Biochemical and Biophysical Research Communications 447 (2014) 19–25

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Monitoring of the serum proteome in Kawasaki disease patients before and after immunoglobulin therapy

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ARTICLE INFO

Article history:
Received 3 March 2014
Available online 29 March 2014

Keywords:
Kawasaki disease
Intravenous immunoglobulin
Proteome
Differentially expressed proteins

ABSTRACT

Kawasaki disease (KD) is a systemic vasculitis that mainly affects children younger than 5 years. The causal pathogen is unknown, therefore specific diagnostic biomarkers and therapy are unavailable. High-dose intravenous immunoglobulin (IVIG) is considered as the most effective therapy to reduce the prevalence of coronary artery lesion (CAL) in KD; however, it has side effects. This study aimed to (1) determine whether IVIG therapy is effective at the molecular level; (2) provide the first serum proteomic profile of KD under IVIG therapy; and (3) screen for monitoring biomarker candidates. We extracted serum proteins from samples of healthy individuals and from KD patients before and after IVIG therapy, and employed two-dimensional electrophoresis and MALDI-TOF/TOF mass spectrometry to identify differentially expressed proteins. The identifications were validated by Western blotting. We identified 29 differentially expressed proteins in KD patients and found that IVIG therapy restored most of these proteins to near-normal levels. Tracing the protein levels of single patients before and after IVIG therapy showed that the proteins, especially Transthyretin (TTR), are potential markers for therapeutic monitoring. Functional analyses of these proteins by PANTHER and String suggested that the key influence of KD lay in the immune system, which was targeted by IVIG.

1. Introduction

Kawasaki disease (KD), first reported by Tomisaku Kawasaki in 1967, is a systemic vasculitis that mainly affects children younger than 5 years [1]. KD has been recognized worldwide and diagnosed in over 60 countries and is the leading cause of acquired pediatric heart disease in the U.S. and Japan [2,3]. The incidences of KD gradually increased for a decade, forming an endemic disease that reached nationwide occurrence in some regions [4–6]. However, the causal pathogen of KD remains unknown, making specific molecular diagnosis and therapy thus far impossible. Patients with KD typically develop a fever lasting 5 days or longer; bilateral conjunctival injection; changes in the lips, oral cavity, and peripheral extremities; cervical lymphadenopathy; and a polymorphous exanthema [3]. Coronary artery abnormalities (CAAs), including dilatations and aneurysms, are the most serious complications of KD [7]. Coronary artery lesion (CAL) develops in 15–25% of KD patients without early therapy [3], and some may lead to large aneurism [8]. High-dose intravenous immunoglobulin (IVIG) is the most effective therapy for KD, as it reduces the prevalence of CAL [9]. However, the side effects of IVIG have been intensively reported, including mild responses such as tachycardia, headache, facial flushing, nausea, diarrhea, and rash, as well as serious and even fatal side effects such as anaphylaxis, acute renal failure, and thromboembolic events [10].

Considering these significant side effects, one would question whether IVIG is a proper therapy—indeed, it seems that IVIG is a lesser evil. It is necessary to assess the effectiveness of the therapy, not by observing the clinical appearance, but by monitoring molecular biomarkers, ideally from serum samples. A proper therapy is supposed to restore homeostasis, especially at the protein level. There have been some earlier proteomics studies on Kawasaki disease in urine and plasma [11,12], but the proteomics studies on

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http://dx.doi.org/10.1016/j.bbrc.2014.03.108
0006-291X/

evaluating the efficacy of IVIG in KD patients have not been reported. To investigate whether immunoglobulin does indeed restore the serum proteome back to a normal state, we employed two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) to compare proteomes in serum samples collected from normal children and KD patients before and after IVIG therapy. Twenty-nine differentially expressed proteins were identified. IVIG therapy restored the serum proteome to near-normal levels, confirming the healing power of IVIG. Tracing the protein levels of single patients showed that these proteins may serve as biomarkers for surveillance of disease progression and therapy effectiveness. Gene function classification analyses indicated that injected immunoglobulin modulated KD most likely by regulating the host immune system. To our knowledge, this is the first proteomic analysis of KD in the context of IVIG treatment, providing a comprehensive atlas of the serum proteome, and providing clues for further biomarker discovery and studies on the disease mechanism of KD.

2. Materials and methods

2.1. Preparation of serum samples

Blood samples from 10 KD patients (5 before IVIG and 5 after IVIG) were randomly selected according to the American Heart Association and the Japanese Ministry of Health and Welfare criteria [13–15]. All the diagnoses of these patients were confirmed by more than 2 pediatric cardiologist and other possible diseases were excluded. Blood samples from five healthy children were used as control group. For each group, the blood samples were pooled with equal amount to reduce the individual variability prior to 2-D gel electrophoresis. To validate the results of the 2-DE analysis, we randomly collected blood samples from another 4 KD patients before and after IVIG therapy according to the diagnostic guidelines as described above and performed Western blotting analysis with another 4 blood samples from healthy children as controls. In addition, in order to detect the change of protein candidates, we collected blood samples from four non-responder KD patients before and after IVIG therapy and 4 healthy children (controls) to carry out Western blotting analysis. All of these children are under 3 years of age. Study approval was granted by the Ethical Committee of Guangzhou Women and Children's Medical Center [2013]077 and the guardians of all children enrolled in the study provided written informed consent.

Blood samples were separated by centrifugation at 1000*g* for 10 min. Aliquots of serum were collected and stored at −80 °C. Serum samples were processed using the ProteoExtract® Albumin/IgG Removal Kit (Merck, New Jersey, USA) according to the manufacturer's instructions. To purify the protein and determine the final protein concentration, the 2-D Clean-up Kit (GE Healthcare, UK) and Bradford protein assay kit (Bio-Rad, USA) were used.

2.2. Two-dimensional electrophoresis (2-DE)

Total proteins were mixed in 250 μL rehydration solution (8 M urea, 20 mM DTT, 2% CHAPS, and 0.5% IPG buffer). Samples containing 300 μg total protein were used for the experiment. 2-DE gel electrophoresis was performed with an Amersham Biosciences IPGphor IEF System and Hoefer SE 600 (GE Healthcare) electrophoresis units using IPG strips (13 cm, pH 3–10 non-linear). The rehydration step was performed for 10 h at 30 V. IEF was performed by following a stepwise voltage gradient: 500 V and 1000 V for 1 h and 5000–8000 V for about 10 h with a total of 64 kVh. Before the second dimension, the strips were subjected to two-step equilibration in equilibration buffers (6 M urea, 30% glycerol, 2% SDS and 50 mM Tris–HCl pH 6.8) with 1% DTT w/v for the first step and 2.5% iodoacetamide (w/v) for the second step. IPG strips were laid on top of a 12.5% SDS–PAGE gel and sealed with 0.5% agarose containing a trace amount of bromophenol blue. SDS–PAGE was performed at a constant current of 15 mA/gel for 30 min and then 30 mA/gel until the bromophenol blue reached the bottom of the gels. Proteins were detected by silver nitrate staining.

2.3. Image analysis

The gels were analyzed in ImageMaster 2D Platinum software (GE Healthcare). The normalized protein amount for each spot was calculated as the ratio of that spot volume to the total spot volume on the gel. A threshold of 1.5-fold change was used to determine significant differences between groups.

2.4. In-gel digestion

Protein spots of interest were excised manually from 2-DE gels; each gel piece was rinsed twice with deionized water, destained with a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, and then equilibrated in 50 mM ammonium bicarbonate to pH 8.0. After hydrating with acetonitrile, digestion was performed in a minimal volume of trypsin (Promega, USA; 20 μg/mL in 25 mM NH_4HCO_3) and incubated at 37 °C overnight. The supernatants were transferred into a 200 μL microcentrifuge tube. Gels were extracted once with extraction buffer (67% ACN containing 2.5% TFA). The peptide extract and the supernatant of the gel spot were combined and completely dried in a Speed Vac centrifuge.

After digestion, protein digestion extracts were lyophilized and resuspended in 2 μL of a 30% ACN/0.1% TFA solution. The samples (0.8 μL) were spotted onto the MALDI sample target plate, followed by 0.4 μL of a saturated matrix solution of a-cyano-4-hydroxycinnamic acid prepared in 50% ACN/0.1% TFA.

2.5. Protein identification and data analysis

Protein identification was performed with a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) in positive ion reflection mode. After external calibration with a mixture of Gradykinin (Mr, 904.458), angiotensin I (Mr, 1296.685), Glul-Fibrinopeptide (Mr, 1570.677), ACTH clip 1–17 (Mr, 2093.08), ACTH clip 18–39 (Mr, 2465.199), ACTH clip 7–38 (Mr, 3657.929), mass spectra were obtained in the mass range between 900 and 3500 Da with 500 laser shots. For each sample spot, a data-dependent acquisition method was created to select the seven most intense peaks (S/N > 50) for each sample spot, excluding those from the matrix due to trypsin autolysis or acrylamide peaks, for subsequent MS/MS data acquisition. MS/MS spectra were acquired with 1200 laser shots in the mass range from 10 Da to the mass of parent ion using the interpretation method in the instrument software, where the seven most intense peaks were selected, and MS/MS spectra were generated automatically.

Database search spectra were interpreted and processed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), which uses internal Mascot (Matrix Science Ltd, UK) software to search the peptide mass fingerprints and MS/MS data. Searches were performed against the uniProt database with the taxonomy of *Homo sapiens*. Mass searches were performed using MS tolerance settings of 100 ppm for the precursor and 0.2 Da for the fragment masses. The following parameters were used: carbamidomethyl (cysteine) as fixed modification, oxidation (methionine) as variable modification,

and up to one missed trypsin cleavage was allowed. To ensure reliable identification, results from both the MS and MS/MS spectra were used in the database search. Protein identification was accepted when the score reported by the Mascot search routine was greater than 62 and, whenever possible, confirmed with MW/pI values.

2.6. Protein categorization

Differentially expressed proteins were classified on the basis of Protein Analysis Through Evolutionary Relationships (PANTHER) system (http://www.pantherdb.org), which classifies genes and proteins by their functions. The PANTHER ontology, a highly controlled vocabulary (ontology terms) by molecular function and biological process was used to categorize proteins into families and subfamilies with shared functions.

2.7. Predication of protein–protein interaction

Identified proteins were processed by the Functional Protein Association Networks (STRING) system (http://www.string-db.org). STRING is a database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations.

2.8. Western blot analysis

Protein extracts from serum of normal children and KD patients before or after IVIG treatment, were separated by SDS–PAGE (12–15% acrylamide), and then transferred onto PVDF membranes. The membranes were incubated with Retinol-binding protein 4 (RBP4), Serum amyloid P-component (APCS) and Transthyretin (TTR) antibodies at 4 °C overnight, followed by incubation with secondary antibodies at room temperature for 2 h. The bands were visualized using the SuperSignal chemiluminescence system (ECL, Pierce, USA).

2.9. Statistical analysis

All data were analyzed using Statistical Package for Science Software (SPSS) version 16.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical observations before and after IVIG therapy

KD patients received IVIG 2 g/kg day. Blood tests and echocardiography were repeated after 2 days IVIG therapy. In general, body temperature returned to normal range, and symptoms such as

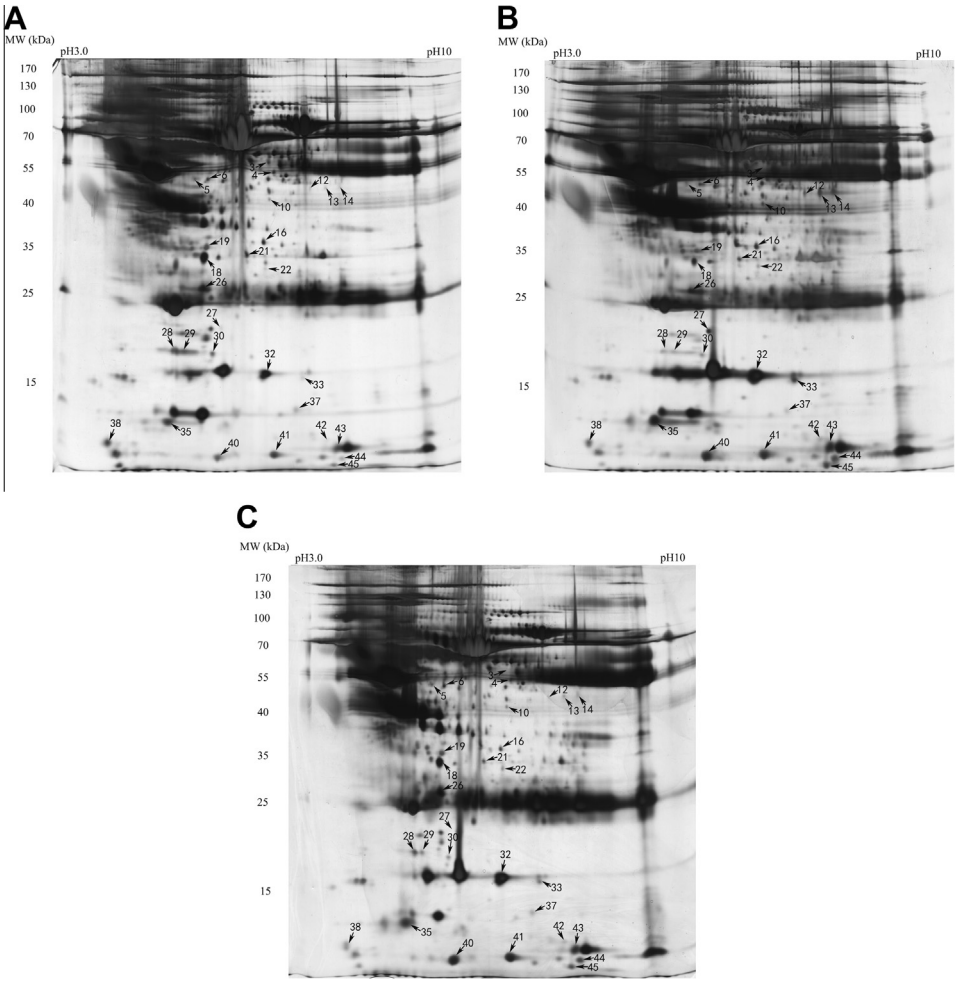

Fig. 1. 2-DE analysis of the serum proteome. Gels were visualized by silver staining. Arrows indicate differentially expressed proteins in KD patients before/after IVIG therapy and healthy controls. Spot numbers correspond to proteins in Supplementary Tables S1. (A) Healthy children, (B) KD patients before IVIG therapy, (C) KD patients after IVIG therapy.

conjunctival hyperemia, rash, and hand and foot swelling were significantly improved. Leukocytes and neutrophils were significantly reduced within 48 h. Biochemical indicators like C-reactive protein, ALT (alanine transaminase), and AST (aspartate aminotransferase) were remarkably improved. Coronary artery decreased after therapy. These findings confirm that the IVIG therapy is effective. Clinical observations before and after IVIG therapy are listed in STable 1.

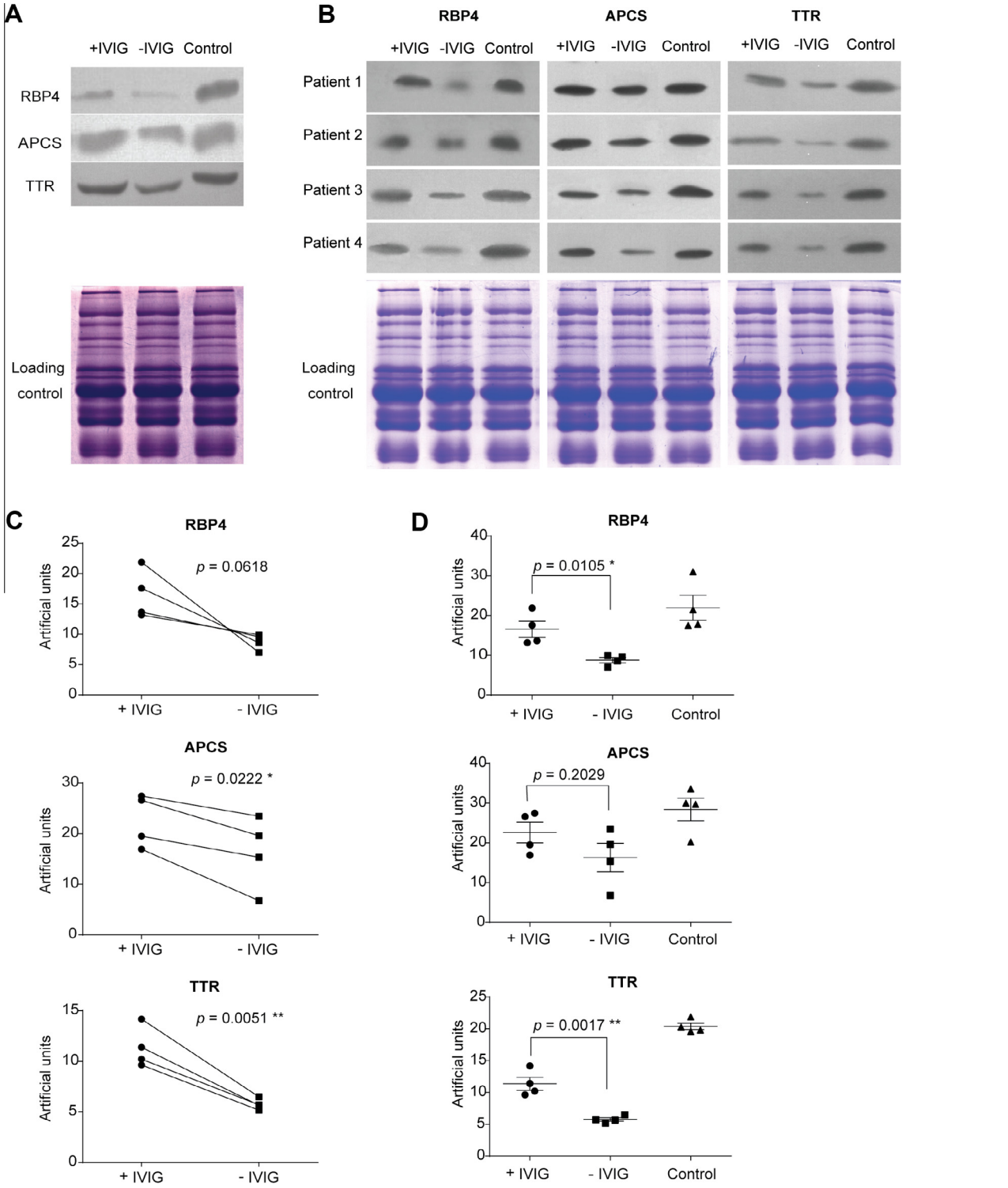

Fig. 2. (A) Western blots of RBP4, APCS, and TTR in pooled serum samples from five healthy persons (control) and five KD patients before therapy and five KD patients after IVIG therapy (−IVIG and +IVIG, respectively). The Coomassie-stained gel was used as the loading control. (B) Western blots of RBP4, APCS, and TTR of four individual patients before and after IVIG therapy. Band quantification is shown in panels C and D. (C) Paired analysis of the band intensities in B. Band intensities in the same patient are linked. Paired *t*-test was performed and *p*-values are shown. Significance was accepted when $P < 0.05$. (D) Grouped analysis of the band intensities of B. Unpaired two-sample *t*-test was performed. Significance was accepted when $P < 0.05$.

3.2. Identification of differentially expressed proteins in KD patients before and after IVIG therapy

Pooled serum proteins from five untreated KD patients, five IVIG-treated patients, and five healthy controls were separated by 2-DE (Fig. 1A–C). Fifty differentially expressed protein spots before and after IVIG therapy were detected on the ImageMaster 2D Platinum (GE Healthcare) software. Twenty-nine spots were further uniquely identified using MALDI-TOF/TOF MS (STable 2). The unidentified proteins are likely due to their special physical and chemical properties [16], or low Mascot scores, or ambiguous identifications. To validate the 2-DE quantification and identification, three proteins (RBP4, APCS, and TTR) were subjected to Western blot. The results of Western blot confirmed the pattern in the 2-DE gels. The Coomassie-stained gel was used to confirm that each lane was equally loaded (Fig. 2A). To examine the utility of these proteins in therapeutic monitoring, we performed Western blots of proteins from the same patients before and after IVIG therapy; the Western blots revealed that the expression levels of these proteins were especially consistent with the 2-DE results (Fig. 2B). In all 4 individuals, IVIG therapy increased the levels of RBP4, APCS, and TTR (Fig. 2C). Of these, TTR were remarkably stable and significantly differed before and after IVIG therapy, for both paired *t*-test ($P = 0.0051$) and unpaired *t*-test ($P = 0.0017$) (Fig. 2C and D). This indicates that TTR might be a good candidate for monitoring disease and therapy effectiveness.

3.3. IVIG restores protein levels to a near-normal state

We found that the quantities of these proteins after IVIG therapy were much closer to the normal control than they were before IVIG (Fig. 3). The overall standard deviation of these protein expression levels was reduced from 1.70 to 0.91. Many proteins such as HPX and CCDC150 reverted almost completely to normal levels after IVIG therapy. These results suggest IVIG restored protein expression to normal, indicating the healing power of IVIG at the molecular level.

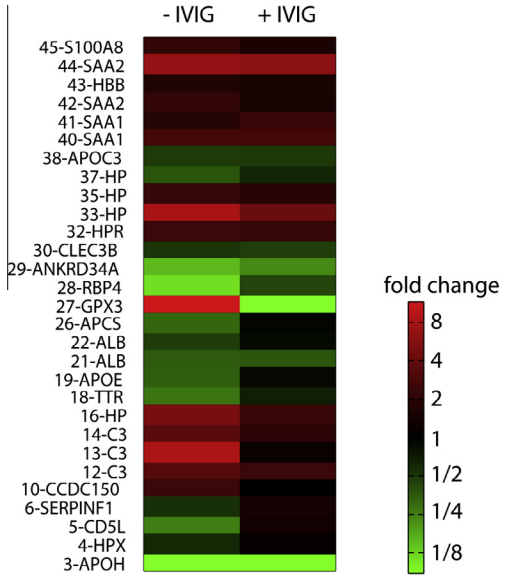

Fig. 3. Heat map of differentially expressed proteins, quantified from the 2-DE. KD patients before and after IVIG therapy were compared to healthy controls. Green color indicates downregulation; red indicates upregulation.

3.4. The change of TTR protein in non-responder KD patients before and after IVIG therapy

To detect the change of expression levels of TTR in non-responder KD patients before and after IVIG therapy, we performed Western blots of four non-responder KD patients before and after IVIG therapy. The Western blots analysis showed that the expression levels of TTR increased insignificantly in non-responder KD patients before and after IVIG therapy (Fig. 4A). This insignificance were valid in both paired test and unpaired test (Fig. 4B and C). Together with the results above, TTR change can be used to predict the efficacy of IVIG therapy.

3.5. Functional classification

To reveal the potential biological functions represented by the differentially expressed proteins, we performed Gene Ontology (GO) analysis using PANTHER [17] according to their molecular functions (SFig. 1A) and biological processes (SFig. 1B). Serum proteins are predominantly classified as extracellular component in Cellular Component of GO analysis, thus this analysis is neglected. Indeed, the most prominent functions and processes in molecular function and biological processes were transporters and metabolic processes, confirming alterations in substance exchange and metabolism. Immune system process was another major category, suggesting the immune system is activated in KD patients, leading to a series of changes of cellular metabolism. Receptors and antioxidant proteins were a minority of the differentially expressed proteins, indicating that cell signaling and oxidative response played minor roles in these KD patients. Proteins related to apoptosis, cell cycle, and cell adhesion were almost undetectable, indicating that the cellular alterations in KD were totally different from cancer.

String analysis revealed protein interaction networks in the KD patients (SFig. 1C). The nodes with highest connectivity were complement C3, TTR (Transthyretin), and ALB (serum albumin). Complement C3 is an important component of the complement system, involved in immune response. TTR is a transporter of thyroxine and is involved in retinol metabolism in immune cells [18]. ALB binds metal ions in plasma and is a zinc transporter, correlated with immune/inflammatory response [19]. In sum, these results suggested the key influence of KD lays in the immune system, which was targeted by IVIG.

4. Discussion

We confirmed that immunoglobulin restored the serum proteome of KD patients almost back to normal. This suggests immunoglobulin has a comprehensive healing power, not by controlling the symptoms temporarily, but probably by a more fundamental mechanism regulating the immune system. Indeed, the central nodes of the differentially expressed proteins in KD were related to the immune response (complement C3, TTR, and ALB), and can be reverted by IVIG. Epidemiologic and clinical observations suggest that the causal pathogen of KD may trigger the inflammatory process in genetically susceptible hosts and then develop the clinical syndrome [20], which supports our speculation. Thus, immunoregulation may be the most important and effective therapy for KD before the pathogen is found. The side effects of immunoglobulin may be unrelated to its healing properties. Novel treatments should remain within the scope of immunoregulation, but avoid the side-effect-causing properties of immunoglobulin.

This study identified 29 differentially expressed proteins in serum samples, offering a list of biomarker candidates. This largely favors molecular diagnosis and progression monitoring of KD. There is no specific diagnostic test for KD; diagnosis is based on clinical

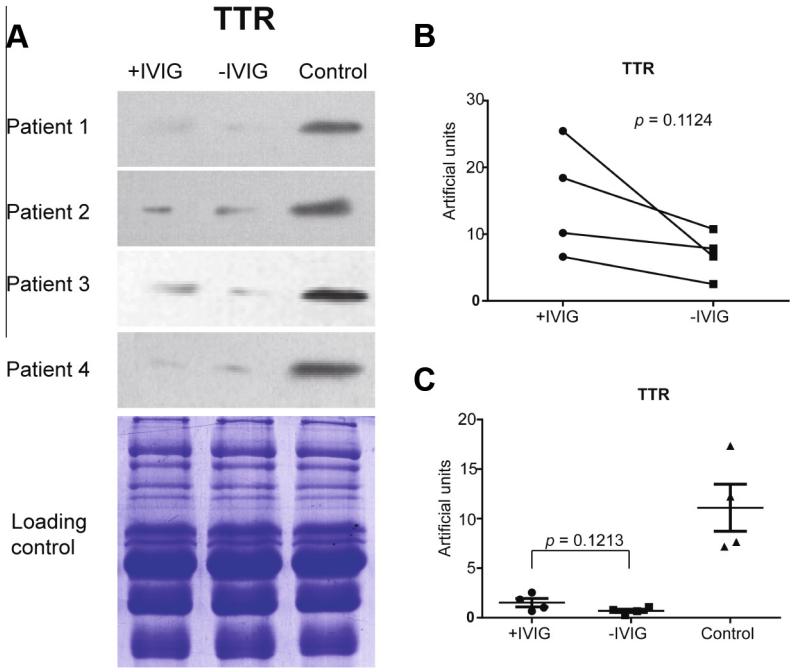

Fig. 4. (A) Western blots of TTR of four non-responder KD patients before and after IVIG therapy. Band quantification is shown in panels B and C. (B) Paired analysis of the band intensities in A. Band intensities in the same patient are linked. Paired *t*-test was performed and *p*-values are shown. Significance was accepted when $P < 0.05$. (C) Grouped analysis of the band intensities of A. Unpaired two-sample *t*-test was performed. Significance was accepted when $P < 0.05$.

signs and supportive non-specific laboratory tests [21,22]. Recently, KD cases with limited and milder phenotypes have increased in frequency. These mild KD cases occur more commonly in young infants than in older children [23]. The initial phase of mild KD may present as insignificant clinical symptoms, and a fraction of these patients will develop severe, life-threatening KD symptoms. As the cause of KD remains unclear, these factors complicate diagnosis, delaying therapy. Considering the remarkable side effects of IVIG, a molecular biomarker indicating the potential of the disease is important. Indeed, we validated TTR as a potential biomarker with the following features: (1) the signal intensity of each protein is almost independent of specific healthy individuals, thus a universal reference can be established; (2) the amount of these proteins changes significantly in KD patients; (3) after successful IVIG therapy, these proteins return to almost normal levels. This biomarker may serve as a monitor for KD diagnosis and therapy. Moreover, TTR level can quickly distinguish KD patients who respond and do not respond to the IVIG therapy. For the non-responders, we may timely adjust the treatment plan to avoid the occurrence of coronary artery aneurysms.

TTR is mainly secreted by liver, choroid plexus and retina cells, and exists mainly in the blood and cerebrospinal fluid. It transports thyroid hormone thyroxine (T4) and retinol-binding protein bound to retinol [24]. Numerous studies revealed the role of TTR in neurobiological disorders and neurodegenerative diseases [25–29]. However, no direct connection between TTR and KD has been reported, to our best knowledge. The function and role of TTR in KD still remains elusive and is worth further investigation.

In sum, we have identified a potential monitoring biomarker TTR for KD. Although validation with a larger sample size is necessary, our proteome profiling provides the first hints of potential KD biomarkers.

Conflict of interest

None declared.

Acknowledgments

This work was supported by National Clinical Key Specialty Project Foundation, Guangdong Natural Science Foundation (9151008901000033 to L.D.), Social Development Project Foundation of the Department of Science and Technology Guangdong Province (2012B031800013 to L.Z.), Key Project of Chinese Ministry of Education (212207 to G.Z.) and Institutional Grant of Excellence of Jinan University, China (50625072 to G.Z.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.108.

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