

Molecular basis of coronary artery dilation and aneurysms in patients with Kawasaki disease based on differential protein expression

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Received September 6, 2017; Accepted October 10, 2017

DOI: 10.3892/mmr.2017.8111

Abstract. Kawasaki disease (KD) is an acquired cardiac disease with a high incidence that affects children. KD has various complications, including coronary artery dilation (CAD) and coronary artery aneurysms (CAA). The identification of differentially expressed proteins and the underlying mechanisms may be the key to understanding differences between these KD complications. In the present study, isobaric tags for relative and absolute quantitation were used to identify variations in serum proteins between KD patients with CAD and CAA. In total, 87 (37 upregulated and 50 downregulated) and 65 (33 upregulated and 32 downregulated) significantly differentially-expressed proteins were identified in comparisons between control samples (healthy individuals)

and those obtained from patients with KD and with CAD or CAA. Investigation into the underlying biological process revealed that variations between the two complications were associated with the wound healing response, as well as lipoprotein- and cholesterol-associated processes. Important proteins involved in the formation of the wound healing signaling network were identified via enriched biological processes and pathway analysis using ClueGo and ReactomeFIViz software. In the present study, 5 significantly differentially-expressed proteins, including mannose binding lectin 2 (MBL2), complement factor H (CFH), kininogen 1 (KNG1), serpin family C member 1 (SERPINC1) and fibronectin 1 (FN1), were selected and confirmed by western blotting. Analysis indicated that these proteins were associated to immunity, inflammation and metabolism, serving a key role within each module, which has never been reported previously. The present study proposed that MBL2, CFH, KNG1, SERPINC1 and FN1 may be a potentially excellent indicator group for distinguishing the two major KD complications, CAD and CAA.

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Key words: Kawasaki disease, coronary artery dilation, coronary artery aneurysms, potential indicators

Introduction

Kawasaki disease (KD), first described in 1967 by Kawasaki (1), is a type of acute vasculitis with unknown etiology; however, KD is known to induce pathological alterations in medium-sized arteries, particularly coronary arteries. Some patients with KD develop coronary artery lesions and exhibit similar clinical outcomes to those of patients with coronary atherosclerosis (2). Several studies have demonstrated that patients without coronary artery lesions still exhibit a high risk of various diseases, such as atherosclerosis, in adulthood (2-4). KD is a main cause of acquired heart disease in children, predominantly affecting coronary arteries (5).

Without routine therapy, ~25% of patients with KD develop coronary artery dilation or aneurysms (CAD or CAA, respectively) (6). Mild CAD can regress, while CAA and severe CAD are difficult to restore to a normal state. Mueller *et al* (7) reported that patients with coronary artery sizes of ≤ 5.0 mm exhibited regression to the normal state, while patients with coronary artery sizes of >5.0 mm exhibited persistence or even increases in size. CAA with a diameter of ≥ 8.0 mm is strongly associated with the development of coronary artery stenosis and myocardial infarction (7).

These results indicate that KD may exert long-term effects on the coronary artery and cause pathophysiological insults of the cardiovascular system leading to CAD or CAA. To further understand complications in patients with KD, it is important to investigate the mechanisms of CAD and CAA formation, and to characterize the differences among normal coronary arteries, CAA and CAD. Coronary artery specimens are optimal for CAD and CAA studies; however, they are difficult to collect from patients with KD. Thus, blood samples were used in the present study.

Rapid advances in mass spectrometry (MS) for peptide identification and improved protein sequence coverage of complex biological samples (8) has provided highly effective proteomic technologies for biomarker identification and therapeutic target discovery in serum samples (9,10). In order to obtain accurate and reliable results, isobaric tags for relative and absolute quantitation (iTRAQ) and MS were employed for quantitative proteomics analyses, with increased proteome coverage and labelling efficiency to compare proteomes among serum samples from healthy individuals, and patients with KD and CAD or CAA (6). Western blotting was employed to confirm the significantly expressed proteins following bioinformatics analysis. The objective of the present study was to investigate proteomic variations to further our current understanding of the pathogenesis of coronary artery lesions, and provide a basis for the establishment of indicators for these KD complications.

Materials and methods

Preparation of serum samples. Blood samples were collected from 51 children (≤ 6 years old) who recruited to the present study (Table I). Written informed consent was obtained from the guardians of each participant and the present study was approved by the Ethical Committee of Guangzhou Women and Children's Medical Center [(2013)077; Guangdong, China] based on the Japanese Ministry of Health and Welfare criteria, and the American Heart Association (6,11). A total of 17 age- and sex-matched samples obtained from normal children, served as the controls; individuals were physically examined in Guangzhou Women and Children's Medical Center. The other 34 samples (17 CAA and 17 CAD) were obtained from patients who were diagnosed and excluded of other possible diseases by ≥ 2 pediatric cardiologists. Blood samples were separated by centrifugation at $1,000 \times g$ for 10 min at 4°C and aliquots of serum were collected and stored at -80°C . Serum samples were processed to remove albumin and immunoglobulin G (IgG) using a ProteoPrep Blue Albumin and IgG Depletion kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The concentrations of these proteins

were determined using a Bradford protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). These protein samples were randomly combined into 17 comparison groups, each of which included 1 control sample, 1 CAA sample and 1 CAD sample. Of the 17 comparison groups, 5 were randomly chosen and pooled together according to each type of sample; 3 pooled samples of the control, CAA and CAD groups were produced, and were subsequently analyzed by iTRAQ (Sciex, Framingham, MA, USA) experiments (Table IA). The other 12 groups out of the total 17 were analyzed via western blotting. Information regarding the groups and diagnosis is presented in Table IB.

Protein identification and analysis. iTRAQ labeling was performed according to the manufacturer's instructions (Sciex). Briefly, 100 μg of each protein sample was reduced with Tris(2-carboxyethyl)phosphine hydrochloride reducing reagent (Sciex) at 60°C for 1 h, and alkylated with methyl methanethiosulfonate cysteine-blocking reagent (Sciex) for 30 min at room temperature. Then, proteins were digested with 2% trypsin (Promega Corporation, Madison, WI, USA) at 37°C overnight, at a ratio of 1:50 (enzyme-to-substrate). Each sample was labelled separately with the iTRAQ tags (118 tag for control, 113 tag for CAA and 116 tag for CAD), then dehydrated by centrifugation at $6,000 \times g$ for 4 h at 25°C in a vacuum centrifuge.

iTRAQ-labelled samples were firstly diluted to 100 μl with H_2O buffer ($\text{NH}_3\text{-H}_2\text{O}$, $\text{pH}=10$) prior to high performance liquid chromatography on a Dionex Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) at 25°C on a Gemini-NX 3u C18 110A; 150×2.00 mm Phenomenex column, and Gemini 3u C6-Phenyl 110A; 100×2.0 mm column (all from Phenomenex, Torrance, CA, USA). The flow rate used for reversed-phase column separation was 0.2 ml/min with H_2O (mobile phase A) and 80% acetonitrile (mobile phase B) with the following gradient system parameters: 5-10% B for 0-15 min, 15-28% B for 15-48 min, 25-37% B for 48-60 min, 37-95% B for 60-65 min and 95% B for 65-70 min. The elution was monitored by absorbance at 214/280 nm and the fractions were collected every 50 sec; these were pooled for each sample and dehydrated by centrifugation at $6,000 \times g$ for 4 h at 25°C in vacuum centrifugation.

Peptides were separated with mobile phase A (0.1% formic acid), and 5-40% mobile phase B (0.1% formic acid and 80% acetonitrile) for 99 min (0.3 ml/min flow rate). MS analysis was performed on a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the following parameters: MS spectra were acquired across the scan range of 350-1,800 m/z in high resolution mode ($>35,000$) and 100 msec was accumulated per spectrum. A maximum of 20 precursors per cycle were chosen for fragmentation, with 120 msec set as the minimum accumulation time for each precursor and dynamic exclusion for 10 sec.

The database searching of MS raw data was conducted against the human protein database by ProteinPilot Software 5.0 (Sciex). The search parameters were set as: 'Cys alkylation', 'Methyl methanethiosulfonate', 'Digestion', 'Trypsin', ≤ 1 missed trypsin cleavage was allowed and the false discovery rate (FDR) was <0.01 .

Table I. Clinical indicators of patients with Kawasaki disease.

Patient	Age (years)	Sex	WBC count (10 ⁹ /l)	N/L	CRP (mg/l)	ALT (U/l)	AST (U/l)	Coronary change (mm)
A, Patients for iTRAQ experiments								
Patient-1 CAA-1	11 months	Female	11.3	0.50	13.31	50	37	LCA=5.8, RCA=5.7
Patient-2 CAA-2	2 years, 5 months	Male	17.7	3.13	88.80	26	18	LCA=9.7, RCA=7.5
Patient-3 CAA-3	1 year, 11 months	Male	10.5	0.88	0.21	19	35	LCA=12, RCA=8.3
Patient-4 CAA-4	1 year, 3 months	Male	22.8	1.20	27.37	8	18	LCA=5.6, RCA=6.0
Patient-5 CAA-5	2 months	Male	24.8	1.75	71.51	36	24	LCA=4.0, RCA=4.6
Patient-1 CAD-1	2 years, 7 months	Male	23.4	5.25	211.13	7	12	LCA=3.2, RCA=3.7
Patient-2 CAD-2	3 years	Male	14.6	11.35	9.50	10	23	LCA=3.2, RCA=2.8
Patient-3 CAD-3	3 years	Male	11.6	4.15	45.12	14	30	LCA=4.0, RCA=3.7
Patient-4 CAD-4	1 year, 1 month	Male	10.0	4.72	93.30	161	32	LCA=2.9, RCA=3.4
Patient-5 CAD-5	3 months	Female	20.5	1.26	98.60	18	25	LCA=3.5, RCA=3.9

B, Patients for western blotting experiments

Patient	Age (years)	Sex	WBC count (10 ⁹ /l)	N/L	CRP (mg/l)	ALT (U/l)	AST (U/l)	Coronary change (mm)
Patient-6 CAA-6	1 year 3 months	Male	24.3	1.71	175.60	19	26	LCA=4.0, RCA=4.5
Patient-7 CAA-7	4 months	Male	34.7	2.95	75.05	20	26	LCA=4.8, RCA=3.3
Patient-8 CAA-8	6 years	Female	15.5	3.77	68.57	26	21	LCA=6.6, RCA=3.1
Patient-9 CAA-9	6 months	Male	13.31	1.09	46.06	17	20	LCA=3.7, RCA=4.6
Patient-10 CAA-10	8 months	Male	15.6	1.60	146.70	20	16	LCA=3.3, RCA=4.2
Patient-11 CAA-11	2 months	Male	17.0	1.62	39.90	19	17	LCA=4.3, RCA=4.0
Patient-12 CAA-12	2 months	Male	15.4	1.85	18.18	14	17	LCA=3.8, RCA=4.6 m
Patient-13 CAA-13	3 years	Male	7.0	0.48	0.23	22	31	LCA=10.4, RCA=9.8
Patient-14 CAA-14	1 year, 2 months	Female	19.6	2.82	202.37	16	19	LCA=4.6, RCA=5.3
Patient-15 CAA-15	4 months	Female	23.1	1.42	110.05	40	42	LCA=3.8, RCA=4.5
Patient-16 CAA-16	7 months	Male	17.5	1.67	87.36	8	19	LCA=5.0, RCA=5.4
Patient-17 CAA-17	1 year	Female	20.8	1.76	58.00	11	21	LCA=5.0, RCA=3.6
Patient-6 CAD-6	1 year, 1 month	Male	12.6	0.43	73.13	35	30	LCA=3.1, RCA=2.8
Patient-7 CAD-7	4 months	Male	13.7	2.12	113.75	27	23	LCA=3.2, RCA=2.0
Patient-8 CAD-8	4 years	Male	19.8	7.26	59.22	73	20	LCA=3.4, RCA=3.1
Patient-9 CAD-9	6 months	Male	21.5	5.37	71.00	50	38	LCA=2.8, RCA=1.9
Patient-10 CAD-10	7 months	Male	12.1	0.99	78.10	14	19	LCA=2.5, RCA=2.0
Patient-11 CAD-11	3 months	Female	9.7	0.47	15.57	84	66	LCA=2.2, RCA=1.5
Patient-12 CAD-12	2 months	Male	24.5	12.26	209.63	24	24	LCA=2.5, RCA=2.1
Patient-13 CAD-13	2 years, 9 months	Male	7.3	1.58	29.98	23	23	LCA=3.4, RCA=2.8
Patient-14 CAD-14	7 months	Male	24.1	1.91	55.72	13	20	LCA=2.7, RCA=2.8
Patient-15 CAD-15	7 months	Male	17.6	0.83	44.60	18	19	LCA=3.0, RCA=2.4
Patient-16 CAD-16	4 years	Male	22.3	9.16	147.85	136	39	LCA=3.0, RCA=2.6
Patient-17 CAD-17	1 year, 1 month	Male	17.5	7.79	144.10	82	44	LCA=3.5, RCA=2.9

CAA, coronary artery aneurism; CAD, coronary artery dilation; WBC, white blood cell; N/L, neutrophils/lymphocytes; CRP, C-reactive protein; ALT, alanine transaminase; AST, aspartate aminotransferase; LCA, left coronary artery; RCA, right coronary artery; iTRAQ, isobaric tags for relative and absolute quantitation.

Gene Ontology (GO) term and pathway analysis. The Cytoscape plug-in ClueGO (12) and ReactomeFIViz (13) were applied for the differentially expressed proteins to process the Biological Process GO terms, and Kyoto

Encyclopedia of Genes and Genomes (KEGG) pathway analysis (www.genome.jp/kegg/). $P < 0.05$ and FDR < 0.01 were selected as threshold values for GO and KEGG pathway enrichment.

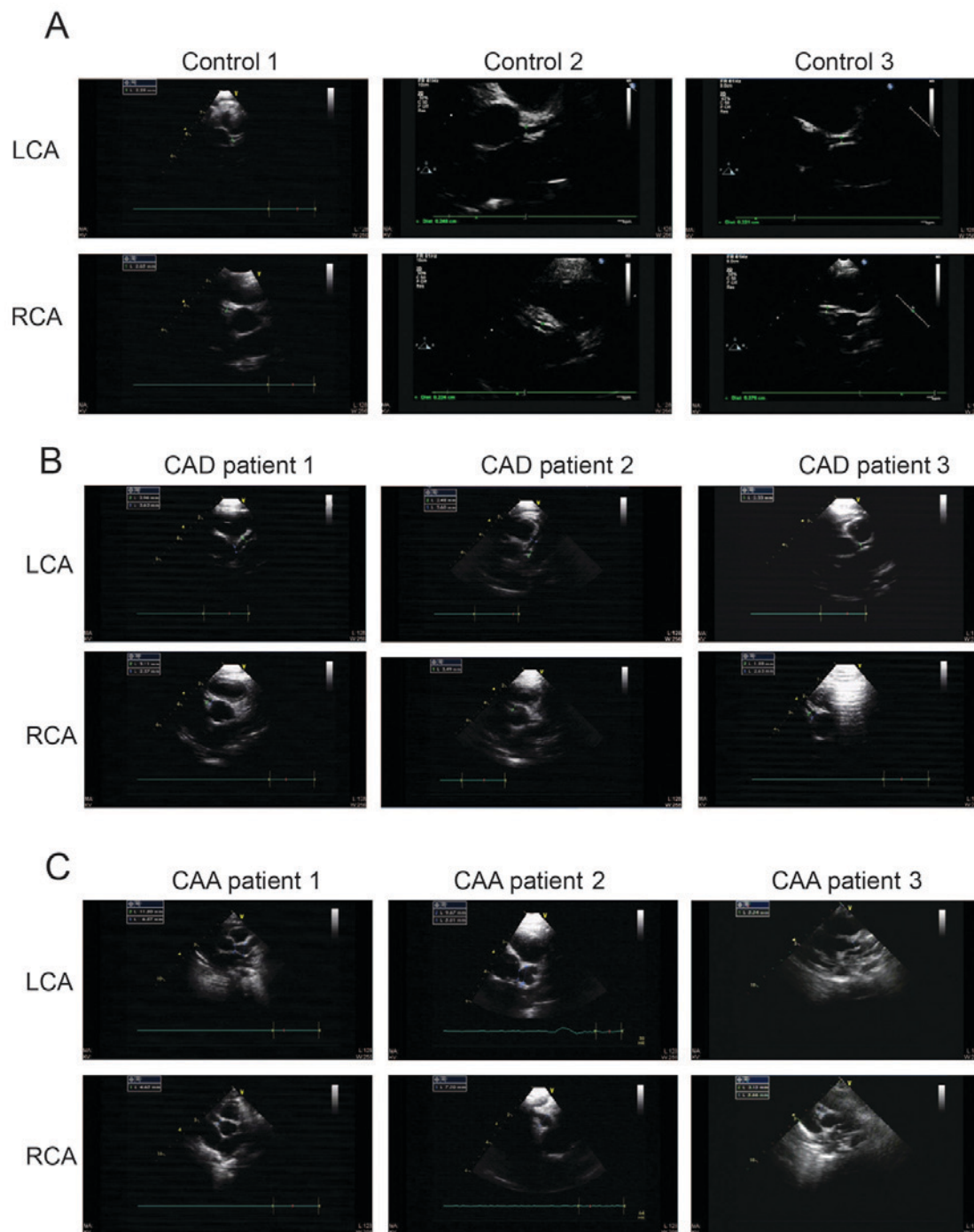


Figure 1. ECG images of coronary arteries from healthy individuals and patients with Kawasaki disease. Representative ECG images from (A) three children with normal left and right coronary arteries (controls); (B) three patients with CAD; and (C) three patients with CAA. ECG, echocardiography; CAA, coronary artery aneurysm; CAD, coronary artery dilation; LCA, left coronary artery; RCA, right coronary artery.

Western blot analysis. Serum samples from 36 children (12 control, 12 CAA and 12 CAD) were randomly grouped into 12 sets (for Control, CAA and CAD; details are presented in Table IB) for validation. Proteins from the sera of these patients were extracted and the concentrations were measured using lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and a Bradford protein assay kit (Bio-Rad Laboratories, Inc.). Following electrophoresis with 20 μ g protein per lane via 12-15% SDS-PAGE, the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich; Merck KGaA) and blocked with

5% skimmed milk for 1 h at room temperature, then incubated with primary antibodies (1:1,000) against: Complement factor H (CFH; cat. no. ab133536), mannose-binding lectin 2 (MBL2; cat. no. ab203303), kininogen 1 (KNG1; cat. no. ab170475), fibronectin (FN1; cat. no. ab2413), antithrombin-III (SERPINC1; cat. no. ab126598) and β -actin (cat. no. ab8227) (all from Abcam, Cambridge, UK) overnight at 4°C. Subsequently, horse radish peroxidase goat anti-rabbit IgG (cat. no. ab6721, 1:2,000; Abcam) was applied to the primary antibody-treated PVDF membranes at room temperature for 2 h. The specific bands on these membranes were

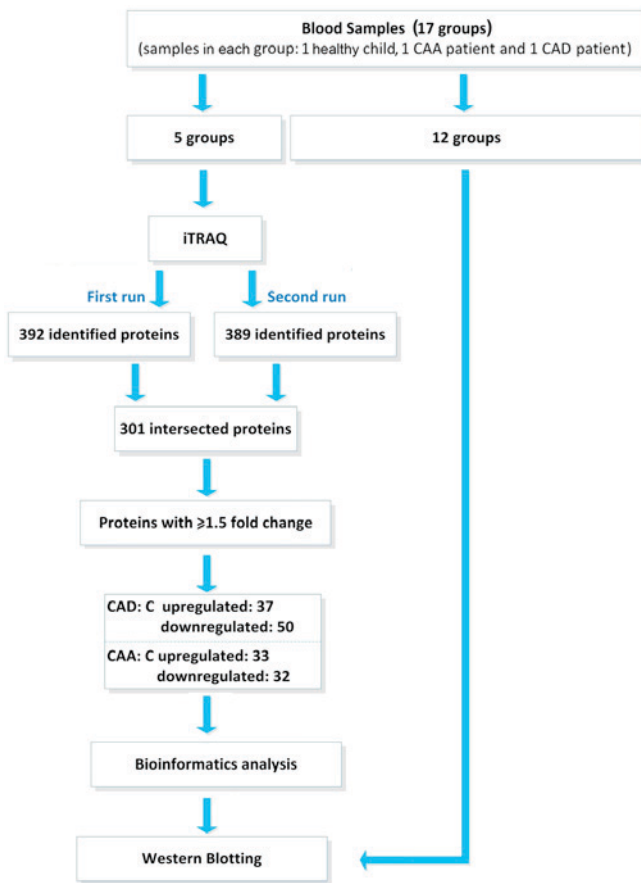


Figure 2. Flow diagram showing the trial profile and study design. CAA, coronary artery aneurism; CAD, coronary artery dilation; iTRAQ, isobaric tags for relative and absolute quantitation.

visualized using the SuperSignal chemiluminescence system (Promega Corporation).

Results

Characteristics of patients with KD. Blood samples were obtained from 51 children (including 17 controls, 17 patients with CAA, and 17 patients with CAD); basic diagnostic information is shown in Table IA and B. The patients with KD were ≤ 6 years and had clear clinical features; the abnormal degree of coronary alteration revealed that some rare CAA samples were included. Representative echocardiographic images of patients with KD and CAD or CAA, and healthy individuals are presented in Fig. 1. Fig. 1 shows that the diameter of the normal coronary artery was <3 mm and the diameter of CAD was <4 and >3 mm and the diameter of CAA was >5 mm. The maximum diameter of the coronary artery was indicated in the images.

Trial profile and study design. A total of 51 samples were divided into 17 groups, each of which contained one control, one CAD and one CAA sample. Of the 17 groups, 5 were randomly selected and the samples from these groups were pooled based on the sample type for iTRAQ analyses in order to detect significantly differentially expressed proteins. The other 12 groups were analyzed via western blotting. All experiments and validation procedures are summarized in Fig. 2.

Identification of differentially expressed proteins in KD patients with CAA or CAD by iTRAQ. Three pooled samples of the control, CAA and CAD groups, were subjected to iTRAQ. As presented in Fig. 2, MS analysis was performed twice using the iTRAQ technique to ensure that reliable results were obtained; 392 and 389 proteins were identified as a result of the two independent experiments. In total, 301 proteins overlapped in the two experiments and were considered the final accurate detected, high-quality proteins for the differential expression analysis. A threshold change of 1.5-fold was established to identify differentially expressed proteins in comparisons between CAD and control samples or CAA and control samples. For comparisons between CAD and control samples, 37 upregulated proteins and 50 downregulated proteins were identified as differentially expressed. For comparisons between CAA and control samples, the numbers of upregulated and downregulated differentially expressed proteins were 33 and 32, respectively (Tables II and III).

Differentially expressed protein categorization and network modelling. The functions (i.e., biological processes) of these differentially expressed proteins were analyzed using ClueGO, a Cytoscape plug-in that integrates GO terms (12). Pie charts were used to display the differences in the distribution of functional categories for differentially expressed proteins between KD patients with CAD and CAA (Fig. 3A). For the CAD vs. control comparison, 9 enriched processes were observed; however, only 4 processes were enriched in the CAA vs. control comparison. These results are presented as bar charts in Fig. 3B, which use the same color scheme as that of the pie charts. Analysis of the biological processes, as summarized in the two pie charts, notably demonstrated that the wound healing response and lipoproteins are involved in CAD and CAA. In a combined analysis of the corresponding CAA/CAD bar charts and pie charts, the main differentially expressed proteins were involved in the wounding response, and lipoprotein and cholesterol-associated processes, indicating that KD patients with CAA may suffer more harmful attacks in the coronary arteries or surrounding areas.

To comprehensively characterize the associations among significantly differentially expressed proteins in CAA and CAD, KEGG pathway enrichment analysis and signaling network modelling were employed using the Cytoscape plug-in ReactomeFIViz (13) (Fig. 4A). The thresholds for pathway enrichment were defined as $P < 0.05$ and $FDR < 1\%$. These enriched pathways were associated with bacterial and viral infection (e.g., *Staphylococcus aureus* infection), inflammatory responses (e.g., complement cascades and platelet activation) and other metabolic processes. Various bacteria and viruses have been reported as infectious agents of KD, such as *Staphylococcus aureus* (14), which may explain the activation of the inflammatory response pathways. However, KD is a highly-complicated disease, and its etiology and pathology are still unclear. The infection and inflammatory response-associated sub-networks were extracted from the whole differentially expressed protein networks in KD and the sub-network was highly enriched for proteins in infectious and inflammatory pathways. Some of these have demonstrated a high correlation with KD; the expression levels of the fibrinogen γ chain, which belongs to

Table II. Identification of differentially expressed proteins between the Kawasaki disease with coronary artery dilation and control groups by isobaric tags for relative and absolute quantitation technology.

Protein name	Gene name	Accession nos.	95% CI	Peptides (95%)	Unused ProtScore	Ratio (Con/CAD)
Complement C4-A	C4A	P0C0L4	59.46	263	176.2	1.9953
α -1-antitrypsin	SERPINA1	P01009	87.32	357	147.8	3.4041
Haptoglobin	HP	P00738	83.25	426	125.86	6.4269
Uncharacterized protein	N/A	B4E1Z4	47.47	105	100.21	3.4356
Hemopexin	HPX	P02790	74.24	155	82.36	2.3121
α -1-antichymotrypsin	SERPINA3	P01011	67.38	119	66.07	9.0365
α -1-acid glycoprotein 1	ORM1	P02763	55.22	90	44.86	7.5162
Plasma protease C1 inhibitor	SERPING1	P05155	47.80	61	44.05	3.5318
Complement component C9	C9	P02748	38.82	34	40.08	9.6383
Inter- α -trypsin inhibitor heavy chain H3	ITIH3	Q06033	29.10	53	34.19	2.4434
Ig α -1 chain C region	IGHA1	P01876	58.36	92	33.33	2.5119
Leucine-rich α -2-glycoprotein	LRG1	P02750	43.80	27	23.89	4.9204
Hemoglobin subunit β	HBB	P68871	88.44	41	21.25	3.0200
Angiotensinogen	AGT	P01019	25.77	21	20.46	9.7275
Fibrinogen α chain	FGA	P02671	25.17	21	19.87	9.3756
Plastin-2	LCP1	P13796	21.37	11	18.50	1.6596
Lipopolysaccharide-binding protein	LBP	P18428	23.70	10	18.31	3.6983
Fibrinogen γ chain	FGG	C9JEU5	33.26	11	17.56	2.5119
α -1-acid glycoprotein 2	ORM2	P19652	49.75	43	17.51	6.4269
Hemoglobin subunit α	HBA1	P69905	67.61	40	16.75	5.2966
Fibrinogen β chain	FGB	P02675	27.09	11	13.20	2.8314
Monocyte differentiation antigen CD14	CD14	P08571	32.00	7	12.14	2.0512
Serum amyloid A-1 protein	SAA1	P0DJI8	54.92	20	11.73	3.8019
Serum amyloid P-component	APCS	P02743	31.39	12	11.02	2.4889
Complement C4-B	C4B	P0C0L5	59.12	257	10.00	1.7865
Polymeric immunoglobulin receptor	PIGR	P01833	10.34	6	9.25	2.4210
C-reactive protein	CRP	P02741	16.52	8	8.53	8.3176
Mannose-binding protein C	MBL2	P11226	16.53	4	7.33	2.5586
Ig Δ chain C region (fragment)	IGHD	A0A0A0MS09	16.05	5	6.96	5.2966
Keratin, type I cytoskeletal 10	KRT10	P13645	11.82	5	6.66	2.2284
Transgelin-2 (fragment)	TAGLN2	X6RJP6	27.27	5	6.57	3.0479
Protein S100-A9	S100A9	P06702	30.7	3	6.39	4.9204
α -1-antitrypsin	SERPINA1	A0A024R6I7	87.32	348	5.19	46.1318
Lymphatic vessel endothelial hyaluronic acid receptor 1	LYVE1	Q9Y5Y7	5.59	2	4.00	2.0701
Protein S100-A8	S100A8	P05109	23.66	2	2.14	4.4463
Protein IGLV2-11 (fragment)	IGLV2-11	A0A075B6K3	6.723	3	2	5.7544
Resistin	RETN	Q9HD89	10.19	1	2	1.8707
Apolipoprotein B-100	APOB	P04114	51.92	374	399.96	-2.5586
Serotransferrin	TF	P02787	78.08	349	172.35	-1.8707
Serum albumin	ALB	P02768	74.88	271	139.74	-5.2966
Fibronectin	FN1	P02751	36.5	101	108.76	-30.4790
Complement factor H	CFH	P08603	50.93	85	92.38	-2.4660
Complement C5	C5	P01031	30.91	58	78.08	-2.0893
Apolipoprotein A-I	APOA1	P02647	78.28	162	65.7	-5.0582
Inter- α -trypsin inhibitor heavy chain H1	ITIH1	P19827	45.77	81	56.95	-2.7040
Plasminogen	PLG	P00747	52.59	63	55.98	-4.4875
Apolipoprotein A-IV	APOA4	P06727	65.4	52	45.59	-1.5996
Afamin	AFM	P43652	42.9	27	37.22	-2.4889
Heparin cofactor 2	SERPIND1	P05546	42.48	43	35.9	-3.3420

Table II. Continued.

Protein name	Gene name	Accession nos.	95% CI	Peptides (95%)	Unused ProtScore	Ratio (Con/CAD)
Histidine-rich glycoprotein	HRG	P04196	38.67	27	35.14	-9.2897
α -2-HS-glycoprotein	AHSG	P02765	57.49	51	34.96	-1.9231
Apolipoprotein A-II	APOA2	V9GYM3	49.62	66	30.77	-5.1051
Serum paraoxonase/arylesterase 1	PON1	P27169	59.44	35	30.46	-2.7040
Apolipoprotein E	APOE	P02649	56.78	38	26.73	-2.0512
Gelsolin	GSN	P06396	25.45	24	24.19	-3.8371
Complement component C8 β chain	C8B	F5H7G1	19.48	14	22.71	-2.5823
Coagulation factor XII	F12	P00748	26.5	21	21.87	-2.0893
Coagulation factor XIII B chain	F13B	P05160	23.45	15	21.37	-1.9231
Pigment epithelium-derived factor	SERPINF1	P36955	30.14	12	18.6	-1.9231
Lumican	LUM	P51884	41.42	17	18.42	-1.5417
β -2-glycoprotein 1	APOH	P02749	43.19	16	18.2	-2.1677
N-acetylmuramoyl-L-alanine amidase	PGLYRP2	Q96PD5	38.72	17	17.6	-2.3988
Kallistatin	SERPINA4	P29622	30.91	13	17.58	-3.1333
Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	P35858	18.18	10	17.48	-2.6546
Extracellular matrix protein 1	ECM1	Q16610	22.04	10	16.77	-2.1878
Sex hormone-binding globulin	SHBG	P04278	39.55	10	16.18	-2.2699
Transthyretin	TTR	P02766	72.11	27	15.31	-3.0479
Apolipoprotein L1	APOL1	O14791	34.42	11	14.3	-2.3335
Preylcysteine oxidase 1	PCYOX1	Q9UHG3	18.02	8	14	-3.1623
Retinol binding protein 4, plasma, isoform CRA_b	RBP4	Q5VY30	58.79	14	11.92	-3.6983
Phosphatidylinositol-glycan-specific phospholipase D	GPLD1	P80108	10.95	8	11.54	-3.6308
Apolipoprotein M	APOM	O95445	36.7	11	10.76	-3.5318
Tetranectin	CLEC3B	E9PHK0	55	10	10.58	-2.0893
Protein IGHV3-53 (fragment)	IGHV3-53	A0A087WSX4	60.34	21	10.43	-1.9770
Phospholipid transfer protein	PLTP	P55058	12.58	5	10.05	-1.8030
Apolipoprotein(a)	LPA	P08519	11.65	7	9.86	-2.8576
Uncharacterized protein	N/A	A0A0G2JPD4	71.04	150	9.19	-1.9055
Vitamin K-dependent protein C	PROC	E7END6	10.1	5	8	-1.7378
Cholesteryl ester transfer protein	CETP	P11597	10.14	6	6.52	-4.9659
Fetuin-B	FETUB	Q9UGM5	13.87	4	6.26	-2.7040
Apolipoprotein C-III	APOC3	B0YIW2	34.19	11	6.18	-2.8840
Uncharacterized protein	N/A	A0A0G2JN06	55.05	79	6.03	-3.3729
Protein IGHV1-69-2 (fragment)	IGHV1-69-2	A0A0B4J2H0	58.12	11	6	-1.7061
Apolipoprotein C-IV	APOC4	P55056	15.75	2	4	-2.5823
Apolipoprotein C-I (fragment)	APOC1	K7ERI9	23.38	2	2.84	-2.2699
Urea transporter 2	SLC14A2	Q15849	1.522	3	2	-3.4674
Extracellular superoxide dismutase (Cu-Zn)	SOD3	P08294	4.583	1	1.77	-1.6144

95% CI, percentage of matching amino acids from identified peptides having confidence intervals $\geq 95\%$, divided by the total number of amino acids in the sequence; Unused ProtScore, a measure of the protein confidence for a detected protein, calculated from the peptide confidence interval for peptides from spectra that are not already completely used by higher scoring winning proteins. Con, control group; CAD, coronary artery dilation group.

the fibrinogen family, are associated with KD according to a previous study (15). Thus, the sub-network generated was deemed to be associated with KD (Fig. 4B). The network

proteins were divided into 5 modules (presented as nodes of different colors in the figure) according to different clusters; proteins that serve a core role in each module and have either

Table III. Identification of differentially expressed proteins between the Kawasaki disease with coronary artery aneurysm and control groups by isobaric tags for relative and absolute quantitation technology.

Protein name	Gene name	Accession nos.	95% CI	Peptides (95%)	Unused ProtScore	Ratio (Con/CAA)
α -1-antitrypsin	SERPINA1	P01009	87.32	357	147.80	1.5996
Haptoglobin	HP	P00738	83.25	426	125.86	7.1121
Uncharacterized protein	N/A	B4E1Z4	47.47	105	100.21	2.8576
Ig μ chain C region	IGHM	A0A087WYJ9	64.94	98	68.73	1.6749
α -1-antichymotrypsin	SERPINA3	P01011	67.38	119	66.07	4.3251
α -1-acid glycoprotein 1	ORM1	P02763	55.22	90	44.86	6.7920
Complement component C9	C9	P02748	38.82	34	40.08	6.3096
Pregnancy zone protein	PZP	P20742	25.98	146	24.62	5.3951
Leucine-rich α -2-glycoprotein	LRG1	P02750	43.80	27	23.89	3.6983
Angiotensinogen	AGT	P01019	25.77	21	20.46	2.2909
Fibrinogen α chain	FGA	P02671	25.17	21	19.87	25.3513
Lipopolysaccharide-binding protein	LBP	P18428	23.70	10	18.31	4.4463
Fibrinogen γ chain	FGG	C9JEU5	33.26	11	17.56	21.2814
α -1-acid glycoprotein 2	ORM2	P19652	49.75	43	17.51	6.1376
Hemoglobin subunit α	HBA1	P69905	67.61	40	16.75	2.6303
Galectin-3-binding protein	LGALS3BP	Q08380	18.63	10	14.74	1.8535
Fibrinogen β chain	FGB	P02675	27.09	11	13.20	9.2897
Monocyte differentiation antigen CD14	CD14	P08571	32.00	7	12.14	1.7219
Serum amyloid A-1 protein	SAA1	P0DJ18	54.92	20	11.73	3.8371
Complement C4-B	C4B	P0C0L5	59.12	257	10.00	2.3335
Apolipoprotein(a)	LPA	P08519	11.65	7	9.86	3.6644
C-reactive protein	CRP	P02741	16.52	8	8.53	3.6644
Hemoglobin subunit γ -2	HBG2	P69892	43.54	6	7.70	4.0179
Transgelin-2 (fragment)	TAGLN2	X6RJP6	27.27	5	6.57	3.3420
Protein S100-A9	S100A9	P06702	30.70	3	6.39	5.2966
Protein IGHV1-69-2 (fragment)	IGHV1-69-2	A0A0B4J2H0	58.12	11	6.00	2.4660
α -1-antitrypsin	SERPINA1	A0A024R6I7	87.32	348	5.19	29.1072
Protein IGLV3-19 (fragment)	IGLV3-19	A0A075B6J8	70.54	7	4.66	2.9107
Protein IGLV7-46 (fragment)	IGLV7-46	A0A075B6I9	15.38	3	3.04	1.6596
HCG1782423 (fragment)	IGLV2-18	A0A075B6J9	24.17	2	2.63	3.3113
Protein S100-A8	S100A8	P05109	23.66	2	2.14	4.8306
Ras-related protein Rap-1b (fragment)	RAP1B	F5H823	17.48	2	2.00	2.2699
Serglycin	SRGN	P10124	8.23	1	2.00	1.6444
Serotransferrin	TF	P02787	78.08	349	172.35	-8.5507
Serum albumin	ALB	P02768	74.88	271	139.74	-3.6983
Fibronectin	FN1	P02751	36.50	101	108.76	-3.8726
Apolipoprotein A-I	APOA1	P02647	78.28	162	65.70	-3.1623
Inter- α -trypsin inhibitor heavy chain H1	ITI1	P19827	45.77	81	56.95	-2.0137
Plasminogen	PLG	P00747	52.59	63	55.98	-2.5119
Kininogen-1	KNG1	P01042	45.19	73	54.56	-2.3121
Apolipoprotein A-IV	APOA4	P06727	65.40	52	45.59	-2.7290
Antithrombin-III	SERPINC1	P01008	43.75	46	44.44	-2.0893
Afamin	AFM	P43652	42.90	27	37.22	-1.9953
Histidine-rich glycoprotein	HRG	P04196	38.67	27	35.14	-2.8054
α -2-HS-glycoprotein	AHSG	P02765	57.49	51	34.96	-2.2699
Apolipoprotein A-II	APOA2	V9GYM3	49.62	66	30.77	-3.3420
Serum paraoxonase/arylesterase 1	PON1	P27169	59.44	35	30.46	-2.0512
Apolipoprotein E	APOE	P02649	56.78	38	26.73	-1.7378
Gelsolin	GSN	P06396	25.45	24	24.19	-1.8707
Coagulation factor XII	F12	P00748	26.50	21	21.87	-1.8030

Table III. Continued.

Protein name	Gene name	Accession nos.	95% CI	Peptides (95%)	Unused ProtScore	Ratio (Con/CAA)
Protein AMBP	AMBP	P02760	31.25	24	18.34	-2.2284
Kallistatin	SERPINA4	P29622	30.91	13	17.58	-2.1281
Extracellular matrix protein 1	ECM1	Q16610	22.04	10	16.77	-1.6596
Transthyretin	TTR	P02766	72.11	27	15.31	-2.8576
Apolipoprotein L1	APOL1	O14791	34.42	11	14.30	-1.8030
Prenylcysteine oxidase 1	PCYOX1	Q9UHG3	18.02	8	14.00	-1.7061
Retinol binding protein 4, plasma, isoform CRA_b	RBP4	Q5VY30	58.79	14	11.92	-2.6303
Phosphatidylinositol-glycan-specific phospholipase D	GPLD1	P80108	10.95	8	11.54	-1.8880
Apolipoprotein M	APOM	O95445	36.70	11	10.76	-2.1086
Tetranectin	CLEC3B	E9PHK0	55.00	10	10.58	-1.8030
Cholinesterase	BCHE	P06276	10.96	7	10.56	-2.1281
Keratin, type I cytoskeletal 10	KRT10	P13645	11.82	5	6.66	-1.9953
Apolipoprotein C-III	APOC3	B0YIW2	34.19	11	6.18	-2.7040
Uncharacterized protein (fragment)	N/A	A0A0C4DH43	24.37	3	4.00	-2.3768
Urea transporter 2	SLC14A2	Q15849	1.52	3	2.00	-10.0000

95% CI, percentage of matching amino acids from identified peptides having confidence intervals $\geq 95\%$, divided by the total number of amino acids in the sequence; Unused ProtScore, a measure of the protein confidence for a detected protein, calculated from the peptide confidence interval for peptides from spectra that are not already completely used by higher scoring winning proteins; Con, control group; CAA, coronary artery aneurysm.

not been investigated or have rarely been investigated in previous studies were identified. Specifically, CFH, MBL2, KNG1, FN1 and SERPINC1 were identified as primary candidates involved in CAA and CAD (presented as bigger nodes with red circles). CFH and MBL2 were associated with classical complement proteins (yellow and pink clusters), KNG1 and FN1 were associated with proteins correlated to KD (grey and green clusters), and SERPINC1 is as an inhibitor in a complement protein associated pathway (green cluster). These results clarified the differentially expressed proteins in CAA and CAD samples and the associations among proteins in the network.

Validation of proteomics results. Alterations in the expression levels of proteins were validated by western blotting using CAD and CAA samples, and normal samples for comparison. Five proteins (CFH, MBL2, KNG1, FN1 and SERPINC1) were validated in 12 groups of samples (each including 1 control, 1 CAD and 1 CAA sample); the results for 5 of 12 validated groups are presented in Fig. 5. The protein expression results for the 5 proteins exhibited high specificity and were consistent with the proteomics results observed in the iTRAQ. In particular, MBL2 and CFH exhibited reduced expression levels within the CAD samples, and the opposite results were obtained for KNG and SERPINC1, in which the expression levels were downregulated within CAA samples. The expression levels of FN1 were downregulated in the CAD and CAA samples when compared with the control samples.

Discussion

The increased risk of coronary artery complications in patients with KD results in increased morbidity and mortality. The pathological processes of coronary artery lesions include endothelial dysfunction, the destruction of the internal elastic lamina, thrombocytosis and hypercoagulability. CAD and CAA are the main complications of KD, and may cause cardiovascular events (16). It is highly important to determine the mechanisms of CAD/CAA to prevent and treat these complications. In the present study, serum protein level differences between healthy volunteers, patients with CAD and patients with CAA were investigated. The results suggested that several pathways, including those involved in the response to wounding, lipoprotein remodeling, platelet activation and blood coagulation, may differ between healthy volunteers, patients with CAD and patients with CAA.

In the present study, differentially expressed proteins associated with the two coronary artery abnormalities, CAD and CAA, within patients with KD were identified using proteomics methods. A total of 51 samples were divided into 17 groups. Each group contained 1 control sample, 1 CAD sample and 1 CAA sample. Three pooled samples (5 control, 5 CAD and 5 CAA) were generated by combining samples from 5 groups and were used for iTRAQ analyses to detect significantly differentially expressed proteins. The other 12 groups were used for western blot validation. In total, 87 and 65 differentially expressed proteins for the CAD and CAA were obtained, respectively, compared with the control.

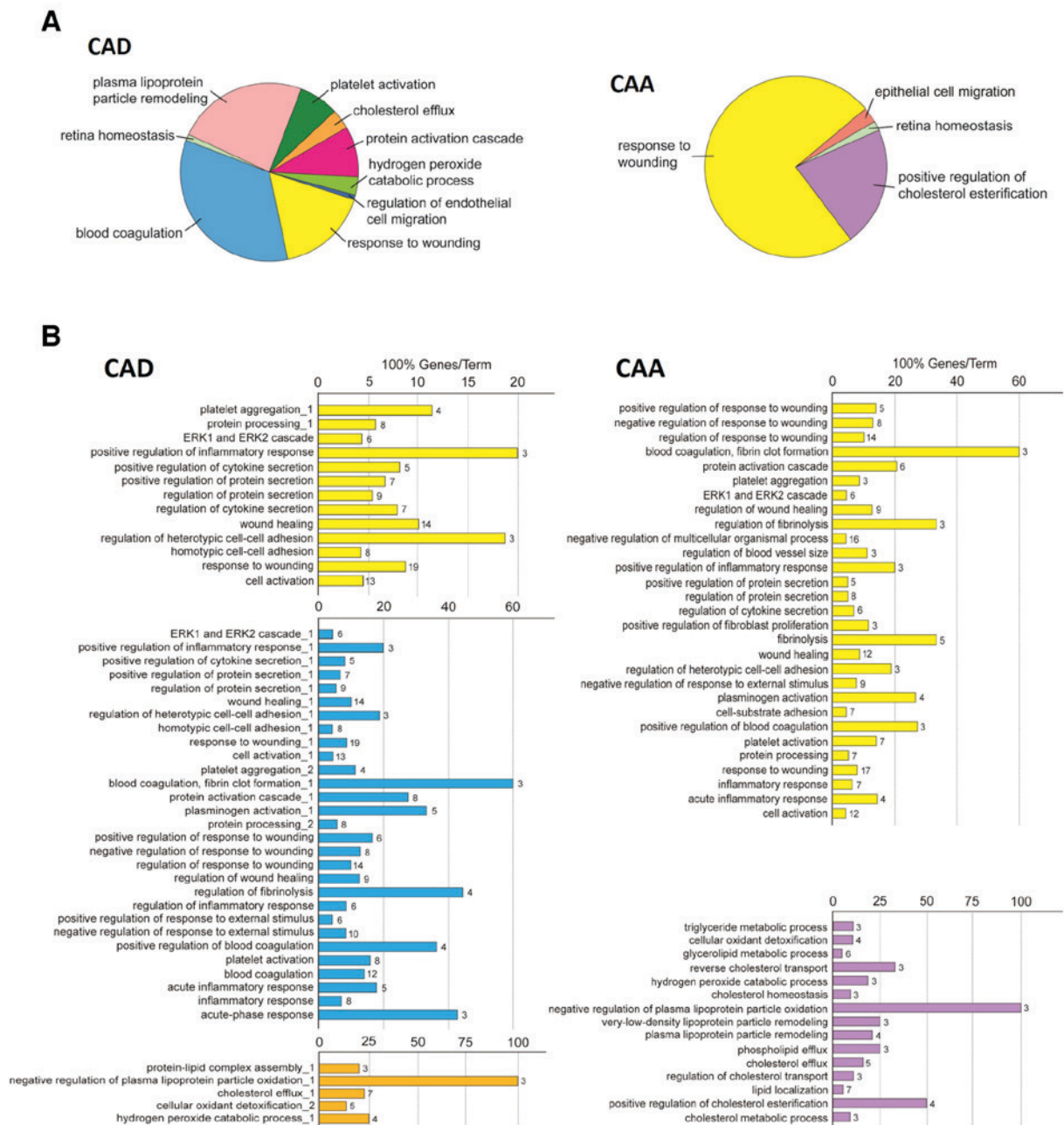


Figure 3. Biological process of differentially expressed proteins in KD with CAD and CAA. (A) Pie charts representing the distribution of identified differentially expressed proteins according to their biological process. (B) Bar charts demonstrating the specific processes that correspond to the classification; the same color key that was used in the pie charts has also been applied in these charts. $P < 0.05$ was used as a threshold for gene ontology categories enrichment. KD, Kawasaki disease; CAA, coronary artery aneurysm; CAD, coronary artery dilation.

The biological processes associated with differentially expressed proteins in CAD and CAA were also analyzed. Variations between the two conditions were identified in the present study; lower representation of proteins associated with the wounding response, lipoprotein modelling, platelet activation and blood coagulation, revealed that patients with KD and CAD may exhibit stress responses associated with coronary artery injury. However, within CAA patients, the representation of proteins involved in the response to wounding and the corresponding cholesterol components increased significantly with respect to control samples, indicating aggravated injury in coronary arteries. All of the identified significant proteins were used to construct a signaling network

to clarify the overall pathway associations. The main pathways were enriched for functions in infection and immune associated diseases, inflammation and metabolism. These results are consistent with a previous hypothesis (17,18). Thus, the present study focused on the infection, inflammation and coagulation pathways, which were extracted from the above network as a novel network for further analyses.

As presented in Fig. 3B, a few proteins in this network have been reported in previous studies associated with KD, including complement C3 (C3) (19), apolipoprotein A-I (20), and fibrinogen β chain (21). Accordingly, the focus of the present study was on significant proteins that have not been reported previously and served key roles in each cluster of the

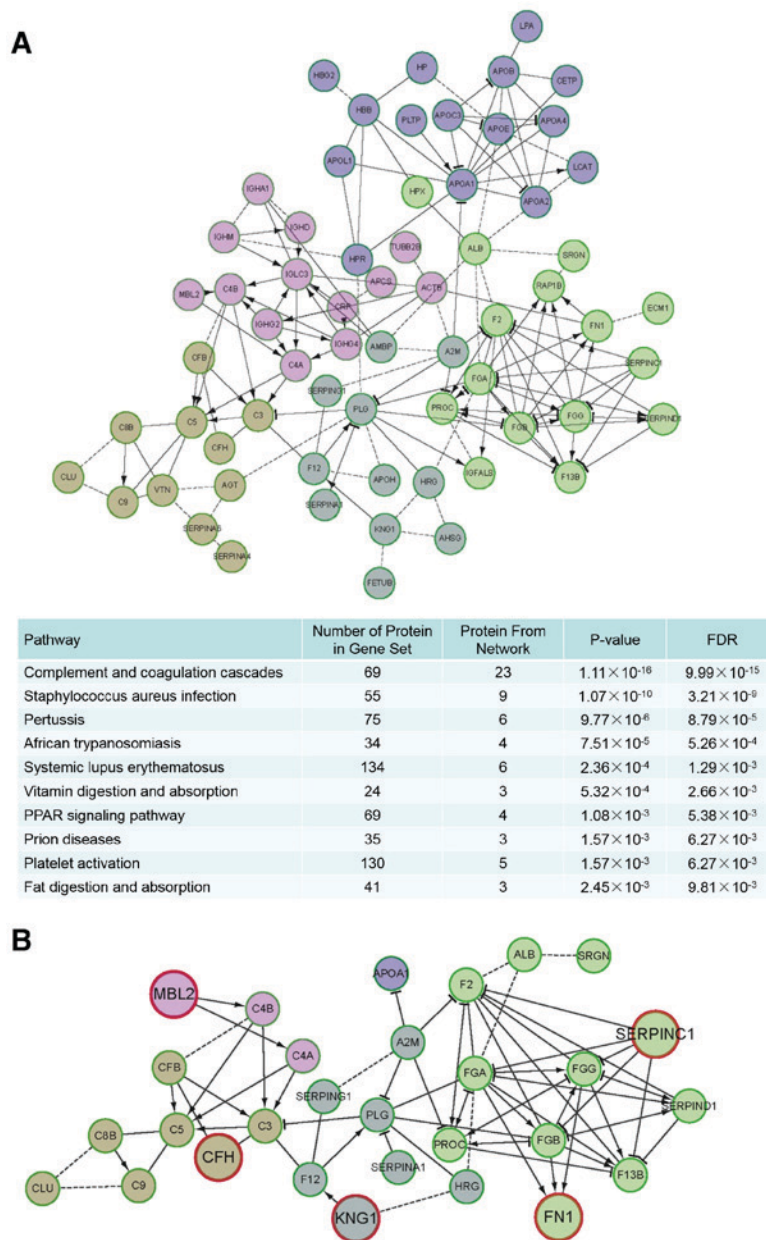


Figure 4. Network of differentially expressed proteins in KD with CAD and CAA. (A) Network based on functionally-enriched KEGG pathways. The proteins in the network were differentially expressed proteins in the present study. (B) Network associated with the variations within patients with KD and CAD/CAA. CFH (yellow cluster), MBL2 (pink cluster), KNG1 (grey cluster), FN1 (green cluster) and SERPINC1 (green cluster) were identified as primary candidates involved in CAA and CAD (presented as bigger nodes with red circles). $P < 0.05$ was a threshold for KEGG pathway enrichment. KD, Kawasaki disease; CAA, coronary artery aneurism; CAD, coronary artery dilation; KEGG, Kyoto Encyclopedia of Genes and Genomes; FN1, fibronectin 1; CFH, complement factor; KNG1, kininogen 1; MBL2, mannose binding lectin 2; SERPINC1, serpin family C member 1; FDR, false discovery rate.

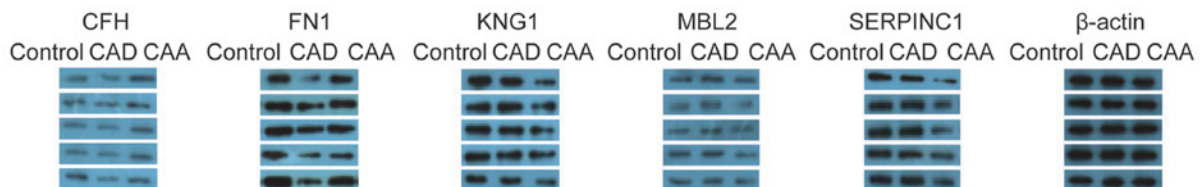


Figure 5. Protein expression confirmation of MBL2, CFH, KNG1, SERPINC1 and FN1 by western blotting in 5 representative groups comprised of healthy children (control), patients with KD and CAD, and patients with KD and CAA. CAA, coronary artery aneurism; CAD, coronary artery dilation; KD, Kawasaki disease; FN1, fibronectin 1; CFH, complement factor; KNG1, kininogen 1; MBL2, mannose binding lectin 2; SERPINC1, serpin family C member 1.

network. Thus, the five proteins, CFH, MBL2, KNG1, FN1 and SERPINC1 were selected as candidate proteins associated

with CAD and CAA. The western blot results for the five proteins were consistent with those of the iTRAQ analyses.

MBL2 and CFH were reduced in KD with CAD compared with controls. KNG1 and SERPINC1 were downregulated in KD with CAA. FN1 was differentially expressed in both diseases; FN1 decreased in CAA and CAD when compared with controls.

CFH and MBL2 were in the complement protein-associated part of the network. Complement proteins, similar to the defense and clearance systems, serve an important role in the innate immune system. Pathogens activate the main complement protein C3 via one of the classical complement proteins, such as lectin, or alternative pathways to form C3b. The process is regulated to avoid excessive C3b production, which damages host cells, or a C3b deficiency, which induces an immune-compromised state via the main regulatory complement factor CFH (22). MBL2 is a macromolecule that contains sugar-binding lectin and collagenous domains, and forms MBL-associated serine proteases to cleave complement proteins C2 and C4. The complex process is also known as the MBL pathway. A reduced level of MBL is associated with increased bacterial or viral infections in children, and MBL is known to serve a dual role in KD. Within children <1-year-old, MBL may be associated with the resistance of infections, and in older children, MBL may be associated with endothelial damage (23). Decreases in CFH and MBL2 were only observed in KD with CAD samples, which suggested a dysfunction in the complement system.

KNG1 and SERPINC1 are located in a coagulation-associated network. To the best of our knowledge, the direct genetic interactions of KNG1 with histidine-proline rich glycoprotein (HRG) and coagulation factor XII have never been reported in previous KD studies. However, some studies have indicated that they may interact with each other in the coagulation pathway. KNG1 and HRG have similar structures and are adjacent to each other on chromosome 3, which suggests that these proteins may exhibit similar functions (24,25). HRG deficiency is associated with enhanced blood coagulation, which indicates that decreased KNG1 is a potential cofactor to enhance coagulation (26). The potent anticoagulant SERPINC1 is a coagulation protease inhibitor and also possesses independent anti-inflammatory properties. Overexpressed SERPINC1 stimulates the expression of Coagulation Factor II and other proteases (27). The protein is mainly associated with the diseases, inherited thrombophilia (28) and SERPINC1 deficiency (29), by affecting the anticoagulation pathways. The downregulation of KNG1 and SERPINC1 observed in KD with CAA samples may increase blood coagulation to enhance thrombosis.

FN1 is a multifunctional glycoprotein that serves in the plasma and extracellular matrix as a soluble dimer, or at the cell surface as a dimer or multimer. It participates in multiple biological processes, including cell adhesion, migration, wound healing, and blood coagulation (30). Both CAD and CAA are characterized by endothelial dysfunction and stress responses to wound healing. Therefore, the decrease in FN1 expression levels in patients with coronary artery lesions may be associated with the aforementioned pathways.

In conclusion, the present study identified five candidate proteins differentially expressed in patients with KD and CAD/CAA: CFH, MBL2, KNG1, FN1 and SERPINC1. These five proteins provide a basis for understanding the differences

between CAD and CAA in patients with KD. The alterations in **MBL2 and CFH indicated that responses to pathogen infections** and the innate immune system may be closely associated with the development of CAD. Decreased levels of KNG1 and SERPINC1 revealed that dysfunctions in coagulation accompanied the development of CAA. Decreased FN1 expression levels in the two conditions indicated that the pathways, including cell adhesion, migration, wound healing and blood coagulation may serve a role in the occurrence of CAD and CAA. The five proteins investigated in the present study may also serve to distinguish CAD and CAA in the early diagnosis of KD.

Acknowledgements

The present study was supported by the Natural Science Fund of China (grant no. 81500275 to H.-L.J), Guangdong Natural Science Foundation (grant no. 2016A030313080 to H.-L.J), Guangdong Natural Science Foundation (grant no. 2016A030310080 to W.-T.L), Fundamental Research Funds for the Central Universities (grant no. 21616305 to W.-T.L), Guangzhou City Scientific Research Project Foundation of Technology and Information Bureau (grant no. 201510010287 to L.Z), the Natural Science Fund of China (81300124 to M.-G.X), Guangdong Medical Science Foundation (grant no. A2016012 to M.-G.X) and Shen Zhen Scientific Plan (grant no. JCYJ20140416141331478 to M.-G.X).

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