Whole transcriptome microarrays identify long non-coding RNAs associated with cardiac hypertrophy

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Abstract

Long non-coding RNAs (lncRNAs) have recently emerged as a novel group of non-coding RNAs able to regulate gene expression. While their role in cardiac disease is only starting to be understood, their involvement in cardiac hypertrophy is poorly known. We studied the association between lncRNAs and left ventricular hypertrophy using whole transcriptome microarrays. Wild-type mice and mice overexpressing the adenosine A2A receptor were subjected to transverse aortic constriction (TAC) to induce left ventricular hypertrophy. Expression profiles of lncRNAs in the heart were characterized using genome-wide microarrays. An analytical pipeline was specifically developed to extract lncRNA data from microarrays. We identified 2 lncRNAs up-regulated and 3 lncRNAs down-regulated in the hearts of A2A-receptor-overexpressing-mice subjected to TAC compared to wild-type mice. Differential expression of these 2 lncRNAs was validated by quantitative PCR. Complete microarray dataset is available at Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE45423. Here, we describe in details the experimental design, microarray performance and analysis.

1. Direct link to deposited data
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE45423.

2. Experimental design, materials and methods

2.1. Biological samples

Six male FVB mice were subjected to transverse aortic constriction (TAC) as an experimental model of pressure overload inducing left ventricular hypertrophy. Three of these six mice carried a transgene allowing cardiac-restricted and inducible overexpression of the human adenosine A2A receptor (A2A-Tg) and the three other mice were wild type (Wt) littermates. Generation of the transgenic line was previously described [1]. Over-expression of A2A receptor was induced from 3 weeks of age by removal of doxycycline from mice chow. TAC was performed at 8 weeks of age, as described [2]. In brief, an aortic band was created by placing a ligature (7-0 nylon suture) between the origin of the right innominate and left common carotid arteries using a 27-gauge needle as a guide. After 4 weeks, mice were sacrificed and...
hearts were harvested and deep frozen. At sacrifice, mice displayed signs of left ventricular dysfunction as documented by decreased fractional shortening [2].

Animal experiments received approval of the Institutional Animal Care and Use Committee of Thomas Jefferson University (Philadelphia, USA) and were performed following the regulations of the Animal Welfare Act of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

2.2. Microarray hybridization and scanning

Total RNA was extracted from the left ventricular myocardium of each mouse and its quality was verified using a Bioanalyzer 2100 (Agilent Technologies). The RIN (RNA Integrity Number) value of all samples was above 8.5. 250 ng of each RNA sample was processed using Ambion® WT Expression Kit (Life Technologies). 10 μg of cRNA was used as an input to the second cycle of cDNA reaction. 5.5 μg of single-stranded cDNA was used as input for fragmentation reaction.

Table 1
Public databases used for microarray probe reannotation.

| Database | Release | URL | Filter | # of sequences |
|----------|---------|-----|--------|----------------|
| RefSeq   | 55      | ftp://ftp.ncbi.nih.gov/refseq/M_musculus/mRNA_Prot/mouse.rna.fna.gz | Only NR prefixed RNAs | 2645 |
| Ensembl  | 68      | ftp://ftp.ensembl.org/pub/release-68/fasta/mus_musculus/nrRNA/ | MicroRNA, small nucleolar RNA and small nuclear RNA were excluded | 3210 |
| IncRNAdb | –       | http://incrnadb.com | MtRNA, Mt-rRNA, rRNA, snoRNA, miRNA and Mt_tRNA were excluded | 126 |

Fig. 1. Effect of adenosine A2A receptor overexpression on cardiac transcriptome. Three A2A-Tg mice and 3 Wt littermates were subjected to TAC and were sacrificed after 4 weeks. Hearts were harvested and gene expression was profiled using Affymetrix Mouse Gene 1.0 ST microarray. (a) and (b): Heat-map and M-A plot of differentially expressed transcripts as determined by significance analysis of microarrays with a q-value < 5% as significance threshold. (c) Principal component analysis. Wt: wild-type; A2A-Tg: adenosine A2A receptor overexpressing mice; PC: principal component. Adapted from [4].
Each cDNA fragment was labeled with biotin using GeneChip® WT Terminal Labeling Kit (Affymetrix) and then hybridized to Affymetrix Mouse Gene 1.0 ST Array which interrogates 28,853 genes with 770,317 distinct probes. Microarray design was based on the mouse genome sequence of February 2006 (UCSC mm8, NCBI build 36). Details on sequence coverage can be found at http://media.affymetrix.com/support/technical/datasheets/gene_1_0_st_datasheet.pdf. Hybridization was performed in a hybridization oven at 45 °C and 60 rpm for 17 h. Scanning of the slides was performed using Affymetrix GeneChip Scanner 3000 7G with 2.5 μm resolution. Raw data were acquired and processed with Partek® Genomics Suite® (version February 2011 Copyright; 2015 Partek Inc., St. Louis, MO, USA) using Robust Multiarray Averaging with GC correction and quantile normalization. Probesets were summarized with mean values for one transcript cluster.

### 2.3. Differential expression

Normalized data were analyzed using the t-test procedure within Significance Analysis of Microarrays software (version 3.09) [3] to identify differentially expressed transcripts. Transcripts with q-value <5% were considered as statistically significant. Over-expression of adenosine A2A receptor resulted in significant changes in gene expression as shown in the heat-map and M-A plot of Fig. 1a–b. Significance analysis of microarrays revealed that 709 transcripts were differentially expressed between A2A-Tg mice and Wt littermates. Principal

### Table 2

Quantitative PCR primer pairs.
Adapted from [4].

| Gene name     | Accession no. | Forward primer 5′–3′ | Reverse primer 5′–3′ | Annealing temperature (°C) |
|---------------|---------------|----------------------|----------------------|-----------------------------|
| Dancr         | NR_015531     | AATGTATCTGGACTTCGTTAG | AGAATTGACACAGGAAGC   | 54                          |
| Gm10400       | NR_035555     | TCACCTCTGCTTTATATAG  | TTACGTCTTGCACTGAC    | 52                          |
| Gm14005       | NR_028390     | ACCCACTACACTGACGTT   | AAATCCTCCAGGAAAGAC   | 56                          |
| 290005520Rik  | NR_045177     | TAAGTGTGGAATCATTCTTT | TTGCGGAGAAAGAATGCTTT | 56                          |

![Diagram](image_url)
component analysis showed the ability of gene expression data to discriminate A2A-Tg mice from Wt mice (Fig. 1c).

2.4. Microarray probe re-annotation

To identify the probes on the array corresponding to long non-coding RNA (lncRNA) sequences, we re-annotated all transcripts differentially expressed with a threshold q-value of 5%. Probe sequences were obtained from Affymetrix. Differentially expressed transcripts annotated as mature messenger RNAs were discarded. Probe sequences were obtained from RefSeq database. Perfectly matched probes were discarded. Third, transcript clusters missing at least one probe were discarded. After filtering, 5 lncRNAs were identified as being differentially expressed between A2A-Tg mice and Wt mice (Fig. 2).

2.5. Quantitative RT-PCR

Total RNA was extracted from left ventricular samples of mouse hearts. One microgram of total RNA was reverse-transcribed using the Superscript II RT kit (Life technologies). Controls without enzyme were performed to check for the absence of genomic DNA contamination. Real-time quantitative PCR was performed in a CFX96 thermocycler (BioRad) using IQ SYBR Green Supermix (BioRad) and primer pairs designed with the Beacon Designer software (Premier Biosoft). Primer sequences are displayed in Table 2. PCR amplification cycles were as follows: 3 min at 95 °C, 30 s at 95 °C and 1 min annealing-extension (40-fold). Optimal annealing-extension temperature was determined for each primer pair. PCR specificity was confirmed by melting curve analysis. GAPDH was used as a housekeeping gene for normalization purpose. Expression levels were obtained by the relative quantification method (ΔΔCt) within the CFX Manager 2.1 software (Bio-Rad).

Expression levels of the 5 lncRNAs identified by microarrays as differentially expressed between A2A-Tg mice and Wt mice were assessed in left ventricular samples from the 6 mice used in microarray experiments. As shown in Fig. 3, the lncRNAs Gm14005 and 2900055J20Rik were down-regulated (1.7- and 3.5-fold, respectively) in A2A-Tg mice as compared to Wt mice, consistently with microarray results. The lncRNAs Dancr and Gm10400 followed the same trends as in microarray experiments, albeit these differences did not reach statistical significance. BC016548 lncRNA was undetected.

3. Conclusion

We describe the gene expression profiling of left ventricular samples from adenosine A2A-receptor overexpressing mice using Affymetrix Mouse Gene 1.0 ST Array. We have developed a microarray re-annotation pipeline allowing the investigation of lncRNAs from widely-used whole transcriptome microarrays. These microarray data provide an important resource for exploring and identifying genes involved in cardiac hypertrophy and heart failure.

Acknowledgments

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References

[1] H. Funakoshi, T.O. Chan, J.C. Good, J.R. Libonati, J. Pihlaja, X. Chen, S.M. MacDonnell, L.L. Lee, D.E. Herrmann, J. Zhong, J. Martini, T.M. Palmer, A. Sanbe, J. Robbins, S.R. Houser, W.J. Koch, A.M. Feldman, Regulated overexpression of the α1-adenosine receptor in mice results in adverse but reversible changes in cardiac morphology and function. Circulation 114 (2006) 2240–2250.
[2] E.A. Hamad, W. Zhu, T.O. Chan, V. Myers, E. Gao, X. Li, J. Zhang, J. Song, X.-Q. Zhang, J.Y. Cheung, W. Koch, A.M. Feldman, Cardioprotection of controlled and cardiac-specific over-expression of A2a-adenosine receptor in the pressure overload. PloS ONE 7 (2012) e39919.
[3] V.G. Tusher, R. Tibshirani, G. Chu, Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 5116–5121.
[4] L. Zhang, E.A. Hamad, M. Vausort, H. Funakoshi, A.M. Feldman, D.R. Wagner, Y. Devaux, Identification of candidate long noncoding RNAs associated with left ventricular hypertrophy. Clin. Transl. Sci. 8 (2015) 100–106.