

# Comparative proteomics analysis of myocardium in mouse model of diabetic cardiomyopathy using the iTRAQ technique

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## Abstract

**Background.** Diabetic cardiomyopathy (DCM) is one of the most harmful diseases with high morbidity and mortality rates. However, the underlying pathological mechanism of the disorder still remains unclear.

**Objectives.** The purpose of our study was to identify differentially expressed proteins associated with DCM.

**Material and methods.** C57BLKS/J db/db (diabetes mellitus group – DM group) and db/m mice (normal control group – NC group) were acclimated in cages for 15 weeks. The general state was recorded. After 15 weeks, the heart tissues were used for histological examination. In addition, quantitative mass spectrometry using isobaric tags for relative and absolute quantitation (iTRAQ) was used to identify differentially expressed proteins in the heart tissues. SEQUEST software was used to identify proteins with data derived from liquid chromatography–tandem mass spectrometry (LC-MS/MS) spectra by searching ipi.MOUSE.v3.72.REVERSED.fasta database. Expert Protein Analysis System (ExPASy) was used to calculate the theoretical parameters. One upregulated protein (sorbin and SH3 domain containing 2 – Sorbs2) and 1 downregulated protein (myosin-3) was measured by western blot to validate the iTRAQ data.

**Results.** The mice in the NC group were active and grew well, while the mice in the DM group presented with obvious polydipsia, polyphagia and polyuria. The results of histological examination revealed that, compared to the NC group, the DM group showed significant myocardial hypertrophy and myofiber disarray accompanied by damaged nuclei. A total of 73 differentially expressed proteins were identified, including 44 upregulated and 29 downregulated proteins. Western blot analysis confirmed that the expression of Sorbs2 was significantly increased ( $p < 0.01$ ), while the expression of myosin-3 was statistically decreased in the DM group compared to the NC group ( $p < 0.05$ ).

**Conclusions.** These results suggest that DCM shows differences in its proteomics compared to normal controls. Our quantitative proteomic analysis may provide a new insight into the distinct molecular profile of DCM.

**Key words:** proteomics, diabetic cardiomyopathy, isobaric tag for relative and absolute quantitation

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## Introduction

The number of patients with diabetes mellitus (DM) has been increasing worldwide at an alarming pace for the past 2 decades. These patients are predisposed to serious cardiovascular complications, including heart failure (HF), causing mortality and morbidity.<sup>1</sup> The development of diabetic cardiomyopathy (DCM) is a prominent contributing factor to HF.<sup>2</sup> The term DCM was first introduced by Rubler et al., and it is characterized by adverse structural and functional changes in the myocardium and coronary vasculature in the absence of coronary artery disease (CAD) and hypertension.<sup>2–4</sup> Among the diabetic population, the prevalence of DCM has been estimated between 30% and 60%.<sup>5,6</sup> Although remarkable progress has been made in recent years in the diagnosis and treatment of DCM, at present, there is no specific therapy for myocardial damage induced by DCM due to the unclear molecular etiologies. Therefore, it is urgent to understand the underlying pathological mechanism of DCM and then further improve the treatment strategy.

Many potential mechanisms have been proposed and studied, and the development and progression of DCM is involved in cellular and molecular perturbations.<sup>7</sup> It has been reported that fibrosis, inflammation, apoptotic and necrotic cell death, the activation of renin-angiotensin-aldosterone system (RAAS), increased fatty acid (FA) utilization, lipotoxicity, autophagy, and oxidative stress are the most important contributors to the onset and progression of DCM.<sup>8–15</sup> To gain new insights into the molecular mechanisms involved in the development and progression of DCM, we have performed proteomic profiling analysis of heart tissue samples from diabetic mice and normal mice to identify the differentially expressed proteins altered in diabetic hearts. Our proteomic profiling study suggests that heart tissue samples from diabetic mice exhibit significant differences in proteomics compared to normal controls. Our study might provide a new insight into the distinct molecular profile of DCM.

## Material and methods

### Animal testing

Seven-week-old male C57BLKS/J db/db mice ( $n = 8$ ) and nondiabetic db/m littermates ( $n = 8$ ) were purchased from the Model Animal Research Center of Nanjing University (China). All mice were housed in independent plastic cages in an air-conditioned room (room temperature 22–24°C, room humidity 50–60%) under a 12-hour light/12-hour dark cycle. They all received laboratory pellet chow and tap water ad libitum. All animal experimental protocols were performed according to the Principles of Laboratory Animal Care provided by the National Institutes of Health (NIH; Bethesda, USA) and approved by the Animal Ethics Committee at our University.

All animals were acclimated in the cages for 15 weeks before being euthanized. C57BLKS/J db/db mice were used as a DM group, while db/m mice were used as a normal control (NC) group. All the animals were weighed each week and the general state was recorded. Following 15 weeks of feeding, all mice were sacrificed and their hearts were immediately dissected. Heart tissue samples were fixed with 10% formalin for histological examination, or washed with normal saline (NS), and then they were kept at –80°C until further analysis.

### Histological examination

For the histological examination, the tissue samples from both the DM and NC group were fixed with 10% formalin and embedded in paraffin, and 4-millimeter-thick sections were prepared. The sections were then stained with hematoxylin-eosin (H&E) and examined by light microscopy (Axioskop 40; Zeiss, Oberkochen, Germany).

### Isobaric tags for relative and absolute quantitation proteomic analysis

The tissue samples (50 mg) from both the DM and NC group were prepared, homogenized in liquid nitrogen and lysed with 500  $\mu$ L buffer containing 4% sodium dodecyl sulfonate (SDS), 100 mM dithiothreitol (DTT) and 150 mM Tris HCl. Then, the tissues were ultrasonically agitated and centrifuged. Total protein was extracted from the myocardium. Thereafter, the proteins were digested with trypsin, using the filter aided proteome preparation (FASP) protocol.<sup>16</sup> DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). A total of 100  $\mu$ g of peptides from each group were labeled with isobaric tags for relative and absolute quantitation (iTRAQ) reagents (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. Extracts from the NC group were labeled with reagent 114 and the ones from the DM group with reagent 117. Afterwards, the labeled samples were centrifuged at 1000 rpm for 30 s and incubated at room temperature for 1 h. Then, the samples were separated into 10 fractions, using Poly-Sulfoethyl A strong cation-exchange (SCX) columns (Poly LC, Murietta, USA).

### Database search and bioinformatics analysis of differentially expressed proteins

Mass spectrometric analysis was carried out using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Agilent Technologies, Palo Alto, USA) and an LTQ-Velos ion trap mass spectrometer (Thermo Finnigan Corp., San Jose, USA).<sup>17</sup> Protein searching was performed in the database of ipi.MOUSE.v3.72.REVERSED.fasta. and the filtration parameter in SEQUEST-HT (Proteome Discoverer 1.4 package; Thermo Scientific, Rockford, USA) was false

discovery rate (FDR)  $\leq 0.01$ . Expert Protein Analysis System (ExPASy) (<http://www.expasy.org>) was used to calculate the molecular weight and isoelectric point (PI).

## Western blotting analysis

After the protein concentrations had been determined, equal amounts of total protein were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF; Bio-Rad, Chicago, USA). The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline-Tween (TBST) and probed with antibodies against sorbin and SH3 domain containing 2 (Sorbs2) (ab213616; Abcam Inc., Cambridge, USA), myosin-3 (Sigma-Aldrich, St. Louis, USA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab8245; Abcam Inc.) overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam Inc.) for 2 h and visualized by enhanced chemiluminescence (ECL; Thermo Scientific).

## Statistical analysis

The data is presented as the mean  $\pm$  standard deviation (SD). Student's t-test or one-way analysis of variance (ANOVA) was used to compare differences between the groups. Statistical analyses were performed using SPSS v. 19.0 (SPSS Inc., Chicago, USA). A p-value  $< 0.05$  was considered statistically significant.

## Results

### General observations

During the observation period, mice in the NC group were active, grew well, presented good mental condition, and had bright and smooth hair coat. On the other hand, mice in the DM group showed dull and shaggy hair coat, were less active, presented obvious polydipsia, polyphagia and polyuria, and gained substantially more weight than mice in the control group.

### Histological observations

After the observation period, mice from both groups were sacrificed and their hearts were collected for histological examination. As indicated in Fig. 1A and 1B, we found that, compared to the NC group, the DM group showed significant myocardial hypertrophy and myofiber disarray accompanied by damaged nuclei.

### iTRAQ proteomics profiling

We analyzed the myocardial protein profile of db/db and db/m mice using the iTRAQ approach. The myocardial protein from the NC group was labeled with reagent 114,

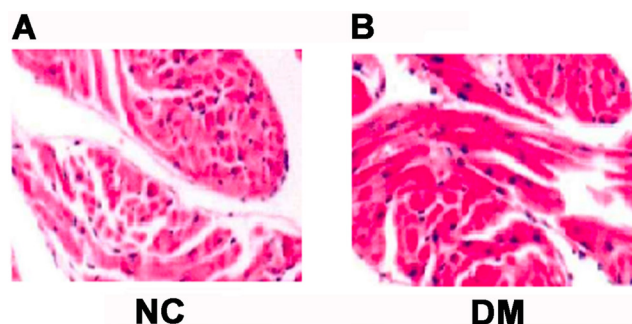


Fig. 1. Histological observation in 2 groups. After 10 weeks of feeding, heart tissue samples were obtained for histological examination. Compared to the NC group (A), the DM group (B) showed significant myocardial hypertrophy and myofiber disarray accompanied by damaged nuclei

NC – normal control; DM – diabetes mellitus.

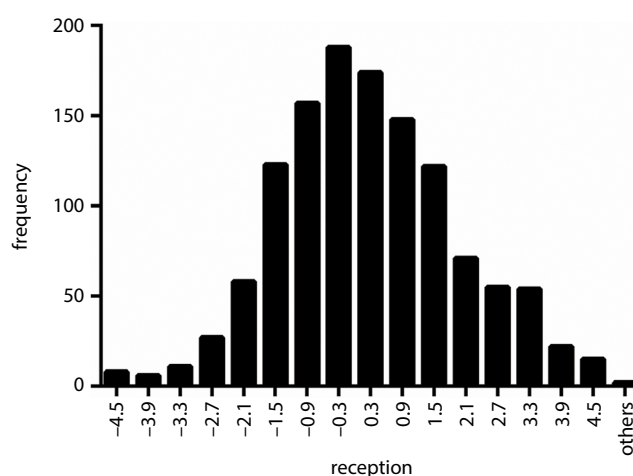


Fig. 2. Frequency distribution histogram of iTRAQ proteomics profiling. Frequency distribution histogram was constructed to analyze iTRAQ quantitative data. We found that the ratio was close to 1, indicating that the data was reliable

iTRAQ – isobaric tags for relative and absolute quantitation.

which was considered as a control. The myocardial protein from the DM group was labeled with reagent 117. Frequency distribution histogram was constructed to analyze iTRAQ quantitative data. The ratio of each label was built by evaluating the logarithm (Log<sub>2</sub>). The group distance was set at 0.6, and the distribution ranged from  $-4.5$  to  $4.5$ . As shown in Fig. 2, we observed that the ratio was close to 1. This data was in line with the normal distribution, indicating the data was reliable.

### Mass spectrometric analysis

A total of 427 proteins were quantified by performing the iTRAQ-based experiments. Of these, 73 proteins were differentially expressed proteins (44 upregulated and 29 downregulated) in the DM group compared to the NC group (Fig. 3). After searching the ipi.MOUSE.v3.72.

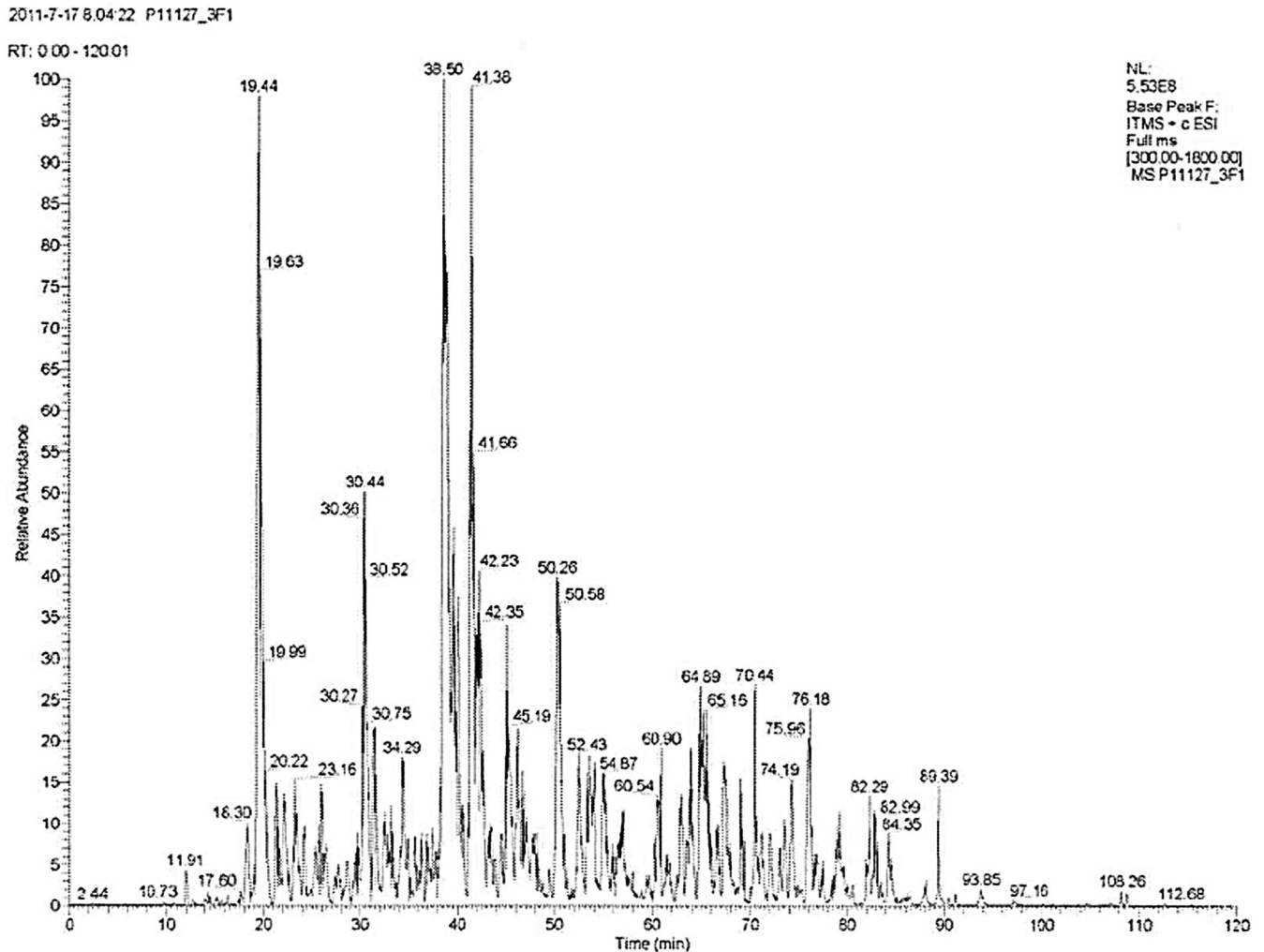


Fig. 3. Mass spectrometric analysis. A total of 73 differentially expressed proteins were identified (44 upregulated and 29 downregulated) in the DM group compared to the NC group

NC – normal control; DM – diabetes mellitus.

Table 1. Some of the differentially expressed proteins in 2 studied groups

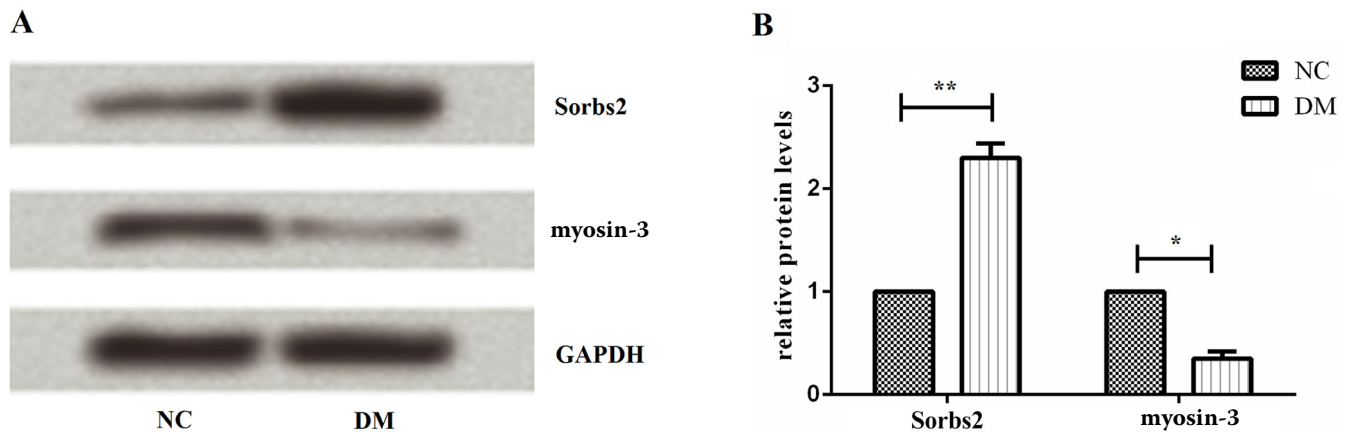
Accession No.	Protein name	Molecular weight [Da]	PI	Protein expression rate (DM/NC)
IP100956780	sorbin and SH3 domain containing 2	78,155.37	9.42	19.67
IPI00880521	solute carrier family 13	14,485.73	6.62	15.33
IPI00128152	multidrug resistance protein	140,993.72	8.51	7.45
IP100885610	secretory carrier-associated membrane protein 3	38,458.31	7.55	5.78
IPI00131334	canon channel sperm-associated protein	78,705.89	6.80	5.53
IP100380895	myosin-3	223,791.03	5.62	0.19
IPI00323035	fibulin-5	50,193.55	4.55	0.29
IPI00130102	desmin	53,497.99	5.21	0.30
IPI04775849	alpha-actinin	59,897.83	5.69	0.35
IPI00116668	integrin-linked protein kinase	51,373.15	8.30	0.18

NC – normal control; DM – diabetes mellitus.

REVERSED.fasta. database and analyzing by SEQUEST-HT the information about proteins was identified. The top-ranked 5 most obviously increased and decreased expression rates were summarized in Table 1.

## Validation of iTRAQ data

To validate the differentially expressed proteins identified by iTRAQ data, we selected 2 proteins for western blot analysis. We chose the highest protein expression rate



**Fig. 4.** Validation of iTRAQ data. We selected 2 proteins, Sorbs2 and myosin-3, to validate the differentially expressed proteins identified by iTRAQ data, using western blot analysis. Representative pictures of western blot (A) and quantitative analysis (B) showed that Sorbs2 was significantly increased in the DM group ( $p = 0.003$ ), whereas myosin-3 was statistically decreased in the DM group compared to the NC group ( $p = 0.026$ ). Data was shown as mean  $\pm$ SD

iTRAQ – isobaric tags for relative and absolute quantitation; NC – normal control; DM – diabetes mellitus; Sorbs2 – sorbin and SH3 domain containing 2; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; SD – standard deviation.

of upregulated and downregulated proteins, Sorbs2 and myosin-3. As shown in Fig. 4A, Sorbs2 was found to be significantly increased in the DM group ( $p = 0.003$ ), whereas myosin-3 was statistically decreased in the DM group compared to the NC group ( $p = 0.026$ ). Quantitative analysis showed the same results, which indicates that the results of iTRAQ data were reliable (Fig. 4B).

## Discussion

In the present study, we used iTRAQ, a recently developed protein quantitation technique, combined with LC-MS/MS, to identify the protein profiles of the myocardium from both DM and normal heart tissues. We found that DCM shows different proteomics compared to normal individuals. Also, a total of 73 differentially expressed proteins (44 upregulated and 29 downregulated) were identified. In addition, we performed western blot to validate the results by measuring 2 representative proteins.

Diabetic cardiomyopathy is a well-known specific cardiomyopathy which develops in patients with diabetes without coronary atherosclerosis and hypertension. It induces changes in cardiac structure/function, leading to significant clinical consequences, such as progressive HF and even cardiovascular death.<sup>18</sup> A prominent characteristic of the DCM is cardiac hypertrophy, which shows increased left ventricular (LV) mass and wall thickness accompanied by compromised systolic and diastolic function.<sup>19,20</sup> Numerous molecular mechanisms have been proposed to be responsible for the development of DCM.<sup>21</sup> For example, elevated FA utilization and lipotoxicity may contribute to mitochondrial dysfunction. Mitochondrial dysfunction and endoplasmic reticulum (ER) stress may promote apoptosis. In addition, oxidative stress, altered myocardial insulin signaling, increased advanced glycation end products (AGEs) signaling, impaired  $Ca^{2+}$  handling,

and inflammation may accelerate increased pro-fibrotic genes expression or promote apoptosis. It is well-known that accurate and absolute protein quantification plays a critical role in better understanding the basic biological responses as well as revealing valuable biomarkers for the diagnosis, treatment and predicting the prognosis of diverse diseases.<sup>22</sup> In the last decades, protein quantification has become an important and significant component of modern MS-based proteomic research.<sup>23,24</sup> A number of quantification strategies have been developed and successfully applied. However, most of them are dependent on the incorporation of stable isotopes for the following MS sorting and quantification.<sup>25,26</sup> The recently established iTRAQ technique has been widely used as a comprehensive and efficient MS-based proteomic research tool for multiplexed protein quantification.<sup>27,28</sup> Isobaric tags for relative and absolute quantitation could compare the protein abundance by measuring the peak intensities of reporter ions released from iTRAQ-tagged peptides.<sup>29</sup> Hence, iTRAQ could be a potential method for studying quantitative proteomics.

In the present study, we used C57BLKS/J db/db mice and nondiabetic db/m littermates. Db/db mice are a well-characterized and widely used type 2 diabetic mouse model, which can show diabetic symptoms, such as hyperglycemia, obesity, insulin resistance (IR), and renal damage, occurring after 10–20 weeks of sustained hyperglycemia.<sup>30</sup> During the 15 weeks of feeding, we found that db/db mice gradually increased their water and food intake, indicative of developing diabetes. After 15 weeks of observation, the heart tissues were harvested to confirm the pathologic changes by using histological examination. As expected, significant myocardial hypertrophy and myofiber disarray accompanied by damaged nuclei were observed in db/db mice compared to the db/m mice, confirming myocardial damage. Thereafter, the proteomics of the myocardium

was quantitatively analyzed in both db/db mice and db/m mice, using the iTRAQ technique, to reveal differentially expressed proteins in the 2 groups. As a result, a total of 73 differentially expressed proteins – 44 upregulated and 29 downregulated – were identified. Some of the up- and downregulated proteins were shown in Table 1. Among them, we determined the expression of Sorbs2 and myosin-3 to confirm the reliability of iTRAQ. Sorbs2, also known as ArgBP2, is a protein encoded by the *Sorbs2* gene in humans.<sup>31</sup> It belongs to a small family of adaptor proteins having sorbin homology domains; Sorbs2 is highly expressed in cardiac muscle cells at sarcomeric Z-disc structures, as well as in other cells at actin stress fibers and the nucleus.<sup>31,32</sup> Sorbs2 is considered an adapter protein that plays a significant role in cytoskeletal organization, cell adhesion and signaling pathways.<sup>33</sup> A proteomic analysis of cardiac tissues from patients has suggested that Sorbs2 is released from injured cardiac tissue into the bloodstream upon lethal acute myocardial infarction (AMI).<sup>34</sup> An animal experiment also revealed that Sorbs2 was increased in the damaged heart tissues.<sup>35</sup> Similarly, our study found that Sorbs2 was highly expressed in the myocardium of db/db mice, implying a destroyed structure of the myocardium. Myosin is a ubiquitous eukaryotic contractile protein which converts chemical energy into mechanical energy by means of hydrolysis of adenosine triphosphate (ATP). Cardiac myosin has been reported to be changed in DM.<sup>36,37</sup> Myosin-3 is a protein that is encoded by the *MYH3* gene in humans.<sup>38</sup> Myosin-3 is a component of the signaling complex, which is required for the adaptation response, and functions as a plus-end directed motor.<sup>39,40</sup> However, no information is available on myosin-3 tissue expression. We first observed that myosin-3 is downregulated in DM compared to normal individuals. Thus, Sorbs2 and myosin-3 might serve as potential protein therapeutic targets for DCM. Still, the functions of other identified candidates, such as the solute carrier family 13 and integrin-linked protein kinase, remain to be further elucidated.

The novelty of this study lies in revealing a new insight into the molecular profile of DCM by using iTRAQ; part of the results was also confirmed by western blot. However, there are limitations of this study, which needs further development. Firstly, although we performed western blot to confirm our results, other experiments such as immunohistochemistry (IHC) or polymerase chain reaction (PCR) should be performed. Secondly, we only selected Sorbs2 and myosin-3 as testing factors in our study. Moreover, the potential mechanisms should be explored. Thirdly, the mouse model of DCM was used in the current study; human studies should also be conducted to verify the results of animal experiments.

In conclusion, the present study suggests that DCM shows protein differences in proteomics by the application of iTRAQ. The quantitative proteomic analysis might provide a new insight into the distinct molecular profile of DCM.

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