis (PCA) of patients with myocarditis vs idiopathic dilated cardiomyopathy (IDCM). To illustrate the significance of each of the 62 genes for phenotypic categorization, we performed PCA with correlation matrix in samples from patients with myocarditis (n=16) or IDCM (n=32) with genes as variables. Genes are labeled with serial numbers, and expression levels of each individual gene are illustrated as eigenvector toward the class in which they are overexpressed. Vectors close to the center with close to vertical direction depict genes that were less robust; genes that were highly specific for a phenotype are illustrated as vectors with an end point distant from the center directing toward the corresponding clustered set of samples of a specific phenotype. A, Encircled genes were repeatedly identified to be the most robust markers of myocarditis when various algorithms of misclassified-penalized posterior classification were applied. Output from PCA places those genes both far from the center and distant from the vertical line, confirming that these are highly robust classifiers for myocarditis. B, Clustered samples from patients with myocarditis are labeled M; IDCM samples are labeled I. All samples from myocarditis except 2 were noticeably grouped together, suggesting that a small set of 62 genes enable clear distinction between patients with inflammatory heart disease and IDCM. Importantly, those 2 samples were also misclassified in our heat map analysis, whereas prediction analysis of microarrays identified both of them correctly.

Table 5. Real-Time Reverse-Transcription Polymerase Chain Reaction Data of Patients With Lymphocytic Myocarditis (n=10) Versus Idiopathic Dilated Cardiomyopathy (n=10)

Probe Set	Gene Symbol	Fold Change by SAM	Fold Change by qPCR	$P<0.05$ by SAM	$P<0.05$ by qPCR
201721_s_at	*CD14*	5.9	6.8	Y	Y
1554899_s_at	*FCER1G*	5.3	5	Y	Y
210146_x_at	*TLR1*	4.5	4.2	Y	Y
204923_at	*TLR2*	3.9	5.9	Y	Y
1555349_a_at	*ITGB2*	3.1	1.95	Y	Y
44673_at	*SIGLEC1*	2.3	4.3	Y	Y
219938_s_at	*TLR7*	2.3	2.8	Y	Y
203741_s_at	*ADCY7*	2	4.2	Y	Y
212830_at	*MEGF9*	1.5	2.3	Y	Y
217777_s_at	*PTPLAD1*	1.5	1.7	Y	Y
209307_at	*SWAP70*	1.4	2.1	Y	Y
206333_at	*MSI1*	−1.8	−8.4	Y	Y
1559224_at	*LCE1E*	−2.3	−2.6	Y	Y

SAM indicates significance analysis of microarrays; qPCR, quantitative polymerase chain reaction.

there is growing consensus that early identification and treatment of myocarditis are crucial for positive outcome.

Our diagnostic biomarker also performed accurately in patients with secondary cardiomyopathies associated with inflammation. For example, patients with systemic lupus erythematosus, sarcoidosis, or peripartum cardiomyopathy

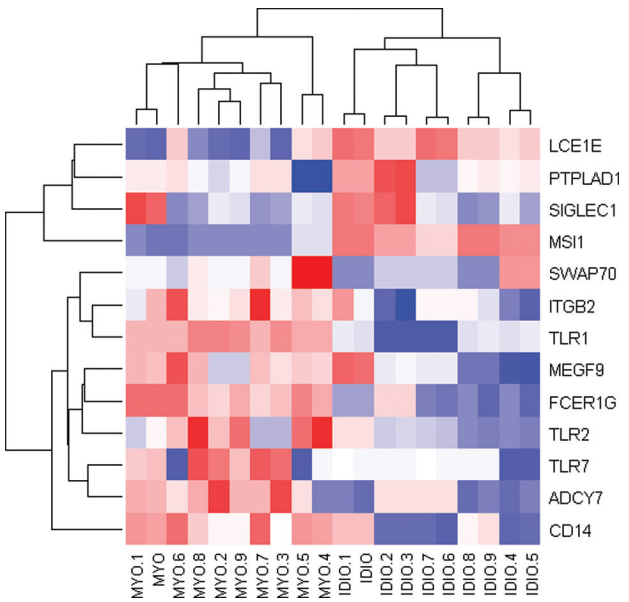

Figure 5. Distinction of patients with idiopathic dilated cardiomyopathy vs lymphocytic myocarditis based on results from quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). This heat map was created with an unsupervised clustering approach based on euclidean distance in R using the detected gene expression levels from quantitative real-time RT-PCR as a confirmatory test. Columns represent samples; rows represent genes labeled with their corresponding gene symbol. Application of the developed 13-gene molecular signature through real-time RT-PCR correctly identified all samples.

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

have significant incidences of myocarditis, which has clinical importance in these conditions. The TBB had a similar degree of accuracy in this population. In patients with giant-cell myocarditis, a very aggressive form of myocarditis, the TBB accurately detected 3 of 3 patients.

Accurate diagnosis is also critical for prognostic assessment because clinical outcome in inflammatory cardiomyopathies correlates with disease origin.[9,10] On the basis of previous findings from others[20,22,34] and our group,[23,24] we argue that TBBs add valuable information to a comprehensive diagnostic evaluation of new-onset heart failure. TBBs obtained from peripheral blood or tissue samples have emerged as highly successful in neoplastic,[20] cardiovascular,[23,24,34,44] and other disease processes.[22]

To achieve an accurate biomarker, we used a broad range of bioinformatic approaches.[20,22–25,29,34,37,44–46] These included significance analysis of microarrays, prediction analysis of microarrays, MiPP, unsupervised hierarchical clustering, and PCA. Using significance analysis of microarrays, we discovered a large number of differentially expressed genes in lymphocytic myocarditis compared with IDCM. Importantly, and predictably, differentially expressed genes involved multiple biological networks with inflammatory components. Using these differentially expressed genes, we identified a subset that functioned as highly accurate biomarker using nearest shrunken centroids.

To find the smallest set of genes for classification, we used svm-rbf, svm-lin, qda, lda, and a combination of lda, qda, and svm-rbf in MiPP. Overall, all rules applied in MiPP consistently revealed 5 classifiers, which were further confirmed with PCA. Interestingly, 2 of those 5 robust predictive genes were not found to be present when quantitative real-time RT-PCR was used for validation. Finally, we developed a highly parsimonious biomarker using *MSI1* and *LSI1* in combination with a subset of biologically relevant genes selected from the prediction analysis of microarrays–derived 62-gene TBB and from significance analysis of microarrays analysis and evaluated this signature using real-time RT-PCR; the 13-gene signature performed with perfect accuracy in the independent test set of our case-control study. The observation that mean FCs obtained from real-time RT-PCR were not entirely identical to the results from significance analysis of microarrays analysis underlines the strength of molecular signature analysis for the development of biomarkers, a classification strategy that emphasizes differentially expressed gene expression patterns rather than individual genes. Because the expression level of an individual gene may vary across a population that shares the same phenotype, the overexpression or downregulation of an entire cluster of genes is more specific for a disease.

From these findings, we conclude that both the transcriptomic biomarker derived from prediction analysis of microarrays analysis and the parsimonious molecular signature that resulted from multiple classification algorithms and testing for biological plausibility performed highly accurately and should be clinically valuable tools for the detection of myocarditis. Although the more comprehensive biomarker of 62 genes performed with slightly higher accuracy, the 13-gene molecular signature is more practical for clinical application.

Because our original data set in which we developed the TBB was matched in a case-control fashion, we further evaluated whether the molecular signature is generalizable or is possibly overfit to this particular study design.[33,47] It has been shown that confounding factors such as gender, age, and therapy can affect gene expression.[25,33,47–49] When the TBB was applied in an additional validation set containing samples from patients with an average ejection fraction that was twice as high as the average ejection fraction of the original data set (65% versus 30%), the biomarker performed with almost perfect accuracy. Furthermore, the transcriptomic biomarker was broadly applicable to various cardiomyopathies and normal heart and performed highly accurately in data derived with a prototype microarray, confirming intraplatform reproducibility.

Both molecular signatures require testing in a clinical trial to evaluate the diagnostic value of those biomarkers compared with a combination of current diagnostic tools such as magnetic resonance imaging, ECG, cardiac enzymes, viral screening, and auto-heart antibodies. Most likely, its addition to current diagnostic standards will dramatically increase sensitivity for myocarditis. The ability to detect inflammatory components such as involvement of the complement cascade toll-like receptor pathway or genes involved in cell adhesion such as *ITGB2* by microarray analysis may explain why this technology is able to identify myocarditis with much greater sensitivity at an earlier stage than standard histology, a method that requires the presence of inflammatory cells.

Although the main goal of this study was to develop a highly accurate biomarker to distinguish lymphocytic myocarditis from IDCM, our results also provide insight into disease pathophysiology at the molecular level. Among overexpressed genes in myocarditis was *CD8*, involved in inflammation and binding and reported to play a fundamental role in myocarditis.[30] Interestingly, a pathway involving the thyroid-stimulating hormone receptor was overexpressed in patients with myocarditis, implicating potential pathophysiological overlap with inflammatory thyroid disease, a finding clinically established for giant cell myocarditis (Graves disease).[50] There was overrepresentation of patients positive for the HLA-DQ1B locus in myocarditis compared with IDCM, suggesting possible susceptibility for lymphocytic myocarditis in this group.

Many transcripts involving structural proteins and muscle development (late cornified envelope 1E, collagen type I) were downregulated in myocarditis, possibly explaining structural defects and consequent dilatation in patients with this type of disease.

Study Limitations

Although the collection of samples and clinical data over a 10-year period is a major strength of this study, a consequent limitation is the diagnosis of our patients according to the Dallas criteria,[26,27] which were standard when the study was initiated but have been suggested to have limited sensitivity. In the meantime, several investigators suggested screening for serum anti-heart antibodies[16] and viral RNA[31] in EMBs. This technical drawback notwithstanding, all patients received comprehensive testing in a highly specialized institution. We anticipate that in the future, the transcriptomic

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

approach coupled with determination of viral persistence and/or use of highly specific imaging techniques might enhance diagnostic accuracy and be used for further diagnostic refinement to distinguish between viral and nonviral causes of myocarditis. Ongoing work is underway to evaluate whether the presented transcriptomic biomarker will also be able to detect samples from patients with myocarditis, in whom comprehensive diagnostic testing was required to detect disease but diagnosis of myocarditis would have been missed by Dallas criteria.

Another limitation of this study that warrants mention is that the number of samples with secondary cardiomyopathy was small owing to the known low incidence of these types of myocardial diseases. Consequently, negative and positive predictive values were estimated on the basis of small sample size.

In short, we discovered a TBB derived from a single EMB that identified samples with lymphocytic myocarditis with very high accuracy. Our findings are highly relevant for clinical application because this novel diagnostic tool exceeds the sensitivity and specificity of any previously applied technology. The molecular signature was highly robust and replicated multiple times by a broad set of established classification algorithms. Validation in 3 independent data sets revealed high diagnostic accuracy, and genes within the transcriptomic biomarker suggest biological plausibility. Altogether, using this approach dramatically increases the diagnostic accuracy of a single EMB, which may be of critical importance to the development and allocation of emerging specific therapies for inflammatory conditions of the heart.

Acknowledgments

We acknowledge the efforts of Gina Edness, RN, Elayne Breton, RN, and the staff of the Johns Hopkins Hospital cardiac catheterization laboratory for support in the collection of patient samples. We thank Francisco Martinez Murillo, PhD, Linda Dorsch, BS, and Ira Maine, PhD, from the Johns Hopkins Microarray Core Facility for consultation and their assistance with sample processing. We dedicate this work to Kenneth Lee Baughman, MD.

Sources of Funding

Dr Hare is supported by National Institutes of Health grant U54-HL081028 (Specialized Center for Cell Based Therapy) and R01s HL084275, AG025017, HL065455, and HL094849, and Dr Heidecker is supported by a fellowship grant from the Myocarditis Foundation.

Disclosures

None.

References

1. Lieberman EB, Hutchins GM, Herskowitz A, Rose NR, Baughman KL. Clinicopathologic description of myocarditis. *J Am Coll Cardiol*. 1991; 18:1617–1626.
2. Cooper LT Jr. Myocarditis. *N Engl J Med*. 2009;360:1526–1538.
3. Kuhl U, Pauschinger M, Seeberg B, Lassner D, Noutsias M, Poller W, Schultheiss HP. Viral persistence in the myocardium is associated with progressive cardiac dysfunction. *Circulation*. 2005;112:1965–1970.
4. Maekawa Y, Ouzounian M, Opavsky MA, Liu PP. Connecting the missing link between dilated cardiomyopathy and viral myocarditis: virus, cytoskeleton, and innate immunity. *Circulation*. 2007;115:5–8.
5. Caforio AL, Keeling PJ, Zachara E, Mestroni L, Camerini F, Mann JM, Bottazzo GF, McKenna WJ. Evidence from family studies for autoimmunity in dilated cardiomyopathy. *Lancet*. 1994;344:773–777.
6. Hannenhalli S, Putt ME, Gilmore JM, Wang J, Parmacek MS, Epstein JA, Morrisey EE, Margulies KB, Cappola TP. Transcriptional genomics associates FOX transcription factors with human heart failure. *Circulation*. 2006;114:1269–1276.
7. Pulerwitz TC, Cappola TP, Felker GM, Hare JM, Baughman KL, Kasper EK. Mortality in primary and secondary myocarditis. *Am Heart J*. 2004; 147:746–750.
8. Cooper LT, Baughman KL, Feldman AM, Frustaci A, Jessup M, Kuhl U, Levine GN, Narula J, Starling RC, Towbin J, Virmani R. The role of endomyocardial biopsy in the management of cardiovascular disease: a scientific statement from the American Heart Association, the American College of Cardiology, and the European Society of Cardiology. *Circulation*. 2007;116:2216–2233.
9. Felker GM, Thompson RE, Hare JM, Hruban RH, Clemetson DE, Howard DL, Baughman KL, Kasper EK. Underlying causes and long-term survival in patients with initially unexplained cardiomyopathy. *N Engl J Med*. 2000;342:1077–1084.
10. McCarthy RE III, Boehmer JP, Hruban RH, Hutchins GM, Kasper EK, Hare JM, Baughman KL. Long-term outcome of fulminant myocarditis as compared with acute (nonfulminant) myocarditis. *N Engl J Med*. 2000; 342:690–695.
11. Hrobon P, Kuntz KM, Hare JM. Should endomyocardial biopsy be performed for detection of myocarditis? A decision analytic approach. *J Heart Lung Transplant*. 1998;17:479–486.
12. Mason JW, O'Connell JB, Herskowitz A, Rose NR, McManus BM, Billingham ME, Moon TE. A clinical trial of immunosuppressive therapy for myocarditis: the Myocarditis Treatment Trial Investigators. *N Engl J Med*. 1995;333:269–275.
13. Kuhl U, Pauschinger M, Schwimmbeck PL, Seeberg B, Lober C, Noutsias M, Poller W, Schultheiss HP. Interferon-beta treatment eliminates cardiotropic viruses and improves left ventricular function in patients with myocardial persistence of viral genomes and left ventricular dysfunction. *Circulation*. 2003;107:2793–2798.
14. Mann DL. Targeted anticytokine therapy and the failing heart. *Am J Cardiol* 2005;95:9C–16C.
15. Frustaci A, Russo MA, Chimenti C. Randomized study on the efficacy of immunosuppressive therapy in patients with virus-negative inflammatory cardiomyopathy: the TIMIC study. *Eur Heart J*. 2009;30:1995–2002.
16. Caforio AL, Calabrese F, Angelini A, Tona F, Vinci A, Bottaro S, Ramondo A, Carturan E, Iliceto S, Thiene G, Daliento L. A prospective study of biopsy-proven myocarditis: prognostic relevance of clinical and aetiopathogenetic features at diagnosis. *Eur Heart J*. 2007;28: 1326–1333.
17. Pauschinger M, Doerner A, Kuehl U, Schwimmbeck PL, Poller W, Kandolf R, Schultheiss HP. Enteroviral RNA replication in the myocardium of patients with left ventricular dysfunction and clinically suspected myocarditis. *Circulation*. 1999;99:889–895.
18. Laissy JP, Hyafil F, Feldman LJ, Juliard JM, Schouman-Claeys E, Steg PG, Faraggi M. Differentiating acute myocardial infarction from myocarditis: diagnostic value of early- and delayed-perfusion cardiac MR imaging. *Radiology*. 2005;237:75–82.
19. Shonk JR, Vogel-Claussen J, Halushka MK, Lima JA, Bluemke DA. Giant cell myocarditis depicted by cardiac magnetic resonance imaging. *J Comput Assist Tomogr*. 2005;29:742–744.
20. Tibshirani R, Hastie T, Narasimhan B, Chu G. Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci U S A*. 2002;99:6567–6572.
21. Pham MX, Teuteberg JJ, Kfoury AG, Starling RC, Deng MC, Cappola TP, Kao A, Anderson AS, Cotts WG, Ewald GA, Baran DA, Bogaev RC, Elashoff B, Baron H, Yee J, Valantine HA. Gene-expression profiling for rejection surveillance after cardiac transplantation. *N Engl J Med*. 2010; 362:1890–1900.
22. Martinez-Llordella M, Lozano JJ, Puig-Pey I, Orlando G, Tisone G, Lerut J, Benitez C, Pons JA, Parrilla P, Ramirez P, Brugera M, Rimola A, Sanchez-Fueyo A. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. *J Clin Invest*. 2008;118:2845–2857.
23. Kittleson MM, Ye SQ, Irizarry RA, Minhas KM, Edness G, Conte JV, Parmigiani G, Miller LW, Chen Y, Hall JL, Garcia JG, Hare JM. Identification of a gene expression profile that differentiates between ischemic and nonischemic cardiomyopathy. *Circulation*. 2004;110:3444–3451.
24. Heidecker B, Kasper EK, Wittstein IS, Champion HC, Breton E, Russell SD, Kittleson MM, Baughman KL, Hare JM. Transcriptomic biomarkers for individual risk assessment in new-onset heart failure. *Circulation*. 2008;118:238–246.

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012

25. Heidecker B, Lamirault G, Kasper EK, Wittstein IS, Champion HC, Breton E, Russell SD, Hall J, Kittleson MM, Baughman KL, Hare JM. The gene expression profile of patients with new-onset heart failure reveals important gender-specific differences. *Eur Heart J*. 2009;31:1188–1196.
26. Aretz HT. Myocarditis: the Dallas criteria. *Hum Pathol*. 1987;18:619–624.
27. Baughman KL. Diagnosis of myocarditis: death of Dallas criteria. *Circulation*. 2006;113:593–595.
28. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003;4:249–264.
29. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A*. 2001;98:5116–5121.
30. Hare JM. The dilated, restrictive and infiltrative cardiomyopathies. In: Zipes DP, Libby P, Bonow R, Braunwald E, eds. *Braunwald's Heart Disease*. 8th Edition. Philadelphia, PA: Saunders Elsevier; 2007:1739–1762.
31. Kuhl U, Pauschinger M, Noutsias M, Seeberg B, Bock T, Lassner D, Poller W, Kandolf R, Schultheiss HP. High prevalence of viral genomes and multiple viral infections in the myocardium of adults with "idiopathic" left ventricular dysfunction. *Circulation*. 2005;111:887–893.
32. Cooper LT Jr, Onuma OK, Sagar S, Oberg AL, Mahoney DW, Asmann YW, Liu P. Genomic and proteomic analysis of myocarditis and dilated cardiomyopathy. *Heart Fail Clin*. 2010;6:75–85.
33. Kittleson MM, Irizarry RA, Heidecker B, Hare JM. Transcriptomics: translation of global expression analysis to genomic medicine. *Handbook of Genomic Medicine.* New York, NY: Elsevier; 2008:1–11.
34. Deng MC, Eisen HJ, Mehra MR, Billingham M, Marboe CC, Berry G, Kobashigawa J, Johnson FL, Starling RC, Murali S, Pauly DF, Baron H, Wohlgemuth JG, Woodward RN, Klingler TM, Walther D, Lal PG, Rosenberg S, Hunt S. Noninvasive discrimination of rejection in cardiac allograft recipients using gene expression profiling. *Am J Transplant*. 2006;6:150–160.
35. Ruppert V, Meyer T, Pankuweit S, Moeller E, Funck RC, Grimm W, Maisch B. Gene expression profiling from endomyocardial biopsy tissue allows distinction between subentities of dilated cardiomyopathy. *J Thorac Cardiovasc Surg*. 2008;136:360–369.
36. Fallach R, Shainberg A, Avlas O, Fainblut M, Chepurko Y, Porat E, Hochhauser E. Cardiomyocyte Toll-like receptor 4 is involved in heart dysfunction following septic shock or myocardial ischemia. *J Mol Cell Cardiol*. 2010;48:1236–1244.
37. Margulies KB, Matiwala S, Cornejo C, Olsen H, Craven WA, Bednarik D. Mixed messages: transcription patterns in failing and recovering human myocardium. *Circ Res*. 2005;96:592–599.
38. Cooper LT Jr, Berry GJ, Shabetai R. Idiopathic giant-cell myocarditis: natural history and treatment: Multicenter Giant Cell Myocarditis Study Group Investigators. *N Engl J Med*. 1997;336:1860–1866.
39. Cooper LT Jr, Shabetai R. Immunosuppressive therapy for myocarditis. *N Engl J Med*. 1995;333:1713–1714.
40. Maisch B, Hufnagel G, Kolsch S, Funck R, Richter A, Rupp H, Herzum M, Pankuweit S. Treatment of inflammatory dilated cardiomyopathy and (peri)myocarditis with immunosuppression and i.v. immunoglobulins. *Herz*. 2004;29:624–636.
41. Robinson J, Hartling L, Vandermeer B, Crumley E, Klassen TP. Intravenous immunoglobulin for presumed viral myocarditis in children and adults. *Cochrane Database Syst Rev* 2005:CD004370.
42. Yuan Z, Kishimoto C, Shioji K. Beneficial effects of low-dose benidipine in acute autoimmune myocarditis: suppressive effects on inflammatory cytokines and inducible nitric oxide synthase. *Circ J*. 2003;67:545–550.
43. Parrillo JE, Cunnion RE, Epstein SE, Parker MM, Suffredini AF, Brenner M, Schaer GL, Palmeri ST, Cannon RO. A prospective, randomized, controlled trial of prednisone for dilated cardiomyopathy. *N Engl J Med*. 1989;321:1061–1068.
44. Morgun A, Shulzhenko N, Perez-Diez, Diniz RV, Sanson GF, Almeida DR, Matzinger P, Gerbase-DeLima M. Molecular profiling improves diagnoses of rejection and infection in transplanted organs. *Circ Res*. 2006;98:e74–e83.
45. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumours. *Nature*. 2000;406:747–752.
46. Bueno-de-Mesquita JM, van Harten WH, Retel VP, van't Veer LJ, van Dam FS, Karsenberg K, Douma KF, van Tinteren H, Peterse JL, Wesseling J, Wu TS, Atsma D, Rutgers EJ, Brink G, Floore AN, Glas AM, Roumen RM, Bellot FE, van Krimpen C, Rodenhuis S, van de Vijver MJ, Linn SC. Use of 70-gene signature to predict prognosis of patients with node-negative breast cancer: a prospective community-based feasibility study (RASTER). *Lancet Oncol*. 2007;8:1079–1087.
47. Heidecker B, Hare JM. Cardiovascular genetic medicine: genomic assessment of prognosis and diagnosis in patients with cardiomyopathy and heart failure. *J Cardiovasc Transl Res*. 2008;1:225–231.
48. Heidecker B, Hare JM. The use of transcriptomic biomarkers for personalized medicine. *Heart Fail Rev*. 2007;12:1–11.
49. Isensee J, Witt H, Pregla R, Hetzer R, Regitz-Zagrosek V, Noppinger PR. Sexually dimorphic gene expression in the heart of mice and men. *J Mol Med*. 2008;86:61–74.
50. Limas CJ, Iakovis P, Anyfantakis A, Kroupis C, Cokkinos DV. Familial clustering of autoimmune diseases in patients with dilated cardiomyopathy. *Am J Cardiol*. 2004;93:1189–1191.

CLINICAL PERSPECTIVE

New diagnostic tools based on gene signatures derived from the entire complement of messenger RNAs in a cell or tissue have become established in the clinical management of certain disorders, particularly cancer. The comprehensiveness of this approach contributes to its accuracy. Myocarditis is a disorder that causes a substantial proportion of patients presenting with new-onset heart failure and left ventricular dysfunction. Typically diagnosed by endomyocardial biopsy and evaluated with histological criteria called the Dallas criteria, clinical management is hampered by low sensitivity and specificity and the need for multiple cardiac biopsies. The present study suggests that the application of a transcriptomic based biomarker can substantially improve the diagnostic accuracy of heart biopsy for myocarditis. Using endomyocardial biopsy tissue obtained at the time of clinical presentation, we developed a molecular signature comprising 62 genes that predicted with high accuracy the presence of myocarditis in a population of 48 patients. Importantly, this required evaluation of tissue from a single endomyocardial biopsy sample and therefore is clinically practical. The present results could provide treating physicians with important and accurate diagnostic information about individual patients and could provide tools for personalized treatment or monitoring. Given emerging treatment strategies for viral and inflammatory myocarditis, accurate diagnostic tools are of increased importance.

Downloaded from http://circ.ahajournals.org/ by guest on January 29, 2012