Gene 586 (2016) 56–61

Contents lists available at ScienceDirect

Gene

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

Research paper

Mitral valve prolapse is associated with altered extracellular matrix gene expression patterns

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ARTICLE INFO

Article history:
Received 5 January 2016
Received in revised form 31 March 2016
Accepted 2 April 2016
Available online 6 April 2016

Keywords:
Whole-transcriptome analysis
Myxomatous mitral valve
TGF-beta signaling

ABSTRACT

Mitral valve prolapse (MVP) is the leading indication for isolated mitral valve surgery in the United States. Disorganization of collagens and glycosaminoglycans in the valvular extracellular matrix (ECM) are histological hallmarks of MVP. We performed a transcriptome analysis to study the alterations in ECM-related gene expression in humans with sporadic MVP. Mitral valve specimens were obtained from individuals undergoing valve repair for MVP ($n = 7$ patients) and from non-beating heart-tissue donors ($n = 3$ controls). Purified RNA was subjected to whole-transcriptome microarray analysis. Microarray results were validated by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Gene ontology enrichment analysis was performed. 2046 unique genes showed significant differential expression (false discovery rate <0.5%). After demonstrating appropriate sample clustering, microarray results were globally validated using a subset of 22 differentially expressed genes by RT-qPCR (Pearson's correlation $r = 0.65$, $p = 0.001$). Gene ontology enrichment analyses performed with *ErmineJ* and *DAVID Bioinformatics Database* demonstrated overrepresentation of ECM components ($p < 0.05$). Functional annotation clustering calculated enrichment of ECM-related ontology groups (enrichment score = 4.1). ECM-related gene expression is significantly altered in MVP. Our study is consistent with the histologically observed alterations in collagen and mucopolysaccharide profiles of myxomatous mitral valves. Furthermore, whole-transcriptome analyses suggest dysregulation of multiple pathways, including TGF-beta signaling.

1. Introduction

Mitral valve prolapse (MVP) is a common disorder which affects over 7 million people in the United States and 144 million people worldwide (Verma and Mesana, 2009). Its clinical effects include the development of progressive mitral valve regurgitation, arrhythmias, congestive heart failure, and thromboembolic disease. Ten to 15% of patients with MVP will require mitral valve surgery, which may consist of mitral valve repair or replacement. In fact, MVP is currently the leading indication for isolated mitral valve surgery in the United States. Yet, despite the broad clinical experience with medical and surgical management of MVP, the cellular mechanisms of the disease are poorly understood.

The histological *sine qua non* of MVP is myxomatous degeneration of the valve leaflets and chordae. Increased glycosaminoglycan and proteoglycan deposition occurs in the zona spongiosa leading to greater water content (Gupta et al., 2009; Tamura et al., 1995; Grande-Allen et al., 2001). Immunohistochemical studies have shown upregulated expression of the proteoglycans versican, decorin, and biglycan (Gupta et al., 2009). Collagen and elastin organization is also highly disrupted (Tamura et al., 1995; Rabkin et al., 2001; Rabkin-Aikawa et al., 2004; Nasuti et al., 2004). Activated valvular interstitial cells (VICs) may secrete matrix metalloproteinases (MMPs) including collagenases and gelatinases, which reduce the structural integrity of the valve (Rabkin et al., 2001; Rabkin-Aikawa et al., 2004; Mulholland and Gotlieb, 1996). These histological changes contribute to a thickened, floppy, dysfunctional valve.

Dysregulation of TGF-β signaling may underlie MVP in several non-idiopathic forms of the disease. For example, in Marfan's disease, fibrillin-1 mutations alter binding of latent-TGF-β to the ECM. Blocking antibodies to TGF-β and the angiotensin II receptor antagonist losartan abrogate the progression of MVP in fibrillin-1 knockout mice (Ng et al., 2004). Similarly, fenfluramine-associated MVP has been linked to

Abbreviations: MVP, mitral valve prolapse; ECM, extracellular matrix; RT-qPCR, quantitative reverse transcription polymerase chain reaction; VICs, valvular interstitial cells; MMPs, matrix metalloproteinases; RMA, robust multi-chip average; SAM, significance analysis of microarrays; FDR, false discovery rate; LVEF, left ventricular ejection fraction; ACE-I, angiotensin converting enzyme inhibitor.

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http://dx.doi.org/10.1016/j.gene.2016.04.004
0378-1119/

serotonergic upregulation of TGF-β signaling (Connolly et al., 2009). Microarray gene expression analysis of degenerative mitral valves from dogs also showed significant serotonin-TGF-β dysregulation (Oyama and Chittur, 2006). Whether dysregulation of TGF-β–ECM interactions underlie sporadic MVP in humans is unknown.

In this study we used a validated, whole-transcriptome microarray and bioinformatics approach to investigate altered patterns of gene expression and identify dysregulated signaling pathways in sporadic human MVP. The results provide gene expression evidence for the known histological alterations of MVP and also highlight new areas for future research.

2. Materials and methods

2.1. Patient characteristics and sample acquisition

Mitral valve specimens were collected from 10 individuals (7 MVP valves, 3 normal valves). All patients presenting to our institution for valve repair of isolated posterior mitral leaflet prolapse with characteristics of fibroelastic deficiency were considered. Patients with known connective tissue disorders, history of myocardial infarction, concomitant valvular disease, endocarditis, prior cardiac surgery, or Barlow's mitral valve morphology were excluded. Patient characteristics are summarized in Table 1. There was no significant difference in age ($p = 0.18$), gender ($p = 1$), and use of beta-blockers ($p = 0.17$) or ACE-inhibitors ($p = 0.08$) between the two groups. Five of the MVP patients had ruptured chordae with flail posterior leaflets. Surgical repair consisted of resection of the P2 scallop with concomitant mitral annuloplasty. The excised leaflet segment was immediately snap-frozen in liquid nitrogen and stored at −80 °C until further processing. Control specimens were obtained from otherwise healthy trauma victims with no known mitral valve pathology through a commercial biorepository (Bioserve, Beltsville, MD). These specimens were snap-frozen within 3 h of death. Institutional Review Board approval was obtained, along with appropriate consent for sample acquisition.

2.2. RNA purification and microarray analysis

Frozen mitral valve specimens were transferred to RNAlater-Ice (Ambion, Austin, TX) and stored at −20 °C overnight prior to processing. Tissues were rapidly homogenized, and RNA was purified using RNeasy Fibrous Tissue Midi Kit (Qiagen, Valencia, CA). RNA integrity was confirmed by a bioanalyzer (Agilent RNA 6000 Nano Kit, Santa Clara, CA). Purified RNA samples were stored at −80 °C until further use.

Whole-transcriptome gene expression analysis was performed on 3 controls and 5 patient valves (C1–3, P1–5) by the NYU Genome Technology Center Core Lab. cDNA synthesis and linear amplification of 5 μl of template RNA were performed using the Ovation® RNA Amplification System V2 (NuGEN, San Carlos, CA). 3.75 μg of amplified cDNA was fragmented and 3′-biotin-labeled using the Encore™ Biotin Module (NuGEN, San Carlos, CA). Fragmented and labeled single-stranded cDNA was hybridized to Affymetrix HG U133 plus 2.0 microarrays (Affymetrix, Santa Clara, Calif) following a standard Affymetrix protocol.

Table 1
Patient characteristics.

Sample #	Valve type	Age (years)	Gender	Race	LVEF	Beta-blocker	ACE-I
C1	Normal	47	M	Vietnamese	N/A	−	−
C2	Normal	49	M	Vietnamese	N/A	−	−
C3	Normal	43	M	Asian	N/A	−	−
P1	MVP	58	F	Asian/Pacific Islander	75%	−	−
P2	MVP	69	M	Caucasian	70%	−	−
P3	MVP	33	M	African	50%	+	+
P4	MVP	78	M	Caucasian	60%	−	−
P5	MVP	54	M	Asian-Indian	60%	+	−
P6	MVP	50	M	Caucasian	60%	+	+
P7	MVP	44	F	Caucasian	60%	+	−

Abbreviations: MVP, mitral valve prolapse; LVEF, left ventricular ejection fraction; ACE-I, angiotensin converting enzyme inhibitor.

2.3. Data analysis

Analysis of microarray data was performed using FlexArray version 1.5 (http://genomequebec.mcgill.ca/FlexArray/). Microarray expression values were read and normalized, and background was subtracted using a robust multi-chip average (RMA) algorithm. Effective normalization of expression values was achieved.

Statistical comparison between groups was performed by the modified t-test, Significance Analysis of Microarrays (SAM) algorithm implemented by *MeV* (http://www.tm4.org/mev/) (Saeed et al., 2003). All possible permutations were used to generate an empirical null distribution. Delta-threshold was adjusted ($\delta = 2.6$) such that 3020 probes were classified as significant; this allowed an estimated 12.6 false-positive probes (yielding a false-discovery rate of 0.004). For comparison, a routine t-test was performed and genes were filtered for $FC > 2$ and $p < 0.05$. A clustering analysis was then performed by k-means and hierarchical clustering methods (*Matlab*, Mathworks, Natick, MA).

Probe ID-tags were annotated and duplicate and non-coding tags were filtered prior to submitting the complete gene set for further analysis. This complete dataset will be made available in the National Center for Biotechnology Information's Gene Expression.

2.4. Gene set analysis

Gene set ontology enrichment analysis was performed with *ErmineJ* (http://www.bioinformatics.ubc.ca/ermineJ/) using a published protocol (Gillis et al., 2010). Gene score re-sampling was performed using genes ranked by best-probe negative-log corrected-p-values. Gene set size-range was limited to values between 5 and 200, and 200,000 iterations were performed.

Additionally, functional anotational clustering analysis was performed using *DAVID Bioinformatics Database* (http://david.abcc.ncifcrf.gov) default settings. The set of 2046 significantly altered genes was divided into up-regulated and down-regulated groups for subset analysis. Significantly altered KEGG pathways were visualized using *KegArray* (http://www.genome.jp/kegg/). Transcription factor target enrichment analysis was performed with *WebGestalt v2* (http://bioinfo.vanderbilt.edu/webgestalt/).

A comparison of the results of the *ErmineJ* and *DAVID* gene-set analyses was achieved by tracing the parent GOSlim gene ontologies of significantly enriched gene-sets.

2.5. Real-time quantitative PCR

In order to validate the microarray data, a subset of 22 genes of interest were selected for RT-qPCR: *ACTN2*, *ADAMTS19*, *ADAMTS9*, *CHSY3*, *COL1A1*, *COL5A1*, *FBN1*, *FN1*, *ITGA2*, *ITGA7*, *ITGB1*, *LTBP2*, *LTBP4*, *MMP14*, *MMP16*, *PDGFA*, *SDC2*, *SPOCK3*, *TGFB2*, *THBS4*, *TNC*, *VCAN*. To select genes of interest, a leading-edge analysis was performed on the significantly enriched functional clusters (as determined by *DAVID*). Genes were chosen to vary over a broad expression range and also overlap several functional clusters. 100 ng of RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Primer pairs were synthesized (Integrated DNA Technologies, Coralville, IA) or purchased (SABiosciences, Frederick, MD), and efficiency curves were generated by 5 log-fold serial dilution. Sso7d Real-Time PCR (SsoFast™ EvaGreen® Supermix, Bio-Rad, Hercules, CA) was performed using 1 μl of cDNA