Genomics 94 (2009) 20–31

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Left ventricular global transcriptional profiling in human end-stage dilated cardiomyopathy

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ARTICLE INFO

Article history:
Received 9 September 2008
Accepted 17 March 2009
Available online 28 March 2009

Keywords:
Idiopathic dilated cardiomyopathy
Global gene expression
Microarray gene expression
Gene up/down-regulation
Mitochondrial function
Apoptotic signaling

ABSTRACT

We employed ABI high-density oligonucleotide microarrays containing 31,700 sixty-mer probes (representing 27,868 annotated human genes) to determine differential gene expression in idiopathic dilated cardiomyopathy (DCM). We identified 626 up-regulated and 636 down-regulated genes in DCM compared to controls. Most significant changes occurred in the tricarboxylic acid cycle, angiogenesis, and apoptotic signaling pathways, among which 32 apoptosis- and 13 MAPK activity-related genes were altered. Inorganic cation transporter, catalytic activities, energy metabolism and electron transport-related processes were among the most critically influenced pathways. Among the up-regulated genes were HTRA1 (6.9-fold), PDCD8(AIFM1) (5.2) and PRDX2 (4.4) and the down-regulated genes were NR4A2 (4.8), MX1 (4.3), LGALS9 (4), IFNA13 (4), UNC5D (3.6) and HDAC2 (3) ($p<0.05$), all of which have no clearly defined cardiac-related function yet. Gene ontology and enrichment analysis also revealed significant alterations in mitochondrial oxidative phosphorylation, metabolism and Alzheimer's disease pathways. Concordance was also confirmed for a significant number of genes and pathways in an independent validation microarray dataset. Furthermore, verification by real-time RT-PCR showed a high degree of consistency with the microarray results. Our data demonstrate an association of DCM with alterations in various cellular events and multiple yet undeciphered genes that may contribute to heart muscle disease pathways.

Background

Idiopathic dilated cardiomyopathy (DCM) is the thinning of one or both ventricle(s) from an unknown cause, with the resultant impaired cardiac contractility often leading to overt congestive heart failure or cardiac arrhythmias. While no clear cause is evident in the majority of cases, DCM is probably an end product of myocardial damage triggered by a variety of toxic, metabolic or infectious agents [1]. Besides, some forms of familial DCM, in particular, also appear to be triggered by mutations in genes encoding cytoskeletal, contractile or other myocardial proteins [2–5]. The ensuing progression of heart failure is associated with left ventricular remodeling, which manifests as a gradual increase in left ventricular end-diastolic and end-systolic volumes, wall thinning and alteration in the shape of the chambers to a more spherical and less elongated form [6]. Several molecular and cellular alterations have been identified that contribute to cardiac muscle contractility and relaxation abnormalities in this process. These include, among others, the cyclic AMP (cAMP)-dependent pathways, calcium (Ca^{2+}) homeostasis, neurohumoral activation and myofibrillar function [7]. Essentially, cAMP-dependent pathways are desensitized due to alterations in β-adrenoceptors (β-AR), β-AR kinases and guanine nucleotide binding proteins (G-proteins) [8]. Calcium ion (Ca^{2+}) homeostasis is impaired, characterized by a reduced sarcoplasmic reticulum Ca^{2+} reuptake rate, elevated Ca^{2+} release channel threshold and an increase in sodium ion (Na^{+})/Ca^{2+} exchanger expression [9,10]. Myofibrillar function may also be influenced by a decrease in Mg^{2+}-ATPase activity and in troponin I phosphorylation, as well as changes in troponin T isoform expression [9,11–13]. Accumulating data also suggests a link between alterations and/or deficiencies in cytoskeletal proteins and the progression of cardiomyopathy to heart failure. Moreover, the remodeling process appears to be regulated by a number of pathways including cytokines and growth factors [14].

Despite great efforts to understand the mechanism involved in the progress of DCM to overt heart failure, the underlying triggering factors for the disease remain to be elucidated. Accumulating evidence from gene profiling and other studies implicates diverse pathways, including among others, the vascular renin–angiotensin system [15], G_i-coupled receptors [16], TGFβ-activin-A/Smad signaling pathway

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doi:10.1016/j.ygeno.2009.03.003

[17], SH2-containing cytoplasmic tyrosine phosphatase (Shp) [18] and apoptotic signaling [15,17–20], to name a few. While classical opinion might argue that several of these alterations occur independently of the underlying etiology of the disease, it has also become apparent that the greater part of the familiar myocardial changes is probably triggered by chronic neurohumoral activation and abnormal mechanical load [21], which greatly promote the progression of heart failure as part of a vicious circle. However, the molecular basis for this link remains unclear. Several studies have been performed using different microarray-based and other techniques to evaluate alterations in gene expression in DCM [22–26], and recently intraplatform consistency in terms of sample sources as well as a high level of interplatform concordance with respect to genes identified as differentially expressed have been demonstrated [27,28]. Hence, deciphering the pattern of alterations in gene expression in DCM using the microarray system provides a valuable basis for elucidating some of the mechanisms involved in this vicious circle. In particular, the ABI high-density oligonucleotide microarray platform allows analysis of a greater number of genes than most platforms, as it includes annotated genes from both public and Celera databases. ABI platform is also a chemiluminescent based array by which signal is enhanced to fentomol sensitivity which may help to detect rare mRNAs. It has also been shown that ABI 1700 platform has substantially higher sensitivity, detecting four times as many changes in an identical experimental design and results are well correlated ($R^2 > 0.7$) with qRT-PCR compared to other microarray platforms [29–31]. In this study, we therefore sought to establish left ventricular differential gene expression in DCM employing the ABI 1700 platform, in order to be able to detect a relatively rare class of mRNAs and obtain further insight into the mechanism of heart muscle disease pathways.

Materials and methods

Study patients

For the gene expression and subsequent experiments, 300 mg of tissue were harvested from left ventricles of five DCM hearts excised from patients (3 male and 2 female; 42.3 ± 6.3 years) with end-stage heart failure undergoing cardiac transplantation at our institution. All samples were procured from identical myocardial loci to ensure optimal uniformity. The patients had New York Heart Association class 3–4 symptoms, and received anti-heart failure treatment and/or inotropic support. None of the patients was on a left ventricle assist device or any other mechanical support. Four healthy hearts procured from organ donors (three male and 1 female; 34.1 ± 4.7 years) who died of traffic accidents with no history of cardiac disease served as controls. The mean age of the controls was not significantly different from that of the patients ($p = 0.37$). These hearts had originally been intended for transplantation, but failed to get suitable matching recipients. At the time of harvesting, whole hearts were explanted after preservation in cold cardioplegia, followed by immediate dissection into small portions, snap-frozen in liquid nitrogen, and maintained at −80 °C until use. Minimum time possible (usually < 3 h) was allowed between harvesting donor hearts and freezing the samples in liquid nitrogen. Fully informed consent was obtained from all patients or family members before participating in the study. This study was performed in accordance with the Declaration of Helsinki as adopted and promulgated by the US National Institutes of Health as well as rules and regulations laid down by our Institutional Ethics Committee.

Expression array analysis

Total RNA was isolated from similar left ventricular biopsies using Applied Biosystems (ABI) Totally RNA Isolation Kit (ABI-Ambion, Foster City, CA, USA), quantified with the NanoDrop® ND-1000 Spectrophotometer (Nanodrop Inc., Wilmington, DE, USA) and further analyzed by RNA 6000 Nano Assay using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Digoxigenin-UTP labeled cRNA was generated and amplified from 1 μg of total RNA using Applied Biosystems (ABI) Chemiluminescent RT-IVT Labeling Kit v 1.0. The array hybridization was performed for 16 h at 55 °C and detection, image acquisition and analysis were performed using ABI Chemiluminescence Detection Kit on ABI 1700 Chemiluminescent Microarray Analyzer (ABI, Foster City, CA, USA).

Real-time RT-PCR

In order to validate our microarray results, confirmatory quantitative real-time RT-PCR (qRT-PCR) was performed using the ABI 7500 Sequence Detection System (ABI, Foster City, CA, USA). For this purpose, 50 ng total RNA procured from the same microarray study samples were transcribed into cDNA using Sensiscript Kit (QIAGEN Inc., Valencia, CA, USA) under the following conditions: 25 °C for 10 min, 42 °C for 2 h, and 70 °C for 15 min in a total volume of 20 μl. Six differentially expressed genes (CRYM, NR4A2, PDK4, RASD1, TNNI3K, and AIFM1) were randomly selected and primers designed using Primer3 software. After primer optimization, the PCR assays were performed in 6 μl of the cDNA using the QIAGEN Quantitet SyBR Green Kit, employing GAPDH as the endogenous control gene. All reactions were conducted in triplicates and the data was analyzed using the delta delta C_T method [32].

Data analysis

Hybridization images were analyzed using the ABI 1700 Chemiluminescent Microarray Analyzer software v 1.1, with the detection threshold set at signal to noise (S/N) ratio > 3 (a value that indicates 99.9% confidence level for the signal being above the background level, "present" probes) and quality flag < 5000. The open source Bioconductor packages, ab1700, limma, multtest and affy (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) and Partek Genomics Suite (Partek Inc.) were employed to normalize the data via quantile normalization and to determine significant differences in gene expression levels between DCM patients and normal controls [33]. When comparing DCM patients and normal controls to identify the differentially expressed genes, we used a combination of three criteria. We considered genes that are "present" in at least half of the samples in either group. Given the nature of the data, and statistical tests selected, adjusting for multiple testing errors is critical. We used Benjamini-Hochberg [34] step-up procedure to control the false discovery rate (FDR). As an alternative approach, we employed the two-class SAM procedure to estimate the FDR [35]. Significantly modulated genes were defined as those with absolute fold change (FC) > 1.8 and controlling FDR at 5%. A validation data set was generated from an independent study by Barth et al. [22] using Affymetrix HG-U133A array, and the raw data was analyzed by using dChip [36] and open source R/Bioconductor packages. The dChip outlier detection algorithm was used to identify outlier arrays (all arrays passed), and probes "present" in at least 50% of the samples in either group were filtered. The data was normalized by the GC Robust Multi-array Average (GC-RMA) algorithm [37,38]. Unpaired *t*-tests were performed to determine significant differences in gene expression levels between patients and normal controls, Multi Experiment Viewer (MeV4.0) [39] was used to perform two-dimensional hierarchical clustering employing Euclidean distance as well as Pearson correlation with average linkage clustering. Functional annotation and biological term enrichment analysis were performed using DAVID Bioinformatics Resources [40], Expression Analysis Systematic Explorer (EASE) [41], Protein ANalysis Through Evolutionary Relationships (PANTHER™) classification systems [42], and