Online Appendix

Proteomic architecture of valvular extracellular matrix: FNDC1 and MXRA5 are new biomarkers of aortic stenosis

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Supplemental Materials and Methods

Collection of Secreted ECM in vitro

Human valve cells, plated onto 6 well plates at 10^5 cells/well, were treated with calcifying medium (2 mM NaH_2PO_4, 0.1 μM insulin, and 50 μg/ml ascorbic acid) for 3 and 7 days. [23] Secretome was collected at baseline and after 3 and 7 days of treatment and concentrated with Amicon ultra-4 centrifugal filter columns (Millipore) according to the manufacturer's protocol. Cells were isolated from 2 different patients and a total of 6 experimental replications was performed.

Proteomics Analysis

a) Sample preparation

Protein samples (100 μg) were collected in guanidine buffer and aliquoted for trypsin digestion. Protifi S-trap MS Sample Prep Columns [24] were utilized following established procedures that included: MTT reduction, TCEP alkylation, trypsin digestion at a substrate ratio of 10:1, and multi-step elution with samples dried to 1 μl in a Thermo CentriVac. Samples were then brought back to volume in 50 μl of 0.1% formic acid in water. Peptides (1 μg each) were analyzed using nLC-MS/MS on a Thermo 1200 EasynLC coupled directly to a Thermo Q-Exactive High Fidelity mass spectrometer.

b) LC-MS/MS analysis

Peptides were separated by reverse phase chromatography utilizing a 15 cm Phenomenex peptide Aeris XBC-18 1.7 μM column at a flow rate of 300 nl/min using a 110 min discontinuous gradient of ACN as follows: 3% B for 1 min, 3% to 5% B over 5 min, 5% to 35% B over 84 min, 35% to 60% B over 9 min, 60% to 90% over 2 min, and finally, 90% mobile phase B maintained for 10 minutes (Mobile phase A: 0.1% formic acid in water, Mobile phase B: 0.1% formic acid in 90% ACN). The mass spectrometer operated in Data-Dependent Acquisition (DDA) mode (TopN). A single acquisition cycle comprised a single full-scan mass spectrum (400 – 1600m/z) in the Orbitrap with mass accuracy set at 60,000, AGC=5e5, and Max IT=50ms. CID fragmentation on the top 15 most intense precursor ions was performed at 15,000 resolution, AGC=5e4, Max IT=50 ms, and 32 eV collision energy. Other parameters included charge state exclusion of unassigned, 1,7,8, >8, and exclusion of former target ions for 15 s.

c) Data processing and analysis

MS/MS spectra from raw files corresponding to single biological samples were extracted/ submitted to Proteome Discoverer (Thermo) for database searching against the UniProt SwissProt sequence human proteome database (Version 2017-10-25) containing the subset of human, herpesvirus, and common contaminant sequences (42,252 sequences). Spectra were searched against indexed peptide databases generated from the forward and reverse protein sequence entries utilizing dynamic modifications of methionine oxidation, asparagine and glutamine deamidation, serine and threonine phosphorylation, and carbamidomethylation set for cysteine. Mass tolerances were set to 10 ppm and 0.02 Da for the precursor and fragment, respectively. False discovery rate (FDR) was set to no greater that 95% for relaxed cutoffs. A maximum of 3 missed cleavages was allowed. For Precursor Ion Quantification, pairwise Ratio Based calculations were used for Analysis of variance (ANOVA) hypothesis testing and data were normalized to total peptide amount.

Peptide spectrum matches (PSMs) were then loaded into Scaffold (ver. 4; Proteome Software, Inc.) and post-search validation performed using X!Tandem (GPM 2010.12.1.1). The high mass accuracy search option was enabled. PSMs from described conditions were loaded onto a single Scaffold session and confidence filters were selected at a FDR < 1%: 99% protein confidence, and a minimum of 2 unique peptides per protein in at least one biological sample. Proteins passing these filters were analyzed with respective "Normalized Spectral Abundance Factor (NSAF)". NSAF was chosen because it accounts for protein variability and size between runs to normalize relative protein abundance between samples. [25]

d) Network and Pathway Analysis

ECM proteins present in the proteome obtained from valve tissue were identified by uploading the list of proteins to the Matrisome Project Website (http://matrisomeproject.mit.edu/). STRING v.11.0 (https://string-db.org/) was used to identify protein-protein interactions between the significant proteins identified in fibrotic and calcified valves utilizing a network analysis. ECM proteins were highlighted in the protein network figures. Pathway analysis was performed with STRING v.11.0 to determine the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched with differentially expressed proteins in different comparisons. KEGG pathways with FDR < 0.05 were considered significant.

Immunofluorescence Staining

Sections (7 μm) cut from OCT embedded valves were fixed in acetone/methanol solution for 10 minutes at 4 °C. Then, they were washed with TBS 1X, incubated in 1% TBS-BSA for 1 hour, and incubated with the following

Fluorescence was visualized using a Zeiss microscope and the relative mean fluorescence was quantified with NIH ImageJ. Region of interest were selected using drawing selection tool in ImageJ, from the analyze menu we selected set measurements and then measure from the analyze menu. The background was subtracted by calculating the mean fluorescence from the regions that have no cells. Six to seven randomly selected microscopic fields per section zone of each sample were examined.

RNA Sequencing

We have previously described the analysis and the procedure for RNA sequencing [28]. Briefly, the study included 18 men aged 18 years and above. Surgically explanted tricuspid aortic valves (tricuspid aortic valve calcified TAVc, n=9) with a fibro-calcific remodeling score of 3 were selected to reduce heterogeneity and variations that could occur as a result of the remodeling process itself. The control valves (tricuspid aortic valve normal TAVn, n=8) were obtained from individuals undergoing orthotopic heart transplantation without evidence of aortic valve disease and with normal aortic valve function determined by echocardiography. Patients were recruited at the Institut Universitaire de Cardiologie et de Pneumologie de Québec (IUCPQ) during surgeries performed between March 2005 and May 2011. The TAVc patients were matched by age±10 years with respect to the TAVn controls. Only nonsmokers without type 2 diabetes, renal insufficiency (defined as a serum creatinine > 150 μmol/l), or an ascending aorta replacement were included in the study. Written informed consent was obtained from all participants. The study was approved by the ethics committee of IUCPQ.

Supplement Table 1: Clinical characteristics of patients used for interstitial cells isolation

	VAHN n=2
Males, %	50%
Age	45±60
Weight, Kg	170.2±6.3
Smoking, %	0%
Metabolic markers	
Cholesterol, mg/dL	143±18
HDL, mg/dL	90.1±08
LDL, mg/dL	68.5±23
Echocardiography	
Peak gradient mmHg	ND
Mean gradient, mm Hg	ND
Peak aortic jet velocity, m/s	1.3±0.40
Ejection fraction, %	52%±30

Continuous data are expressed by mean ±SD. Categorical data are expressed by number (percent). VAHN: Normal aortic valve, VAHC: Calcified aortic valve. HDL: high-density lipoprotein; LDL: low-density lipoprotein. ND: not determined.