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Original Article

Proteomic analysis of mitral valve in Lewis rat with acute rheumatic heart disease

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Abstract: Rheumatic heart disease (RHD) makes a heavy burden in human lives and economy. The proteomic analysis of acute rheumatic heart disease (ARHD) can provide precious data to study RHD at the early stages, but no one has looked into. So based on our early research we applied the method of continuous GAS stimulation on Lewis rats to duplicate the animal model of ARHD. And the mitral valves of rats in control group (n=10) and ARHD group (n=10) were selected for proteomic analysis of ARHD with the iTRAQ labeling based 2D LC-ESI-MS/MS quantitative technology. We identified 3931 proteins in valve tissue out of which we obtained 395 differentially expressed proteins containing 176 up-regulated proteins and 119 down-regulated proteins. Changes in levels of GAPDH (6.793 times higher than the control group) and CD9 (2.63 times higher than the control group) were confirmed by Western blot or immunohistochemistry. The differentially expressed proteins such as GAPDH, CD9, myosin, collagen and RAC1 may be potential biomarkers for ARHD. Moreover, the mitral valve protein profile shed light on further understanding and investigating ARHD.

Keywords: Acute rheumatic heart disease, ITRAQ quantitative proteomics, mitral valve tissue, animal model, mitral valve protein profile

Introduction

The number of people with rheumatic heart disease (RHD) is 15.6-19.6 million worldwide [1]. The incidence, morbidity and mortality of RHD remain high among human population and make a heavy burden in their lives and economy [2-4]. The mitral valve dysfunction is the most common valve lesion in patients with RHD, particularly in the early stages [5, 6].

To date, there are mostly two hypotheses about the development of RHD. Tandon et al. proposed the collagen-mediated autoimmunity in cardiac valve [7]; Another study indicated molecular mimicry between streptococcus and the heart, which may be closely related with the former hypothesis [8, 9]. But the exact molecular pathway is still not completely elucidated.

Changes of protein profiles in RHD have been extensively detected in humans such as, PDIA3, HSPA5, vimentin and HSP60 in valve tissue of patients with chronic RHD [10, 11]. Ago et al. [12] also have detected the altered plasma protein profiles. However, they were based on the chronic RHD in patients who were not diagnosed and treated at the initial stage of a disease. Further investigations with suitable animal model of RHD are required to study acute rheumatic heart disease (ARHD) that is defined as the early inflammatory injury in myocardium or valve without the formation of fibrosis and calcification. Although the animal model mainly on Lewis rats have been widely researched [13-15], the rate of success in animal model of RHD was not very high. In our previous study, we compared three immune methods of RHD and successfully found an optimal animal model that can overcome the defect and we can effortlessly obtain the mitral valve of ARHD.

In this study we used the Group A-hemolytic streptococci (GAS) with continuous stimulation

on Lewis rats to duplicate the RHD model. We initially studied the proteomic analysis of valve tissue in Lewis rats of ARHD with iTRAQ coupled 2D LC-ESI-MS/MS quantitative technology. Ultimately, we aimed to identify putative protein biomarker candidates for ARHD, which can be useful for its molecular diagnosis and monitoring, and to provide the altered mitral valve protein profile for further understanding and investigating the ARHD.

Materials and methods

Animals and materials

Thirty-six 8-week-old female Lewis rats (150-180 g) provided by Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) were used to create the RHD model. They were housed in the animal experiment center of Guangxi medical university, maintained under SPF conditions and 12 h light/dark cycle at 23±2°C, with free access to water and diet. All experimental protocols were carried out according to the ethical guidelines about the Care and Use of Laboratory Animals. Unless indicated, all reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Bacterial culture, inactivation and antigen preparation

GAS were cultivated for 24 h, collected and washed by normal saline. Then inactivated in 10 ml of 40 g/l paraformaldehyde solution for 6 h, washed and suspended in sterile normal saline, adjusted concentration to 1×10^{11} CFU/ml at 600 nm absorbance. Finally emulsified in Complete Freund's Adjuvant (CFA, Sigma, USA) at a 1:1 ratio (v/v) to compound the antigen I.

Induction of animal model of rheumatic heart disease

Rats were randomly assigned to two groups: RHD group and control group respectively had 18 rats. Rats in RHD group were immunized with antigen I at the foot pad firstly. After one week, antigen I was injected in abdominal subcutaneous mass once a week for 4 weeks, then given inactivated GAS until to 24 weeks. Rats in control group were immunized with the same protocol outlined as treatment groups but without GAS. At 12 weeks 24 rats were sacrificed (each group comprised of 12) and at 24 weeks 12 were sacrificed (each group comprised of 6).

Sample collection

Hematoxylin-eosin (HE) staining and Masson staining were used to observe the pathological changes in hearts. The anterior mitral valve of every rats stored with the myocardium were immersed in 10% neutral buffered formalin for 8 hours, then cut four-micrometer sections and stained. The posterior mitral valve was excised without myocardium and preserved at -80°C until analyzed.

Mitral valve tissue protein preparation

Rats sacrificed after 12 weeks of antigenic induction was called the ARHD group. The mitral valves of rats sacrificed at 12 weeks in ARHD (n=10) and control (n=10) group were selected for proteomic analysis. In ARHD group, 10 valves were chosen from the rat succeeded in developing the rheumatic valvulitis and carditis. Total valve proteins of ten rats in each group were pooled at the same amount. The valves were desalted and concentrated. The supernatant was taken for determination of protein concentration by the Bradford method with Bradford reagents (Thermo Fischer Scientific, USA).

iTRAQ labeling

Total protein (100 μg) was taken from each sample solution, digested with Trypsin Gold (Promega, Madison, WI, USA). Then peptides were dried by vacuum centrifugation, reconstituted in 0.5 M TEAB and labeled with iTRAQ reagents according to the manufacture's protocol (Applied Biosystems). Samples were labeled with the iTRAQ tags as follows: 121 for the peptides of control group, 117 for RHD group.

LC-ESI-MS/MS analysis

A LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan) was used to perform the separation with strong cation exchange chromatography. Two iTRAQ labeled peptide mixtures were loaded onto a column (Φ5 μm, Phenomenex) in a gradient of buffer A or buffer B to be eluted. The eluted peptides were collected and pooled into 20 fractions, desalted with a Strata XC18

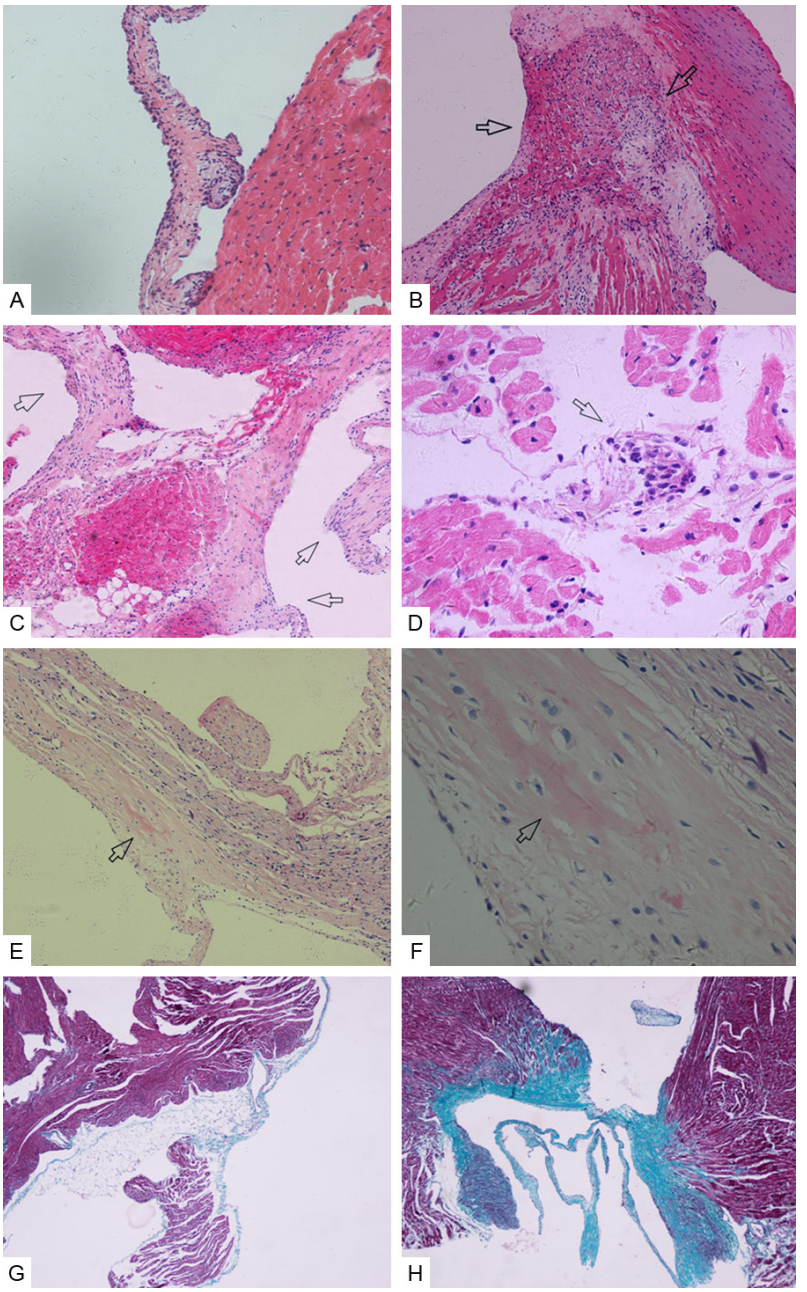

Figure 1. Hematoxylin and Eosin (H&E) staining and Masson staining of myocardial and valvular tissue. A. Normal myocardial and valve tissue in control group. Magnifications, ×200; B. Diffuse inflammatory cells infiltration in myocardium (arrows). Magnification, ×100; C. Diffuse inflammatory cells infiltration in valve (arrows). Magnification, ×100; D. Anitschkow cells (arrows). Magnification, ×400; E, F. Valve fibrosis (arrow) from 24 weeks of rats. Magnification, ×100 and ×400; G. Masson staining of valve in control group at 24 weeks. Magnification, ×40; H. Masson staining of valve in RHD group at 24 weeks. Magnification, ×40.

column. The fractionated peptides re-suspended in buffer A were loaded on a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) by the autosampler onto a 2 cm C18 trap column. Then the peptides were eluted onto a 10 cm analytical C18 column (inner diameter 75 μm) packed in-house. The MS Data acquisition was performed with a Triple TOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA).

Database search

The MS/MS data were searched for the species of Rattus using Mascot search engine (Matrix Science, London, UK; version 2.3.02) with the Uniprot website. For protein identification, we selected search parameters as follows: fragmented mass tolerance, 0.1 Da; peptide mass tolerance, 0.05 Da; max missed cleavages, 1; enzyme: trypsin. Only peptides with significance scores (≥20) at 99% confidence interval by a Mascot probability analysis showing greater than "identity" were considered as identified and every identified protein involved at least one unique peptide. The weighed and normalized quantitative protein ratios were subjected for analysis and the ratios with *P*-values < 0.05, fold changes of > 1.2 were counted as significant. Protein annotations were performed with the Blast2GO program and then applied the kEGG database to classify these identified proteins.

Western blotting and immunohistochemical analysis

The rest of protein samples were used for verifying the up-regulated expression of GAPDH by

Table 1. Up-regulated tissue protein in RHD group compared to control group

Accession number	Protein name	Coverage (%)	RHD group_117: control group_121	*p*-Value
Q4KM66	LOC500183 protein	42.7	2.388	< 0.05
M0R660	Glyceraldehyde-3-phosphate dehydrogenase	47.7	6.793	< 0.05
M0R7B4	Protein LOC684828	17.8	2.357	< 0.05
P58775-2	Isoform 2 of Tropomyosin beta chain	51.1	2.864	< 0.05
M0R6R6	Uncharacterized protein	13.9	2.903	< 0.05
Q5PPG2	Legumain	7.6	2.02	< 0.05
D3ZP98	Histocompatibility 13	2.7	2.111	< 0.05
P47875	Cysteine and glycine-rich protein 1	36.8	2.3	< 0.05
P60203	Myelin proteolipid protein	7.6	2.972	< 0.05
Q62636	Ras-related protein Rap-1b	50	2.155	< 0.05
B0BMS8	Myl9 protein	33.7	2.795	< 0.05
P62912	60S ribosomal protein L32	15.6	2.409	< 0.05
P40241	CD9 antigen	15	2.63	< 0.05
P20767	Ig lambda-2 chain C region	41.3	2.155	< 0.05
Q6RUV5	Ras-related C3 botulinum toxin substrate 1 (RAC1)	11.5	2.826	< 0.05
P62836	Ras-related protein Rap-1A (RAP1)	35.3	2.31	< 0.05
F1LUS1	Uncharacterized protein (Fragment)	17	2.261	< 0.05
Q91V33	KH domain-containing, RNA-binding, signal transduction-associated protein 1	6.5	2.536	< 0.05
B0K040	Sh3bgr protein (Fragment)	17.5	2.221	< 0.05
G3V6S3	Calumenin	32.1	2.001	< 0.05

Western blotting. Immunohistochemistry analysis was used for qualitative analysis of CD9. 10% sodium dodecyl polyacrylamide gel electrophoresis and immunoblotting were performed according to the standard protocol with equal amounts of proteins (20 μg). The antibodies used for testing are as follows: mouse anti-β-ACTIN (1:5000), anti-GAPDH (1:2000) (Vazyme Biotech Co. Ltd) and the goat anti-mouse IgG (1:10000, licor, USA) was used as the secondary antibody. β-actin was used as a normalization control. Blots were scanned with LAS4000 (Fujifilm, Tokyo, Japan) and the data were analyzed with Image J software. Immunohistochemistry was performed with rabbit antibodies for CD9 (1:25) overnight at 4°C, and then sections were incubated with a secondary antibody (MaxVision HRP-Polymer IHC kit, Fuzhou Maixin Biotech. Co. Ltd) for 15 min at room temperature. The DAB substrate system was used for color development.

Results

Growth state observation of Lewis rats

Redness and swelling appeared at the foot joints of all rats after the first immunization and disappeared after 2 weeks. But in treatment group it persisted in other foot joins following successive immunizations, without causing any abnormalities.

Pathological examination of myocardium and valve

HE staining showed that none of the rats in control group had myocarditis or valvulitis (**Figure 1A**). In RHD group at 12 weeks: only two rats were not presented with valvulitis or myocarditis; Diffuse inflammatory cells infiltration and accumulated Anitschkow cells were found in myocardium and valve (**Figure 1B-D**). Fibrosis of valves appeared in five of six rats in RHD group at 24 weeks (**Figure 1E**, **1F**). The results of Masson staining of valve in RHD group at 24 weeks were just similar to the HE staining which is an indication that more collagen fibers accumulated at valves in RHD group than in control group (**Figure 1G**, **1H**).

Differential expression of proteins between the ARHD group and control group

Using LC-ESI MS/MS, we identified 3931 proteins in the mitral valve of Lewis rat. Compared with the control group, a total of 295 proteins showed significant differences in ARHD group with 95% confidence. Since the fold change of 1.2 (fold change is the ratio of intensity of protein expression in ARHD to control group valve tissue) was considered as a threshold to minimize biological and technical errors, of which 176 proteins were found to be up-regulated and 119 proteins were down-regulated. The

Table 2. Down-regulated tissue protein in RHD group compared to control group

Accession number	Protein name	Coverage (%)	RHD group_117: control group_121	*p*-Value
G3V721	WW domain binding protein 2, isoform CRA_b	13	0.478	< 0.05
Q4FZZ3	Glutathione S-transferase	13.5	0.376	< 0.05
F1LM78	Protein LOC100365958	9.8	0.334	< 0.05
F1LQQ1	Malic enzyme (Fragment)	12.2	0.471	< 0.05
D4A1W8	Microsomal triglyceride transfer protein	3.5	0.388	< 0.05
M0RDH0	Glycine N-methyltransferase	18.8	0.164	< 0.05
F1LR47	Uncharacterized protein	4.8	0.231	< 0.05
G3V6C2	Protein Hgd	16.6	0.211	< 0.05
Q68G44	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (Mitochondrial)	27	0.478	< 0.05
D3ZSF3	Uncharacterized protein	17.8	0.392	< 0.05
Q5M897	Otc protein	20.3	0.264	< 0.05
M0RDC5	Acyl-CoA-binding protein (Fragment)	51.2	0.443	< 0.05
P09034	Argininosuccinate synthase	28.6	0.28	< 0.05
P19112	Fructose-1,6-bisphosphatase 1	8.3	0.442	< 0.05
P32755	4-hydroxyphenylpyruvate dioxygenase	14	0.248	< 0.05
F1LS48	Acetyl-CoA acetyltransferase, cytosolic	2.8	0.205	< 0.05
P21213	Histidine ammonia-lyase	4.9	0.152	< 0.05
P11510	Cytochrome P450 2C12, female-specific	3.7	0.183	< 0.05
P80432	Cytochrome c oxidase subunit 7C, mitochondrial	17.5	0.207	< 0.05
P0CC09	Histone H2A type 2-A	35.4	0.208	< 0.05
Q71US7	Cytochrome P450 2E1 (Fragment)	5.5	0.216	< 0.05
P0C169	Histone H2A type 1-C	35.4	0.098	< 0.05
D3ZJ08	Histone H3	43.4	0.138	< 0.05
P09811	Glycogen phosphorylase, liver form	10.4	0.439	< 0.05
D3ZDW1	Protein Uroc1	6.1	0.347	< 0.05
P12928-2	Isoform L-type of Pyruvate kinase PKLR	6.1	0.327	< 0.05
P22734-2	Isoform 2 of Catechol O-methyltransferase	36.7	0.47	< 0.05
G3V885	Myosin-6	62.1	0.106	< 0.05
P19468	Glutamate--cysteine ligase catalytic subunit	4.6	0.463	< 0.05

fold change (> 2.0) of proteins selected in our study described in **Tables 1** and **2**. The two closely related protein GAPDH and CD9 antigen are validated by western blotting or immunohistochemistry in order to check the technical reliability of iTRAQ result. The immunohistochemistry staining results showed significant difference of CD9 expression on mitral valve between ARHD and control group. Expression of CD9 was almost nil in control group (**Figure 2A**) and was increased in cytoplasmic membrane and cytoplasm in ARHD group (**Figure 2B**). The western blotting indicated increased GAPDH and the gray scale in ARHD group is 4.5 of that of the control group (**Figure 3**). The ontology analysis of the identified proteins and their correlation with molecular functions showed that, they were involved in binding (48.78%) and catalytic activity (27.49%) (**Figure 4**). The pathway analysis based on the KEGG database about focal adhesion pathway associated with binding was shown in **Figure 5**.

Discussion

In the past few years, proteomics was widely applied in cardiovascular disease research to make progress in the diagnostic biomarkers and mechanisms of disease [16, 17]. However, the preceding proteomic analysis about RHD was restricted by the technique of 2-DE [10-12]. Moreover, it is worthy to highlight that the previous researches about RHD were either involved with chronic valve tissue or sera from RHD patients [10, 12, 18], which cannot be more intuitive to illuminate the early lesions of mitral valve. So the mitral valves of ARHD rats were selected for proteomic analysis. We obtained 295 different proteins, among them the significantly related protein such as GAPDH and CD9 antigen were confirmed and their expressions were consistent with the iTRAQ result. Moreover, the protein myosin 11, collagen, HSPA12A, RAC1, Myl9 and Mylk protein were also altered in the mitral valve and may

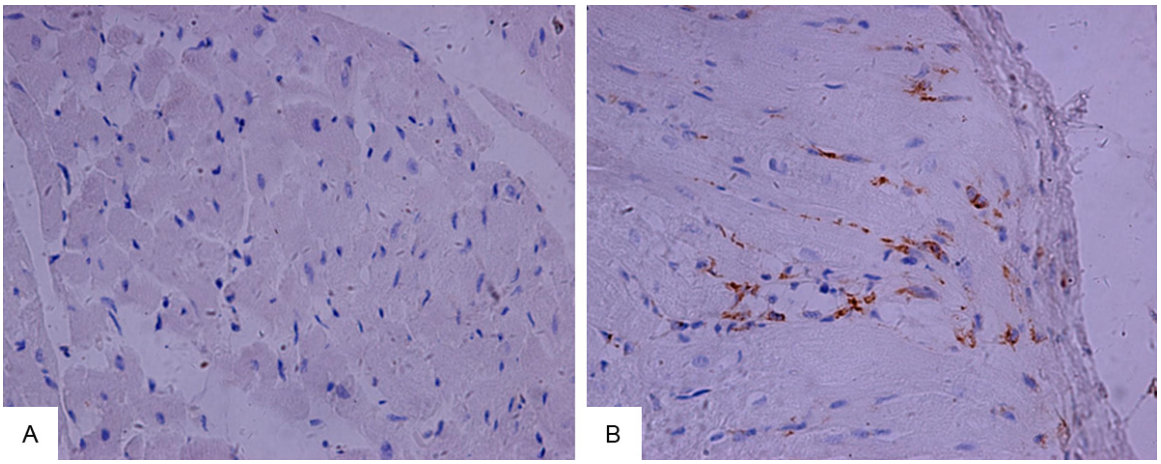

Figure 2. Validation of CD9 using immunohistochemical staining. A. CD9 was almost nil expressed in control group; B. CD9 overexpressed in cytoplasmic membrane and cytoplasm in ARHD group. magnification ×400.

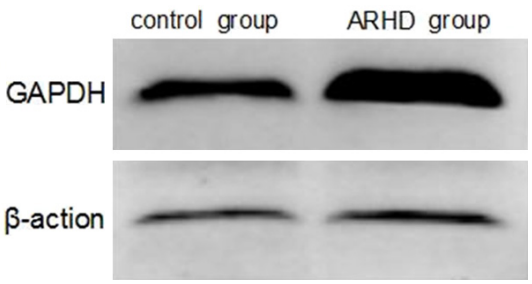

Figure 3. Validation of GAPDH on pooled lysates by Western blotting. GAPDH was up-regulated in ARHD groups and the gray scale in ARHD group is 4.5 of that of the control group, which is consistent with the iTRAQ result.

play an important role in the pathogenesis of ARHD.

In our early research, we compared three immune methods induced in animal model of RHD and selected an optimal one. So in this current study, we applied the method of continuous GAS stimulation on Lewis rats to duplicate the RHD model. In ARHD group, at 12 weeks diffuse inflammatory cell infiltrate and the Aschoff like cells were detected, and at 24 weeks, Masson staining also showed more collagen in valve than in control group. Our animal model proved to be more suitable in terms of the growth state of rats and pathological changes of myocarditis and valvulitis in this study. The pathological changes of valve at 12 weeks were similar to the ARHD. So we choose the valve of rat sacrificed at 12 weeks for the proteomic analysis of ARHD.

Because of the highest fold change of GAPDH (6.793 times higher than the control group), we verified the changes with western blotting. Except their role in glycolysis and as a reference gene, GAPDH plays significant role in processes of apoptosis and cellular signaling [19]. Apoptotic stimuli, for instance oxidative or genotoxic stress, results in GAPDH translocation and accumulation into nuclei [20, 21] to induce apoptosis. And cell apoptosis have essential role in cardiac valve calcification [22-26]. The apoptosis resulted in the initiation of calcification of mitral valve and probably even extended to the chronic phase [27, 28]. Other studies also demonstrated that GAPDH might be a candidate Ag for an autoimmune response to neurons and axons in the autoimmune disease such as MS [29, 30]. Based on these reports, we concluded that GAPDH may play an essential role in the development of ARHD via apoptosis.

CD9 antigen was 2.63 times higher than the control group. The tetraspanin CD9, also called as motility related protein-1 (MRP-1), has been shown to be involved with cell motility, growth, immune response and adhesion [31-33]. It may be ignored that CD9 regulated the formation of human multinucleated giant cell although the precise regulatory mechanism was not clear [34]. However, the appearance of multinucleated giant cells, known as Aschoff body strongly indicated granulomatous inflammation at proliferative stage of ARHD. Moreover, CD9 contacting with the surface-exposed RA-A47 might induce inflammatory reactions and autoanti-

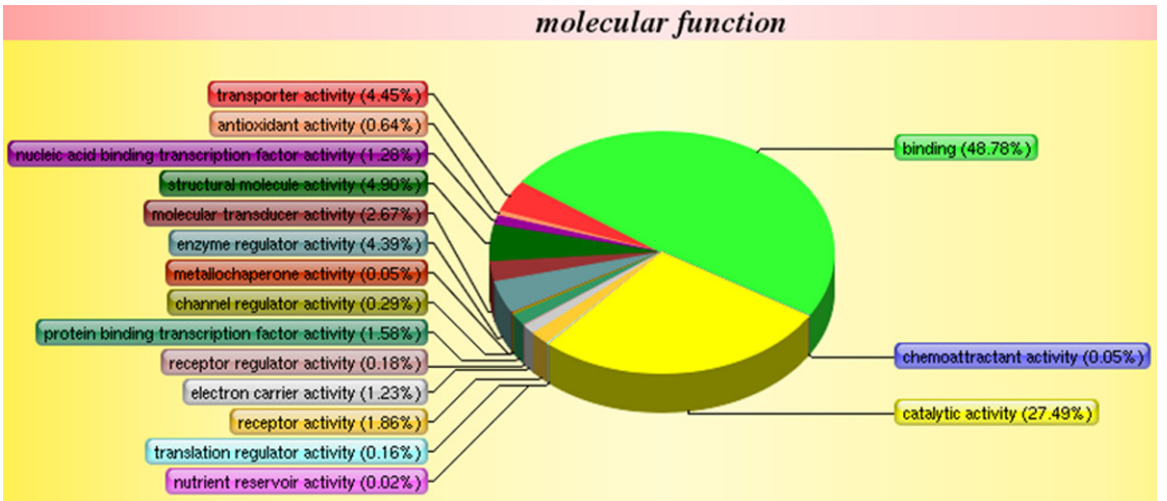

Figure 4. Gene ontology for molecular functions of the identified 3931 proteins between the valve of ARHD and control group. The function about binding accounts for the highest ratio of 48.78 percent.

bodies in autoimmune disease such as rheumatoid arthritis [35]. In our immunohistochemical analysis and study, we noticed overexpression of CD9 in valves, especially in the pericardial inflammatory cells. Therefore, CD9 is possibly associated with the inflammatory reactions in the valvular tissue of ARHD.

In addition, we likewise found elevated Myosin11, collagen I and V with the fold changes of 1.419, 1.665 and 1.392 respectively. It was widely believed for so long that, Myosin and Collagen were autoantigens initiated in the pathogenic progression in RF and RHD [7, 8]. A further study showed that, in acute rheumatic fever with carditis in a patient, collagen I was the major autoimmune response antigen between the putative heart valve components [9]. So our research verified previous work and underlined the role of myosin and collagen in the RF and ARHD as the autoantigen target of antibodies.

Heat shock 70 kDa protein 12A (HSPA12A) is an atypical member of HSP70 family, altered with the fold change of 1.282. HSP70 belonging to the stress protein have also shown to regulate some functions of immune response and also acting as an antioxidant [36]. It was reported that, the antibodies reacting with stress protein hsp70, hsp60 were detected in dilated cardiomyopathy [37]. In other investigation about RHD, HSPA5, hsp78 and hsp60 were considered to induce the autoimmune reaction and eventually lead to valve permanent damage [10, 11, 38]. Combining with the early study, we can speculate that, stress proteins including HSPA12A may be implicated in the inflammatory pathogenesis of ARHD.

In addition, molecular functions of identified proteins were involved in binding (48.78%). The KEGG pathway analysis showed that, the up-regulated RAC1, RAP1, Chondroadherin, Myl9 protein, Protein Mylk and Collagen I were closely related to the focal adhesion. With the development of ARHD, cross-reactive antibody binding with valvular endothelium could provide signal to accelerate the overexpression of adhesion molecules, which in turn could enhance the lymphocytes infiltration to valve [39].

In conclusion, with iTRAQ-coupled 2D LC-ESI-MS/MS we firstly identified 295 different proteins and formed the altered protein spectrum data of ARHD. The significantly altered protein such as GAPDH, CD9, myosin, collagen and RAC1 may be potential biomarkers for ARHD. Moreover, the mitral valve protein profile we delineated in this study will provide useful information for further understanding and investigating ARHD.

Acknowledgements

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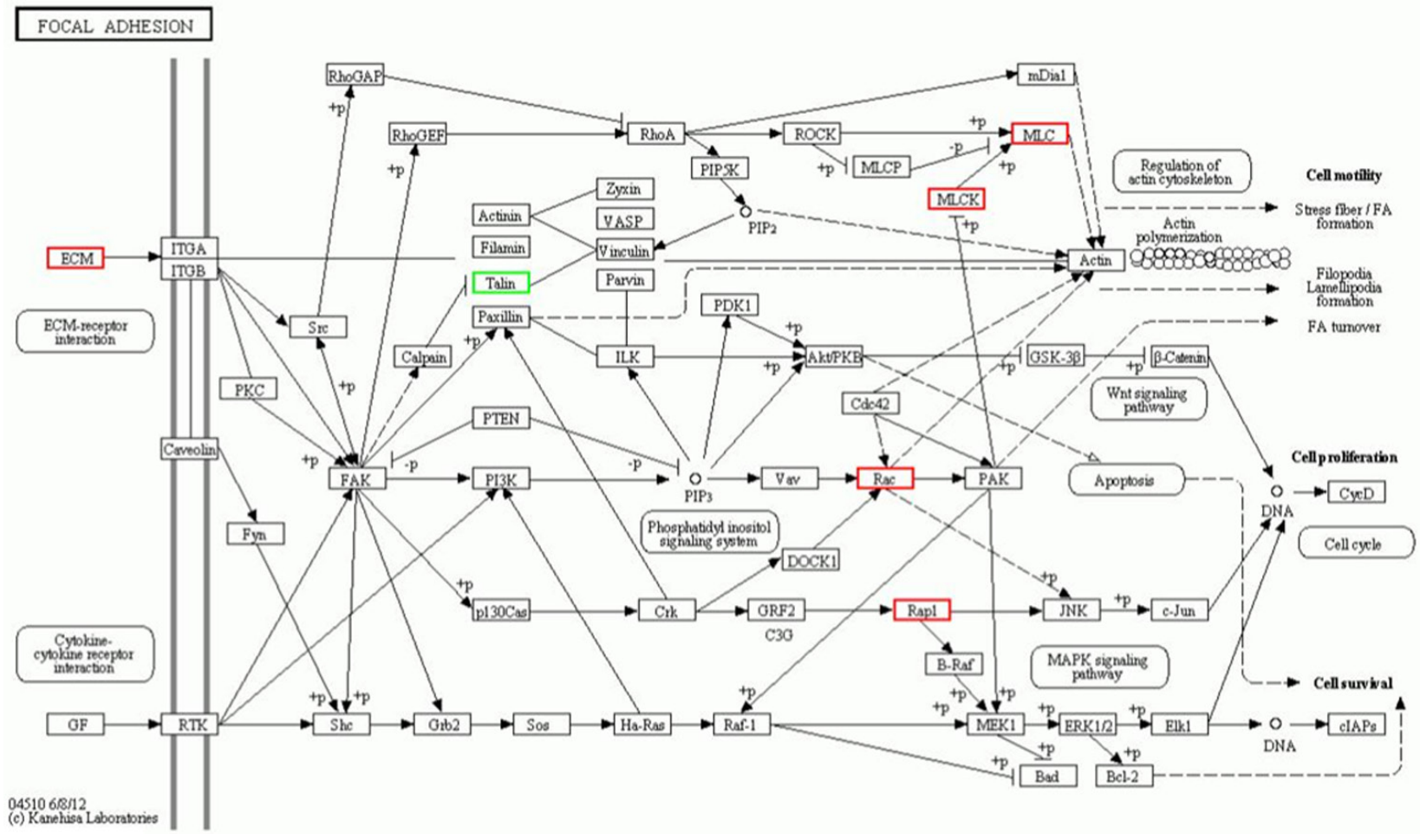

Figure 5. Focal adhesion pathway analysis based on the KEGG database. Ras-related C3 botulinum toxin substrate 1(RAC1), Ras-related protein Rap-1A, Chondroadherin, Myl9 protein, Protein Mylk and Collagen I increased in ARHD group, Talin increased in control group.

Disclosure of conflict of interest

None.

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