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Proteomics discovery of biomarkers for mitral regurgitation caused by mitral valve prolapse

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ABSTRACT

Mitral regurgitation (MR) is a common valvular lesion frequently caused by mitral valve prolapse (MVP). Surgical intervention in MVP patients with significant MR is predicated on symptoms and measures of left ventricular dysfunction. Because these indicators may be subjective or imprecise, serological biomarkers of disease could be a valuable adjunct to standard evaluation. This study aimed to identify such biomarkers by a proteomics approach. Two pooled plasma samples from 24 MVP subjects with MR (MVP/MR) and 24 non-MVP individuals were treated with the combinatorial peptide ligand library (CPLL) beads prior to iTRAQ labeling and ESI-MS/MS. Lower levels of haptoglobin, platelet basic protein (PBP), and complement component C4b were observed in the MVP/MR as compared to the control sample. These findings were verified by ELISA testing of each of the 24 paired samples, and another 42 matched cases and controls. The AUC values, sensitivities and specificities for (i) haptoglobin, (ii) PBP, (iii) C4b, and (iv) all 3 proteins in combination were (i) 0.813, 76%, 74%; (ii) 0.721, 56%, 77%; (iii) 0.689, 83%, 49%; and (iv) 0.840, 89%, 67%, respectively. In conclusion, haptoglobin, PBP, and C4b are down-regulated in MVP/MR. Their value as serological biomarkers of valvular pathology should be further explored.

Biological significance
We report the first study that performed comparative proteomics of clinical human plasma samples to identify novel diagnostic biomarkers for mitral valve prolapse (MVP) patients with moderate to severe mitral regurgitation (MR). MR is a common valvular lesion that can be complicated by heart failure, sudden death and atrial fibrillation, yet many patients with severe MR are asymptomatic. Our results revealed reduced levels of haptoglobin, platelet basic protein (PBP), and complement component C4b in the MVP/MR patients as compared to the matched control cases. The plasma proteomics findings were subsequently confirmed by ELISA. Each of these candidate biomarkers has a putative role in the pathophysiology of MVP/MR, further supporting their roles in detection and possibly surveillance and prognostication of this disease.

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1. Introduction

Mitral regurgitation (MR) is a common valvular lesion, and in the United States and Europe, most frequently caused by mitral valve prolapse (MVP) [1]. When severe, MR can be complicated by heart failure, sudden death and atrial fibrillation [2]. However, many patients with severe MR are asymptomatic, even when left ventricular dysfunction supervenes [3]. The optimal timing of surgical intervention is frequently challenging and requires input from ancillary testing, including echocardiography to assess valve morphology, the degree of MR and extent of left ventricular remodeling. However, echocardiography is costly, operator-dependent, and quantitation of MR is not always reliable, with significant inter-observer variability [4]. Surgery is indicated for symptoms which can be insidious and difficult to interpret in the elderly and deconditioned. This subjectivity is concerning given the attendant risks of surgical repair or replacement. To improve detection, monitoring and management, serological biomarkers with levels correlating with disease pathology would be helpful. While the role of biomarkers in management of MR remains undefined [5], evidence is accumulating that plasma B-type natriuretic peptide may be useful [6].

Plasma proteomics has been applied in identification of biomarkers and therapeutic targets of cardiac dysfunction [7,8]. However, proteomics studies dedicated to biomarker discovery in MVP and MR are limited. A shotgun proteomics analysis of myxomatous canine mitral valves identified more than 300 proteins associated with various stages of valve degeneration, with 117 being differentially expressed [9]. To date, there are no reports of plasma proteomics in human MR. In this study, we aimed to use a comparative proteomics approach to discover novel plasma biomarkers in patients with moderate to severe MR caused by MVP (MVP/MR).

2. Materials and methods

2.1. *Plasma samples from MVP/MR patients and non-MVP individuals*

Plasma samples were collected from 66 MVP/MR patients and 66 individuals without MVP seen as cardiology outpatients at National University Hospital, Singapore. Peripheral venous blood samples were collected into Vacutainer EDTA tubes, separated by centrifugation at 3077 ×*g* for 10 min and the plasma aliquoted and stored at −80 °C until further analysis. The samples were subjected to no more than 2 freeze–thaw cycles before any test. Biological samples were anonymized. All subjects gave written informed consent for the study which was approved by the National Healthcare Group Domain Specific Review Board.

Patients were included if they had no or minimal symptoms (New York Heart Association functional class I–II), prolapsed and/or flail mitral leaflets and moderate to severe MR on echocardiography, defined as a regurgitant volume quantitated by either the proximal isovelocity surface area or continuity equation method to be ≥35 ml. Because there is heterogeneity within the spectrum of degenerative MR, we included only MVP/MR subjects with presumed myxomatous or Barlow's disease characterized by diffuse, multisegmental leaflet involvement and marked annular dilatation, as opposed to isolated chordal rupture in fibroelastic deficiency [10]. Exclusion criteria for MVP/MR patients included greater than mild aortic regurgitation or moderate tricuspid regurgitation, chronic atrial fibrillation, left ventricular ejection fraction <60%, heart failure in the past 3 months, active endocarditis, previous myocardial infarction, coronary revascularization or major regional wall motion abnormality, significant renal impairment (serum creatinine > 200 μmol/l) and pregnancy. Non-MVP individuals were either asymptomatic or had cardiovascular complaints or conditions other than valvular heart disease, and previously documented not to have MVP or valvular dysfunction by echocardiography. They were matched for age and gender with MVP/MR cases.

2.2. *ProteoMiner treatment of plasma samples*

The plasma samples were treated with the combinatorial peptide ligand library (CPLL) beads (ProteoMiner) following the manufacturer's protocol (Bio-Rad, Hercules, CA). 20 μl of plasma samples from each of the 24 MVP/MR patients and 24 non-MVP subjects was pooled separately, and each pooled sample was centrifuged at 10,000 ×*g* for 10 min at 20 °C. 200 μl of the pooled MVP/MR and non-MVP plasma samples was loaded into the small capacity ProteoMiner column (Bio-Rad) separately. The column was washed with 200 μl of 20 mM phosphate buffer (pH7.0) thrice. The bound proteins were eluted from the column with 80 μl of boiling 4% SDS and 25 mM tris(2-carboxyethyl)phosphine (TCEP) twice [11]. Duplicates of each group of pooled plasma samples were processed as outlined in Fig. 1.

2.3. *Protein quantitation*

The protein content was quantitated using the Pierce 660 nm Protein Assay Reagent according to the manufacturer's manual (Pierce Biotechnology, Rockford, IL).

2.4. *iTRAQ 8-plex labeling*

The protein samples eluted from the ProteoMiner columns were labeled with iTRAQ 8-plex reagents (Fig. 1) according to the manufacturer's protocol (AB SCIEX, Foster City, CA). Briefly, 50 μg of each protein samples was topped up to 20 μl with 500 mM triethylammonium bicarbonate (TEAB), and 2 μl of the reducing regent (50 mM mM TCEP) was added to each sample, and incubated at 60 °C for 1 h. Following this, 1 μl of cysteine blocking reagent (200 mM methyl methanethiosulfonate) was added to each sample, and incubated at room temperature in the dark for 10 min. Subsequently, 10 μl of the trypsin solution was added to each sample, and incubated at 37 °C for 16 h. These samples were subjected to SDS-PAGE to confirm digestion efficiency. The peptide digest of each sample was then labeled with the respective iTRAQ reagents (Fig. 1) at room temperature for 2 h. The iTRAQ-labeled samples were pooled and subjected to clean-up and desalting using a strong cation exchange cartridge (AB SCIEX) and a Sep-Pak (Millipore, Bedford, MA) respectively, as described previously [12].