Published in final edited form as:
J Heart Lung Transplant. 2006 May ; 25(5): 579–588. doi:10.1016/j.healun.2006.01.006.

Serial Gene Expression Profiling in the Intact Human Heart

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Abstract

Background—In chronic heart failure due to a dilated cardiomyopathy phenotype, the molecular bases for contractile dysfunction and chamber remodeling remain largely unidentified.

Methods—To investigate the feasibility of measuring global gene expression serially in the intact failing human heart, we performed repeated messenger RNA (mRNA) expression profiling using RNA extracted from endomyocardial biopsy specimens and gene chip methodology in 8 subjects with idiopathic dilated cardiomyopathy. In patients treated with β-blocking agents or placebo, myocardial gene expression was measured in endomyocardial biopsy material and radionuclide ejection fraction was measured at baseline and after 4 to 12 months of treatment. Gene expression was measured for 12,625 gene sequences by using Affymetrix U95 gene chips and commercially available software. For 6 mRNAs, gene chip results were compared with measurements made by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Results—In an unfiltered composite analysis of changes in expression detected in the patients with high-signal intensity chips, 241 genes showed an increase and 331 genes a decrease in mRNA abundance. There was good agreement between changes measured by quantitative RT-PCR and those determined by gene chips. There was less variance between differences in phenotype in patients sampled serially as compared between subjects with similar phenotypes sampled at baseline.

Conclusions—Serial gene expression profiling with association to phenotypic change is feasible in the intact human heart and may offer advantages to cross-sectional expression profiling. This study suggests that the intact failing remodeled human heart is in an activated state of gene expression, with a large net reduction in gene expression occurring as phenotypic improvement occurs. J Heart Lung Transplant 2006;25:579 – 88. Copyright © 2006 by the International Society for Heart and Lung Transplantation.

Data from the Human Genome Project indicate that the human genome contains 20,000 to 25,000 genes.1 Within individual cell types, however, only a small percentage of these genes is actually expressed. It is the selective expression of certain genes that gives rise to individual cell and organ types, and it is the disruption of this expression that creates cellular and, ultimately, tissue and organ pathology. Thus, certain kinds of myocardial failure, such as chronic systolic dysfunction characterized by remodeling without the loss of large amounts of viable myocardium, can be defined as a condition caused by altered expression of contractility-regulating genes in cardiac myocytes. Similarly, pathologic hypertrophy and myocardial chamber remodeling, which involves changes in cardiac myocytes, non-myocytes, and extracellular matrix, can be attributed to changes in the expression of genes

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that include those encoding contractile proteins, components of the cytoskeleton, and interstitial scaffolding proteins.

Our group and others have examined potential mechanisms accounting for systolic dysfunction in end-stage failing human hearts.2– 6 Despite these efforts and extensive work in animal models, the mechanisms responsible for abnormal intrinsic systolic function as well as the progression of left ventricular dysfunction and remodeling that characterizes the natural history of heart failure have not been definitively identified. Accordingly, we designed a study to evaluate the feasibility of serial messenger RNA (mRNA) expression profiling in the intact human heart by using microarray technology and to test the hypothesis that such measurements related to phenotypic change could improve the biologic precision of expression profiling.

METHODS

Clinical Protocol

The right ventricular septal endomyocardial biopsy tissue used in this study was available from a previously described protocol.5 Briefly, human subjects of either gender, 18 to 80 years of age with chronic (≥6 months) symptomatic heart failure caused by idiopathic dilated cardiomyopathy (IDC), were eligible for enrollment in the study. Additionally, subjects required an indication for a baseline endomyocardial biopsy, routinely done at our institution in IDC to rule out inflammatory or other infiltrative processes. To be eligible for randomization, there could be no lymphocytic or any other infiltrate on biopsy, the baseline left ventricular ejection fraction (LVEF) had to be 0.35 or less measured by radionuclide ventriculography, and LV size had to be increased as assessed by 2-dimensional echocardiography (LV end-diastolic diameter > 5.6 cm). Mandatory background therapy, required to be stable for at least 4 weeks, was an angiotensin-converting enzyme inhibitor, digoxin, and diuretics as needed.

Potentially eligible subjects signed written, informed consent approved by the University of Colorado Institutional Review Board. After baseline completion of tests, eligible subjects were randomly assigned to 3 treatments: placebo, metoprolol tartrate, or carvedilol. To maintain a double blind, all tablets were pulverized and placed in gelatin capsules by research pharmacists. For the 8 patients reported here, 2 received placebo, 4 carvedilol, and 2 metoprolol. The average doses (mg/day) of carvedilol or metoprolol were 75 ± 29 (SD) and 100 ± 0, respectively. At the end of 6 months of treatment (EOS) all baseline (BSL) measurements were repeated.

Tissue Sampling and RNA Extraction

Serial endomyocardial biopsies were performed from the right internal jugular vein approach using a Mansfield “Large Bite” bioptome (Mansfield Scientific, Boston, MA) under combined fluoroscopic and echocardiographic guidance. The endomyocardial tissue, 4 to 8 mg from 2 to 3 biopsy specimens, was used for reverse-transcriptase quantitative polymerase chain reaction (RT-QPCR), and 2 to 3 biopsy specimens were used for GeneChip studies. Total RNA was extracted from 2 to 3 right ventricular septal endomyocardial biopsy specimens (a total of 4–8 mg) by the guanidinium thiocyanate phenol-chloroform method by using RNA STAT-60 (Tel-Test, Inc, Friendswood, TX), as previously described.4

Reverse Transcription

Reverse transcription was performed with total RNA as the starting template. Total RNA (1–5 μg) was converted to double-stranded complimentary DNA (ds-cDNA) using the

Superscript Choice System (Life Technologies, Gaithersburg, MD). An oligo-dT primer containing a T7 RNA polymerase promoter (GeneSet, Biomatics, Hokkaido, Japan) was used so that cRNA could subsequently be generated. After second strand synthesis, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol, and ds-cDNA was recovered by ethanol precipitation.

In Vitro Transcription

In vitro transcription was performed to generate biotin-labeled cRNA by using a BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Inc, New York, NY). The entire amount of ds-cDNA template resulting from the previous reverse transcription reaction was transcribed in the presence of a mixture of unlabeled adenosine triphosphate, cytidine triphosphate (CTP), guanosine triphosphate, and uridine triphosphate (UTP) biotin-labeled CTP and UTP (bio-11-CTP and bio-16-UTP). The biotin-labeled cRNA was purified by using an RNeasy affinity column by Qiagen (Valencia, CA). To ensure optimal hybridization to the oligonucleotide array, the cRNA was fragmented such that the cRNA fragments were between 35 to 200 bases in length, accomplished by incubating the cRNA at 94°C for 35 minutes. The sample was then added to a hybridization solution containing 100 mmol/liter 2-morpholinoethanesulfonic acid, 1 mol/liter Na^+ and 20 mmol/liter ethylenediami-netetraacetic acid in the presence of 0.01% Tween 20. The final concentration of the fragmented cRNA was 0.05 μg/μl.

Hybridization

Hybridization was performed by incubating for 18 to 20 hours 200 μL of the sample with the HG-u95a v2 Affymetrix GeneChip (Santa Clara, CA). After hybridization, the hybridization solutions was removed and the GeneChip was washed and stained with streptavidin-phycoerythrin, and read at a resolution of 6 μm with an Hewlett Packard Gene Array Scanner (Palo Alto, CA).

Microarray Analysis

We used the Affymetrix oligonucleotide based approach, which uses photolithography to produce expression arrays containing probesets corresponding to as many as 30,000 known or potential gene sequences. We used the U95A gene chip in this study to evaluate 12,625 probesets. The microarray facility at the University of Colorado has the Affymetrix GeneChip Instrument Station as well as the Hewlett Packard laser reader for Affymetrix products. The Affymetrix data analysis software includes links to the public domain expression databases. Amplification strategies now enable the Core to analyze gene expression in 1 to 5 μg of total RNA, the amount used to generate the data in this study.

Data Analysis

Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described.7 Briefly, each gene is represented by the use of ~20 perfectly matched (PM) and mismatched (MM) control probes. The MM probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals. The number of instances in which the PM hybridization signal is larger than the MM signal is computed along with the average of the logarithm of the PM-to-MM ratio (after background subtraction) for each probeset. These values are used to make a matrix-based decision concerning the presence or absence of an RNA molecule. To determine the quantitative mRNA abundance, the average of the differences (PM minus MM) for each gene-specific probe family is calculated, after discarding the maximum, the minimum and any outliers beyond 3 SD. All of the above calculations are functions performed by the MicroArray Suite (MAS 5.0) (Affymetrix), the

software used to analyze expression data from all Affymetrix expression arrays. This software is specifically designed to determine intensity of hybridization from all features on an array and to provide pair-wise comparison between arrays.

Because of varying quality of hybridization and chip signal intensity for each patient, 2 different levels of fluorometric signal amplification ("scaling") was used in the 16 analyzed GeneChips. Use of low scaling factor (LSF) implies good signal intensity and hybridization, while use of high scaling factor (HSF) implies poor signal intensity and hybridization. Of the 16 analyzed GeneChips, 10 were LSF (including 4 pairs of BSL-EOS chips) and 6 were HSF. Because of the lack of sensitivity of detection of gene expression in HSF chips, only LSF GeneChip pairs were analyzed for changes in gene expression.

We used 2 types of statistical analysis of changes in gene expression vs myocardial phenotypic change in the 4 pairs of LSF chips. In Method A, only those genes present on all 8 LSF chips by Affymetrix "Abs call" and had some Affymetrix "Diff Call" statistical change in at least 2 subjects (MAS 5.0) were analyzed. There were 940 such genes, 7% of the total 12,625. The association between change in LVEF and change in gene expression was then assessed with linear regression in SAS (SAS Institute, Cary, NC), with statistical significance being 2-sided $p < 0.05$ with 2 degrees of freedom.

In Method B, we used the Affymetrix "Diff Call," which is appropriate for higher abundance mRNAs, and the "Present or Absent" designation for expression, which may detect changes in lower abundance mRNAs, to determine changes in mRNA expression that were consistent with phenotypic change. In the 3 patients with improved LVEF who had LSF chips at BSL and EOS, sequences showing changes by Diff Call or by Present/ Absent reading were identified. A consistent change was defined as 3 of 3 patients showing a change in the same direction or 2 of 3 in the same direction, with the third not showing a change. In addition, gene expression in the fourth patient, with a decline in LVEF, was used as a filter; changes in gene expression that were in the same direction, unchanged, or in the opposite direction from gene expression changes in the 3 patients with increased LVEF were considered respectively to be unrelated, possibly related, or likely related to phenotypic change. Finally, to detect patterns of changes in gene expression with phenotypic change, Method B changes were assigned by biologic function into 13 categories that have potential roles in myocardial structure or function.

Repeatability of Microarray Measurements

To assess the repeatability of microarray-based mRNA measurements. we used 3 sets of endomyocardial biopsies taken from the right ventricular distal septum of an end-stage failing, explanted human heart. Three separate sets of microarray experiments were conducted with these samples, which were kept separate during RNA extraction and hybridization. We used the intra-class correlation coefficient to measure correlation across the 12,625 probesets between experiments. Statistical significance was taken as 2-sided $p < 0.05$. Data are presented as mean ± SD. Other comparisons used the unpaired *t*-test and the Wilcoxon's rank sum test.

RESULTS

Phenotypic Change in Dilated Cardiomyopathy

Patient characteristics at BSL and at EOS are presented in Table 1. Seven patients had improvement in LVEF averaging 0.21 ± 0.05. One patient treated with placebo had spontaneous improvement in cardiac function, but the other placebo-treated patient had a decline in LVEF, from 0.26 to 0.15. Histology revealed myocyte hypertrophy and fibrosis in all cases, with no evidence of myocardial inflammation.

RNA Application and Scaling Factors

Three increased LVEF patients and the 1 decreased LVEF patient had LSF chip sets at BSL and EOS. Of the 4 patients with at least 1 HSF chip, 2 also had an LSF chip, both at BSL. The amount of applied RNA ranged from 1.2 to 5.8 μg, and there was no relationship between the amount of RNA applied and the scaling factor necessary to interpret the chip.

Number and Percentage of Expressed Genes

The number and percentage of genes expressed in the 8 patients are summarized by scaling factor in Table 2. In Table 2, detectable expression is defined as a non-Absent ("Present") reading at either BSL or EOS. As seen in Table 2, 44% of the genes were expressed in the 10 LSF chips vs 12% on the HSF chips ($p < 0.001$). These data indicate that an "Absent" call on the HSF chips is unreliable and does not necessarily reflect non-expression of the gene sequence in question. Therefore, the HSF chips were excluded from further analysis.

Comparison of GeneChip Analysis With RT-QPCR

We measured the expression of 6 genes by both RT-QPCR and microarray methods. The comparison of mRNA measurements for these 6 genes in the 3 patients with LSF gene chip sets with improved LVEF is given in Table 3. There is good agreement between GeneChip and RT-QPCR methods of quantitation with the exception of atrial natriuretic peptide (ANP) in 1 patient. ANP decreased in 3 of 5 patients with increased LVEF with at least 1 LSF chip, and increased in the subject with worsened LVEF. ANP expression could not be evaluated in the 2 patients with HSF chips. Because the increased value in the 1 LVEF-increased patient was so obviously disparate (increase by 10.5-fold on GeneChip, decrease by 1.2-fold on RT-QPCR) we interrogated the relevant chips, searching for a technical explanation such as contamination of the chip with an autofluorescing substance. The 2 chips were technically sound, and other measurements in the same chip region did not appear to be anomalous. In addition, the related brain natriuretic peptide was also increased in this patient and showed a similar decreased pattern to ANP in the other chips.

Gene Expression Within Group as Measured Serially, Cross-Sectional Changes Within the Same Phenotype, and Replicate Experiments

In the 3 LSF and improved LVEF patients, we performed a between-patient comparison and a within-patient comparison (Table 4). The number of genes showing different expression was much greater at BSL between subjects with similar LVEFs and dilated cardiomyopathy phenotypes than in the comparison of sequential biopsies within the same subject where the follow-up samples were from ventricles with marked phenotypic improvement.

In these same 3 patients, we used the intraclass correlation coefficient to measure correlation across the 12,625 probesets. At BSL, the average correlation in gene expression between patients was 0.876 ± 0.040. Comparing BSL with EOS within individual patients, the average correlation was 0.951 ± 0.019. Again, interpatient variability in gene expression appeared to be greater than intrapatient variability, because measurements taken in the same patient over time across conditions were closer than between patients with the same condition. By comparison, the average correlation from 3 samples from a single explanted heart processed independently was high (0.991 ± 0.002), suggesting that the lower average correlations, 0.876 and 0.951, truly account for genetic and phenotypic variation respectively, not merely replication error.

Changes in Gene Expression

Genes with significant changes associated with change in LVEF (Method A) in the 4 subjects with LSF chips at BSL and EOS are given in Table 5. After duplicate changes

detected by different sequences of the same gene were eliminated, 22 negative and 22 positive associations were found. Of interest in Table 5 is that 6 ribosomal proteins (22% of the total of increased expression genes) exhibit an increase in gene expression as LVEF increases, with no ribosomal protein genes showing a decrease.

A categorization of changes in gene expression by Method B is given in Table 6. For both the unfiltered changes and changes "likely related to LVEF change," sequences that show a decrease in expression with improved LVEF outnumbered those with an increase. Gene categories showing the greatest differential in favor of decreased expression included cytoskeletal, extracellular matrix, transcription/translation, signal transduction, and growth factors. In contrast, the only category that showed a differential increase in expression with improved LVEF was the metabolic category.

Individual changes in gene expression of interest included those within the contractile protein category; as for unfiltered sequences exhibiting increased expression, all were "adult" isoforms (α-MyHC, ventricular light chain-1, atrial myosin light chain-2), and 3 of 4 genes showing decreased expression were fetal isoforms (skeletal forms of tropomyosin, troponin T, and troponin I). The other contractile protein gene exhibiting decreased expression, skeletal α-actin, is considered to be a fetal isoform in rodent hearts. Additionally, on the unfiltered analysis, Serca 2a and phospholamban, 2 Ca^{2+} handling genes, whose downregulation is part of the failure molecular phenotype, showed increased expression.

Genes with changed expression by Method B filtered criteria (gene expression changes "likely related" to LVEF change) are given in Table 7. Two genes known to be molecular markers of heart failure and whose down-regulation is likely directly involved in contractile dysfunction,8 α-myosin heavy chain and the β_1-adrenergic receptor, exhibited increases in patients with improved LVEFs. The increase in metabolic gene expression included genes involved in glucose and fatty acid metabolism regulation and in oxidative phosphorylation. The only metabolic gene to show decreased expression was apo A1, which encodes a cardioprotective and atherosclerosis-preventing protein that was recently shown to be expressed in the human heart.9

Only modest overlap was noted between the 2 methods of identifying changes in gene expression associated with myocardial phenotypic change. Of the 44 genes in Table 5, 18 were identified in the unfiltered gene expression changes listed in Table 6, and only 6 in the filtered results.

DISCUSSION

Recent advances in genomics and technology have made it possible to evaluate the expression of large numbers of genes on microarray chips. Although this method is challenging, microarray studies appear to be reasonably reliable for detecting biologically important changes in gene expression.10,11 Several previous studies used oligonucleotide microarrays to evaluate gene expression changes in the failing human heart,12–16 typically in cross-sectional designs that compared end-stage failing hearts with organ donor controls. These studies identified hundreds of new genes that are potentially involved in the pathogenesis and progression of heart failure. These studies are limited by multiple factors, however, including the end-stage nature of the myocardial disease process, confounding effects of variably used medications, the lack of a true normal control material, and differences in genetic background in human subjects. As a result, the findings in these studies tend to be inconsistent.17

Another problem, shared with all microarray studies, is that when expression of thousands of genes is measured, a significant number of them can be expected to exhibit changes by chance alone. As a result, in microarray studies with cross-sectional designs, it is difficult to ascertain which changes are of biologic significance and which are random occurrences.

Serial measurements of gene expression in the same patients treated under more controlled conditions would, theoretically, offer advantages to the cross-sectional, end-stage disease approach to expression profiling. Several previously published studies have measured gene expression profiles serially in the intact human heart.18 –20 These studies used tissue samples taken at the time of LVAD implantation and removal but were limited by the reality that assist devices markedly unload the heart and may induce cardiac atrophy or non-physiologic changes in gene expression that are not associated with heart failure.21 Perhaps as a result, the expression of many genes that may be involved in the dilated cardiomyopathy phenotype do not normalize with LVAD unloading.18,19 In addition, the lack of phenotypic data in some of these studies and the absence of controlled medical therapy make these data difficult to interpret.17

In the present study, we assessed the feasibility of performing serial mRNA expression profiling in the intact hearts of patients with non-end-stage, dilated cardiomyopathies by using endomyocardial biopsy specimens, and had the advantage of relating changes in gene expression to changes in myocardial chamber phenotype.

In multiple trials in chronic heart failure, β-blockade has been shown to improve survival and to decrease hospitalization. Therapy with β-blockers has also been shown to consistently improve systolic function and reverse remodeling in patients with both ischemic and idiopathic cardiomyopathies.22 The molecular basis for this improvement in cardiac function and reversal of remodeling by β-blocking agents remains incompletely understood, but partial reversal of a “fetal” pattern of gene expression appears to play a role.5 It is unclear, however, if β-blocking agents more generally alter gene expression and what additional regulators of contractile function and pathologic hypertrophy are affected by this type of treatment. The use of gene chip technology should allow for the extension of the initially reported paradigm of specific changes in gene expression that are associated with myocardial phenotypic change in patients treated with β-blocking agents.

The present study demonstrates that gene expression can in fact be measured serially in the intact human heart with gene chip technology and mRNA extracted from endomyocardial biopsy specimens, with some limitations. Not all patient samples hybridize equally well, affecting the ability to detect expression changes. On LSF chips, approximately 44% of the gene sequences on the U95A chip were expressed in endomyocardial biopsy tissue, whereas on HSF chips, the detectable expression dropped to approximately 12%. Six of the 16 chips required a HSF to be read, compromising data interpretation in 4 of 8 patients.

A comparison of LSF chip data with RT-QPCR results for 6 mRNAs indicated that gene chip measurements were in general agreement with the more quantitative technique. There was, however, one contradictory result with the gene chip technique for ANP that may have been due to an endomyocardial biopsy sampling error.

The value of microarray approaches to the measurement of gene expression is that patterns of expression can be recognized, and previously unrecognized contributors to disease process can be identified and subsequently subjected to hypothesis testing. In the present study, the behavior of gene categories associated with phenotypic change (Method B analysis) suggests that dilated cardiomyopathy is characterized by an overall state of gene activation, with most differentially expressed genes exhibiting decreased expression as LVEF improved. Moreover, the categories of genes exhibiting decreased expression with

phenotypic improvement were not, surprisingly, in the cytoskeletal, extracellular matrix, signal transduction, transcription/ translation, or growth factor categories. Also, as might be expected, improved myocardial function was associated with a differential increase in metabolic category gene expression.

Importantly, the genes that may be intimately involved in producing contractile dysfunction in the failing human heart, α-myosin heavy chain and Serca2a, were identified by Method B as increasing their expression as LVEF improved. Moreover, α-myosin heavy chain, which we have previously reported as the best individual molecular marker of the human dilated cardiomyopathy phenotype,[4,5] was identified by both Method A and Method B as being directly associated with phenotypic improvement.

The purpose of this study was to test the hypotheses that microarray-based gene expression could be measured in the intact heart, and that serial changes that were associated with phenotypic change would add specificity to detection of biologically important changes in gene expression. We used 2 different methods of relating changes in gene expression to changes in LVEF, a measurement of the dilated cardiomyopathy phenotype that includes a contractile function measure (stroke volume) in its numerator and a remodeling measure (end-diastolic volume) in its denominator. Method A used the Affymetrix “Abs call” and “Diff Call” as a filter and then relied on regression analysis against LVEF to determine significant associations with changes in phenotype. Method B used both Diff Call and Present/Absent data from the GeneChips to identify changed expression; this latter method would be expected to be useful for detecting changes in low abundance mRNAs. Because many of the categories (transcription/translation factors, signal transduction, growth factors) that exhibited a decrease in gene expression with improved LVEF would be expected to be represented by low abundance mRNAs, it is not surprising that Method B, but not Method A, was characterized by a differential decrease in gene expression as LVEF improved. In addition, the fact that less than 50% of the LVEF-associated changes in Method A were detected by Method B indicates that each analysis method has unique features.

Serial gene expression profiling, the sampling of the same patient over time, should improve the precision of detecting biologically important changes in gene expression by eliminating genetic and other types of interpatient variability. This theoretical advantage can be observed from our comparative between-subjects analysis at baseline, where a dilated cardiomyopathy phenotype was uniformly present, to the within-subjects comparison with serial sampling, where the phenotype had dramatically improved on the same genetic background. There was more variability (more gene sequences showing a change) between patients within the same phenotype than within patients across large differences in phenotype, suggesting that interpatient variation is a major problem in human microarray analyses.

This study has several limitations. Data were only available on male subjects. Female heart failure patients are known to remodel differently. It is possible that female patients have different changes in gene expression in association with changes in contractile function. It is also feasible that regional changes in gene expression occur; our work only used samples from the right ventricular endomyocardial septum. Finally, this study is limited by its small sample size. Further studies are needed to clarify overall changes in gene expression associated with contractile dysfunction as well as changes that are gender and region specific.

CONCLUSION

The technique of serially measuring changes in gene expression coupled with a change in phenotype allows an emphasis on identification of changes in gene expression changes associated with phenotypic change. In effect, this improves specificity from the standpoint of identifying disease-specific gene expression changes. This approach is feasible in the intact human heart, utilizing endomyocardial biopsy specimens as the starting material. Because of variability in hybridization signal, however, the sample size of such studies should be large enough to account for the loss of as many as 50% of the paired samples.

Serial measurements of gene expression with phenotypic change could be used for any disease process where tissue biopsy is possible and a phenotypic change can be effected and documented. However, the failing human heart is uniquely suited for this approach, based on the ease of obtaining biopsy samples at experienced centers, and the availability of medical therapies that can effect substantial structural and functional improvement in the dilated cardiomyopathy phenotype.

Acknowledgments

This investigation was supported by National Institutes of Health Grants K23 HL068875, 1R01 HL48013 and 1R01 HL71118 from the NHLBI, M01-RR00051 and M01-RR00064 from the National Center for Research Resources, and by GlaxoSmithKline Co.

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J Heart Lung Transplant. Author manuscript; available in PMC 2009 July 13.

Table 1

Patient Characteristics

Parameter	Subj 1	Subj 2	Subj 3	Subj 4	Subj 5	Subj 6	Subj 7	Subj 8
Age	39	57	63	61	64	51	57	39
Sex/race	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C
LVEF BSL→EOS	17→32	26→15	18→37	31→53	8→51	35→51	25→50	19→48
Chip scaling factor	High/high	Low/low	Low/low	Low/high	Low/low	Low/high	High/high	Low/low
Rx	Carv	Pbo	Met	Carv	Pbo	Met	Carv	Carv
Peak VO_2 (ml/kg/min)	20	19	18	15	N/A	14	16	20
NYHA	III	II	II	III	IV	III	II	III
Cl (l/min/m^2)	2.2	2.5	1.2	2.9	2.4	1.8	1.4	2.9

BSL, baseline; EOS, end-of study; Pbo, placebo; Met, metoprolol; Carv, carvedilol; NYHA, New York Heart Association classification.

Table 2

Number of Expressed Genes, By Scaling Factor

Category	Expressed genes (N) (% of 12,625)
Low scaling factors (n = 10)	5544 ± 458 (43.9 ± 3.6)
High scaling factors (n = 6)	1549 ± 554 (12.3 ± 4.4)

Table 3

Comparison of mRNA Measurements by RT-QPCR and GeneChip

Category	β_1-AR	β_2-AR	ANP	α-MyHC	β-MyHC	Serca 2a	Δ LVEF
Δ mRNA*	−0.35	−0.05	−17.4	+6.7*	−11.2	+24.6	+30.3†
± SEM	± .21	± .25	± 10.9	± 1.5	± 3.9	± 12.8	± 7.0
RT-QPCR fold change	−1.9, −1.3, +1.2	−1.5, 1.0, +1.1	−10.0, −2.1, −1.2	−2.1, −1.9, +2.0	−1.3, −1.1, −1.1	−1, −1.2 +2.3	−19, −43, +29
GeneChip change call	NC, NC, NC	NC, NC, NC	D, D, I	MI, I, I	NC, NC, NC	NC, NC, NC	—
GeneChip fold change	—	—	−3.5, −1.5, +10.5	−1.2, −1.8, +1.7	—	—	—
Agree/Disagree GeneChip vs. RT-QPCR	Agree	Agree	Disagree	Agree	Agree	Agree	—

*10^5 molecules/μg total RNA.

†$p < .05$ within-group change. β_1-AR, β_1-adrenergic receptor; β_2-AR, β_2-adrenergic receptor; ANP, atrial natriuretic peptide; α-MyHC, α-myosin heavy chain; β-MyHC, β-myosin heavy chain; Serca 2a, cardiac SR Ca^{2+} adenosine triphosphatase; NC, no change; D, decrease; I, increased; MI, mild increase.

Table 4

Analysis of Gene Expression*

Category of expression	Between-subject (baseline comparisons)	Within-subject (baseline vs. end-of-study) (Rx)
Increased	Subj 2 vs. 1: 952	Subj 1: 904 (M)
	Subj 3 vs. 1: 1325	Subj 2: 153 (P)
	Subj 3 vs. 2: 1116	Subj 3: 557 (C)
Decreased	Subj 2 vs. 1: 561	Subj 1: 675 (M)
	Subj 3 vs. 1: 730	Subj 2: 330 (P)
	Subj 3 vs. 2: 820	Subj 3: 542 (C)
Total # genes with changed expression	5504	3161
		$p = 0.03$ (Wilcoxon) vs between

*Between-subject vs within-subject (Change Calls only).

Table 5

Method A: Correlation of Changes in Gene Expression with Change in LVEF

Rank #	Gene	Accession #	*r*	*p*
	Decreased expression with increasing LVEF			
1	ADP ribosylation factor 4 (ARF4)	M36341	−0.996	0.004
2	Chaperonin-containing TCP-1 β	AF026166	−0.993	0.007
3	SH3 domain binding glutamic acid-rich protein	AI337192	−0.990	0.010
4	Nebulette	Y17673	−0.988	0.012
5	Chromosome 16 BAC clone	U95740	−0.982	0.018
6	Adenylyl cyclase-associated protein 2	(693_G_AT)	−0.979	0.021
7	70kda heat shock protein 8	Y00371	−0.977	0.023
8	Protease inhibitor 12 (neuroserpin)	Z81326	−0.972	0.028
9	Insulin-like growth factor binding protein-2	X16302	−0.972	0.028
10	70kda heat shock protein 2	L26336	−0.972	0.028
11	Chromosome 1q AF1 protein	U16954	−0.971	0.029
12	90 kD heat shock protein	J04988	−0.970	0.030
13	Serine protease with IGF-binding motif	D87258	−0.970	0.030
14	S-adenosylmethionine decarboxylase	M21154	−0.967	0.033
15	Eukaryotic translation initiation factor 4 gamma	AF012072	−0.966	0.034
16	Pitrilysin metalloproteinase 1	AB029027	−0.961	0.039
17	Hypothetical protein FLJ1080	AI765280	−0.960	0.040
18	Carboxypeptidase, vitellogenic-like CPVL	AC005162	−0.958	0.042
19	Hypothetical protein FLJ20986	Z24724	−0.957	0.043
20	Never in mitosis gene A related kinase 7	AL080111	−0.956	0.044
21	ADP-ribosylation factor-like 1	L28997	−0.953	0.047
22	Neuropeptide Y receptor Y6 pseudogene	D86519	−0.952	0.048
	Increased expression with increasing LVEF			
1	Unknown, from clone dkfzp58601318	AL049390	0.995	0.005
2	NADH dehydrogenase (ubiquinone) 1	AC002400	0.994	0.006
3	Ribosomal protein L13	X64707	0.993	0.007
4	Chromosome 9 open reading frame 16	AI885170	0.992	0.008
5	Beta 2 laminin	X79683	0.992	0.008
6	Ribosomal protein L18	LI1566	0.986	0.014
7	TNF-α induced protein 8	AF099935	0.985	0.015
8	Ribosomal protein S15	J02984	0.984	0.016
9	Thioredoxin interacting protein, VDUP1	S73591	0.979	0.021
10	Transformation/transcription domain-associated protein	AF110377	0.978	0.022
11	H1 histone family, member 0	X03473	0.978	0.022
12	α-myosin heavy chain	Z20656	0.974	0.026
13	Karyopherin alpha 4	AB002533	0.971	0.029
14	5-beta tubulin	X00734	0.969	0.031
15	Ribosomal protein L8	Z28407	0.963	0.037

Rank #	Gene	Accession #	*r*	*p*
16	Ribosomal protein P1	M17886	0.962	0.038
17	Heparan sulfate proteoglycan (glypican 3)	U50410	0.957	0.043
18	Thiosulfate sulfurtransferase (rhodanese)	D87292	0.956	0.044
19	JTV1	U24169	0.954	0.046
20	Hypothetical protein FLJ36166	AL049437	0.953	0.047
21	Ribosomal protein S2	XI7206	0.952	0.048
22	Unknown, KIAA0469 gene product	AB007938	0.952	0.048

LVEF, left ventricular ejection fraction; ADP, adenosine diphosphate; TCP, T-complex protein; IGF, insulin-like growth factor; NADH, 1,4-Dihidronicotinamide adenine dinucleotide; TNF-α tumor necrosis factor.

Table 6

Method B: Analysis of Changes in Gene Expression

	Number of gene sequences with consistent changes in ≥2/3 patients with improved LVEF, unfiltered		Consistent changes in ≥2/3 patients with improved LVEF, likely related to phenotypic change (filtered)	
Gene Category	**Increased, *n* = 241**	**Decreased, *n* = 331**	**Increased, *n* = 29**	**Decreased, *n* = 59**
Metabolic	54	10	5	1
Contractile protein	3	4	1	2
Cytoskeletal	7	17	3	5
Extracellular matrix	1	27	0	2
Transcription/translation	21	35	3	3
Ca^{2+} handling	4	1	0	0
Signaling/signal transduction	22	58	2	12
Cell homeostasis	22	15	2	3
Growth factors	7	25	1	4
Cell cycle/apoptosis	3	6	1	0
Immunologic/hematologic	7	12	2	1
Unclassified	20	25	3	4
Unknown function	70	96	6	22

LVEF, left ventricular ejection fraction.

Table 7

Genes Exhibiting Changed Expression by Method B Criteria *

Gene category	Increased expression (n), gene	Decreased expression (n), gene
Metabolic	(5); alcohol dehydrogenase β1 subunit, NAD +-specific isocitrate dehydrogenase β subunit, nuclear- encoded mitochondrial cytochrome c oxidase Va subunit, long-chain acyl-CoA synthetase, pyruvate dehydrogenase kinase isoenzyme 2 (PDK2)	(1); apolipoprotein A1
Contractile protein	(1); α-myosin heavy chain	(2); skeletal tropomyosin, skeletal troponin T1
Cytoskeletal	(3); β2 tubulin, actin depolymerizing factor, plakophilin 2a	(5); non-erythroid α-spectrin, microtubulin associated protein 4, nebulin, plectin, β2 spectrin
Extracellular matrix	(0)	(2); α2 collagen Type 1, thrombospondin-4
Transcription/translation	(3); 18S rRNA, ribosomal protein L3, activator 1	(3); helicase-like protein (DDX13), centromere protein A, forkhead protein (FREAC-1)
Ca^{2+} handling	(0)	(0)
Signaling/signal transduction	(2); β_1-adrenergic receptor, Ser-Thr protein kinase VRK1	(12); neuronal pentraxin receptor, frizzled-1, fibrousheathin 1, dual specificity phosphatase (MKP-5), Arg/Abl-interacting protein, protein tyrosine phosphatase, neogenin, phospholipase A2 RASF-A, calcineurin B, ras associated with diabetes (RAD), phosphatase 2A catalytic subunit-α, diacylglycerol kinase γ
Cell homeostasis	(2); phosphatidyl serine synthase-1, calmegin	(3); inwardly rectifying K^+ channel, Na^+, K^+-ATPase subunit α2, amphiphysin II
Growth factors	(1); transforming growth factor-beta stimulated clone-22	(4); metastasis-associated-1, insulin-like growth factor binding protein 5, insulin-like growth factor II, insulin-like growth factor binding protein-2
Cell cycle/apoptosis	(1); galectin-3	(0)
Immunologic/hematologic	(2); HLA-DR antigen α-chain, MHC class II lymphocyte antigen	(1); interferon regulatory factor-2
Unclassified	(3); TNF-α induced protein 8, acetolactate synthase, prion protein	(4); periplakin, AF1q, immunoglobulin super family four, autoimmunogenic cancer/testis antigen NY-ESO-1
Unknown function	(6)	(22)

* Filtered by requirement of opposite change in the patient with LVEF decline (changes in gene expression “likely related” to LVEF change).