Discovery of Mitochondrial Protein Biomarkers of Atrial Fibrillation Using Unique Human Tissue Samples

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Overview

The objective of this project is to discover mitochondrial protein biomarkers of atrial fibrillation (AF). Our research strategy is based on a comparative mass spectrometric (MS) analysis of mitochondrial protein fractions isolated from lysates of left and right atrial tissue obtained from AF patients and non-AF control subjects. The experimental approach of online liquid chromatography-tandem mass spectrometry (LC-MS/MS) followed by database search and manual data and results evaluation was used to identify peptides and phosphorylated peptides from cardiac tissue mitochondria protein tryptic digests. Potentially differentially abundant peptides/proteins in right atria of AF patients v. controls, and in left v. right atria of AF patients, were determined by spectral counting-based comparative analysis. The initial results of the study include several possibly differentially-abundant proteins and phosphoproteins with potential relevance to AF. Selected candidate biomarkers will be subjected to verification analyses using antibody- (reverse phase protein microarray, RPMA) and MS- (MRM) based analyses.

Introduction

Atrial fibrillation is the most common of sustained arrhythmias encountered in clinical practice and results in significant increase of risk for stroke, premature death and heart failure. More than 2.3 million individuals in the United States are affected with atrial fibrillation with an expected ~6 million patients by 2050. The occurrence of atrial fibrillation is associated with age, race and gender with increasing prevalence with age. Multiple studies suggest that atrial mitochondria have a significant role in the pathophysiological processes of atrial fibrillation, including our finding of mitochondria dysfunction in response to simulated ischemia (1). Despite extensive research, it is unclear whether mitochondrial changes are secondary to the general structural remodeling of atrial tissue, or if mitochondrial dysfunction is related to the occurrence of atrial fibrillation. There is significant potential impact of this study for atrial fibrillation with respect to new biomarkers for the risk to develop arrhythmia as well as markers for new drug therapies, device therapies and hybrid approaches.

AF is an abnormality of the heart electrical system with an occurrence after open heart surgery of >25%. In the Maze procedure incisions are made in the atria to disrupt aberrant signal circuits and thereby create one correct path for the electrical impulse. Even though the Maze procedure success rate is high, 15% of patients develop AF after the procedure.

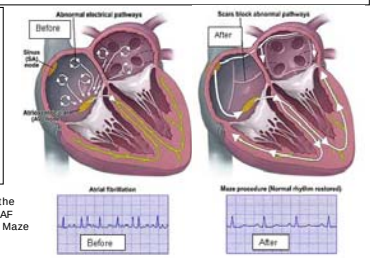

Figure 1: illustration of the electrical circuitry in an AF patient before and after Maze surgery

Methods

Samples and Mitochondria Enrichment: Left and right atrial tissue samples were acquired from 10 patients undergoing the Maze surgery, and right atrial tissue samples were collected from 10 patients undergoing another type of cardiac surgery. The tissue samples were flash frozen in liquid nitrogen in the operating room, and later stored at -80C. 200mg of each tissue sample were used to create a homogenized tissue lysate using hydrostatic pressure cycling technology (Figure 2) (Barocycler, Pressure Biosciences, South Easton, MA). The mitochondria proteins were enriched from the cell lysates using a commercial kit (Mitochondria Isolation Kit, Biochain Inc, Hayward, CA). The protein concentrations of the tissue lysate, mitochondria and cytosolic fractions were measured by Bradford assay. The mitochondria protein yield was highly variable (998.15±773.55 μg/mL).

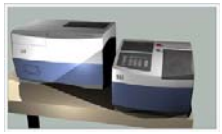

Figure 2: Barocycler NEP 3229

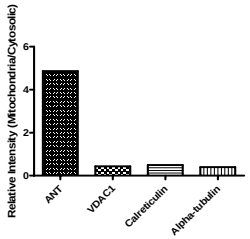

Figure 3: Western assay of mitochondria and cytosol protein markers

Sample QA/QC Assay: As a measure of mitochondria enrichment, aliquots of the fractions were subjected to antibody-based assays for marker proteins of mitochondria (ANT, adenine nucleotide transporter, and VDAC1, voltage-dependent anion channel 1) and cytosol (alpha-tubulin and calreticulin). In addition to initial Western assays, microarray slides were printed with fraction aliquots for SYPRO Ruby staining and RPMA analyses. These assays yielded expected mitochondria:cytosol abundance ratios for ANT, alpha-tubulin and calreticulin; however, a greater abundance of VDAC1 was measured in the cytosol relative to that in the mitochondria fraction (Figure 3).

LC-MS/MS Analysis: An aliquot of each mitochondria fraction was prepared for MS analysis by reduction and alkylation followed by trypsin digestion. For global proteomics analyses, a 10μg digest aliquot was analyzed by LC-MS/MS with a 2-hour HPLC gradient and a top 8 data dependent acquisition method using a Thermo LTQ-Orbitrap mass spectrometer. For global phosphoproteomics analyses, phosphopeptides were enriched from the digest using a TiO_2-based method. Internal protein and peptide standards were used for method quality control. The MS data were searched against a human protein database (NCBI) with SEQUEST, and the results were filtered to obtain high confidence matches (~1% FDR) Comparative analysis was based on spectral counts, which was accomplished using Scaffold (Proteome Software, Inc.). Candidate differentially abundant peptides were evaluated by manual inspection of the raw data to confirm peptide identification and determine signal intensities.

Results

Global Proteomics: Mitochondrial proteins were analyzed from three sets of samples to allow two comparative analyses:

(1) left v. right atrial tissue from each of 10 AF patients (AF left v. AF right) and
(2) right atrial tissue from 10 AF v. 10 non-AF patients (AF right v. non-AF right).

The analyses yielded identification of ~600-700 proteins of which up to ~60% have mitochondrial gene ontological (GO) cell compartment annotations. Analysis of peptide/protein relative abundances based on spectral counts (with manual confirmation) yielded a large number of potentially differentially-expressed proteins (t-test p-value<0.01):

No. of proteins Diff. Abundant	Comparative Analysis
45 proteins	AF left > AF right
19 proteins	AF right > AF left
28 proteins	AF right > non-AF right
4 proteins	non-AF right > AF right

A subset of these proteins and associated spectral counts are shown in Table 1. It is worthwhile to note that several of the above AF v. non-AF potentially differentially abundant proteins are consistent with results of a recent report, e.g., crystallin alpha beta and desmin (2). However, our results indicate an opposite relative abundance of glyceraldehyde 3-phosphate dehydrogenase, which might be due to our targeting of mitochondrial proteins (v. total proteins).

Table 1: Initial Global MS Proteomics Results (Spectral Count Analysis)

	Total Assigned MS/MS Spectra	
Protein	**AF Left**	**AF Right**
mitochondrial aldehyde dehydrogenase 2	98	6
cytochrome c-1	50	8
NADH dehydrogenase (ubiquinone)	66	3
voltage-dependent anion channel 1	90	12
ribonuclease/angiogenin inhibitor	7	53
decorin isoform d	7	25
tubulin alpha 2 isoform 1	16	43
crystallin alpha B	26	102
	AF Right	**non-AF Right**
crystallin alpha B	143	32
desmin	109	38
heat shock 27kDa proteins 1 and 2	94	36
acyl-coenzyme A dehydrogenase	112	28
glyceraldehyde 3-phosphate dehydrogenase	69	120
gelsolin isoform b	51	89

Global Phosphoproteomics: Initial analysis of mitochondria phosphoproteins, performed to assess the performance of our method, yielded identification of 35 phosphopeptides corresponding to 30 proteins. A subset of these results is shown in Table 2.

Table 2: Initial Global MS Phosphoproteomics Results

Protein	Identified Phosphopeptide	Detected Global Proteomics?	Phosphosite Known?
solute carrier 25 (mitochondrial carrier)	DFLAGGVAAAVpSK	yes	not found
myozenin	QLHEGASLELpSDDDTESK	yes	yes
voltage-dependent anion channel 1 (VDAC1)	LTFDSSFpSPNTGK	yes	yes
connexin 43	QApSEQNWANYSAEQNR	no	yes
ATPase Ca++ transporting, cardiac muscle	EFDELNPpSAQR	yes	not found

Several phosphoproteins could be related to AF, e.g., solute carrier 25 is a calcium-dependent carrier of metabolites across the mitochondria membrane, and VDAC1 (a mitochondria marker) has been studied with respect its phosphorylation status and mitochondria membrane permeability (3). In addition, connexin 43 and several of its phosphorylation sites have been studied in regard to ischemia (4).

Conclusion

These first studies in a comparative analysis of mitochondria proteins from unique human atrial tissue samples from AF patients and controls have yielded candidate biomarkers and an initial set of phosphoproteins. The next steps are to identify differentially abundant phosphoproteins (AF v. non-AF) and to verify candidate biomarkers by antibody- (reverse phase protein microarray) and MS- (MRM) methods.

Literature Cited

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