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A study of serum proteome expression in patients with severe hand-foot-mouth disease

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Abstract. – OBJECTIVE: Although numerous studies have been conducted on hand-foot-mouth disease (HFMD), the diagnosis of severe HFMD has not been fully clarified. Hence, it is important to further clarify the diagnosis of severe HFMD. In this study, we conducted a clinical biomarker discovery in patients with severe HFMD.

PATIENTS AND METHODS: In this study, serum samples were isolated from severe HFMD, HFMD, and healthy controls. Each group consisted of 32 cases. Isobaric tagging for relative and absolute quantitation (iTRAQ) combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to detect proteome expression in the serum samples. Then, candidate proteins were screened and verified by ELISA. Protein expressions were significantly different between the HFMD group, severe HFMD group, and healthy control group.

RESULTS: Comparison of the proteins between the three groups showed that serum amyloid A-1 protein (P0DJ18), C-reactive protein (P02741), fibronectin (P02751), plasminogen (P00747) and apolipoprotein A (P08519) were different, so they were selected as candidate proteins. However, the results of ELISA showed that the expression levels of serum amyloid A-1 protein, C-reactive protein, fibronectin, and apolipoprotein a in the severe HFMD group were significantly different from those in the other two groups ($p<0.05$).

CONCLUSIONS: In conclusion, the results showed that serum amyloid A-1, C-reactive protein, fibronectin, and apolipoprotein A may be potential biomarkers for clinical diagnosis of severe HFMD.

Key Words:
Severe hand-foot-mouth disease, Protein expression, iTRAQ combined with MS.

Introduction

Hand-foot-mouth disease (HFMD) is an infectious disease, usually characterized by fever, oral vesicles, and rashes on the hands, feet, and buttocks[1]. In the past decade, large outbreaks of HFMD in children under five years of age have occurred in some provinces of China[2,3], which has become one of the main childhood diseases[4]. Enterovirus 71 (EV71) is the main pathogenic virus causing HFMD. The infection of EV71 is positively correlated to high mortality and incidence rate[5].

Severe HFMD is a major problem worldwide, which causes a heavy burden on children's health and society[6]. The clinical manifestations of severe HFMD include aseptic encephalitis, brainstem encephalitis, myelitis, myocarditis, and pulmonary edema, which cause mortality[7]. However, the degree of HFMD and the causes of severe HFMD remain unclear. Some researchers regard HFMD as the precursor to severe HFMD, and 5% of HFMD cases will progress to severe HFMD without proper diagnosis[8,9].

HFMD is caused by an enterovirus, and different proteins are expressed between HFMD and severe HFMD[10]. Comparative studies between HFMD and severe HFMD may be helpful for further understanding the pathogenesis and biomarkers of HFMD and severe HFMD[11]. Proteomics is a post-genomic biotechnology[12]. Isobaric tagging for relative and absolute quantitation (iTRAQ) is a powerful proteomics technology for protein expression research, which is used for the relative and absolute quantitative detection of proteins. It has many advantages, and the biggest advantage is that it can observe the differences between multiple proteins in a single test compared to traditional experiments[13]. We performed a preliminary study on protein expression differences between HFMD, severe HFMD, and healthy controls by iTRAQ-LC-MS/MS analysis[14]. Then, we screened candidate proteins by enrichment analysis and tested them with ELISA.

Patients and Methods

Participants

Healthy children were randomly selected from the physical examination center of Chengdu Fifth People's Hospital as the healthy control

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group (HC). Patients with HFMD and severe HFMD diagnosed in the pediatric outpatient department of Chengdu Fifth People's Hospital between January 2022 and November 2022 were included in the HFMD and severe HFMD groups, respectively. The diagnostic criteria of HFMD and severe HFMD were from WS-588-2018 diagnosis for HFMD. Severe HFMD is defined as a case of neurological complications such as aseptic meningitis, encephalitis, acute flaccid paralysis, pulmonary edema, or cardiopulmonary failure. The study was approved by the Medical Ethics Committee of the Chengdu Fifth People's Hospital and fully complied with the Helsinki Declaration. Written consent from the parents of all study participants was obtained before any procedure.

Clinical Characteristics

We retrospectively analyzed the clinical characteristics of 64 children with HFMD. The pathogens of 64 patients were confirmed by RT-PCR. The clinical manifestations of these 64 children were mainly rash, fever, lethargy, eclampsia, headache, and restlessness.

iTRAQ-LC-MS/MS Analysis and Identification of Serum Proteins

5 ml of peripheral venous blood was extracted from the children. After centrifugation, the serum was separated, and the proteins in the serum were analyzed and identified by iTRAQ-LC-MS/MS. Protein concentration was detected by BCA Protein Assay Kit (Sangon Biotech, Shanghai, PR China). iTRAQ labeling was carried out according to the manufacturer's protocol (Sciex, Massachusetts, USA). Each sample was individually marked with two of the eight available labels. All labeled peptides were collected. The Ultimate 3000 HPLC system (Dionex, CA, USA) equipped with a 2.00-mm-inner diameter 100-mm-long Gemini-NX 3u C18110A columns (Phenomenex, CA, USA) was used in high-pH fractionation. The peptide was loaded onto the column and washed with equal proportions under 95% eluent A (20 mmol HCOONH4, 2mole NaOH) (pH 10). The peptide was graded linearly by binary gradient using 15-50% B solution (20 mmol HCOONH4, 2 mole NaOH, 80% CAN) (pH 10) at 0.2 ml/min over 45 min. Finally, the column was washed under 90% solution B for 10 minutes and reverted to 95% solution A for 10 minutes. The wavelength of the UV detector was set at 214/280 nm, and the separation solution was collected every minute. A total of 10 fractions were collected and dried in a vacuum centrifuge for subsequent nano-reverse liquid chromatography (nano-LC) classification. Each fraction was resuspended in a loading buffer (0.1% FA, 2% ACN) and separated using an Ultimate 3000 nano-LC system equipped with a C18 reverse phase column (100 μm inner diameter, 10 cm long, 3 μm resin from Michrom Bioresources, Auburn, CA, USA). The peptides were separated. Then, LC eluate was collected from TripleTOF 5600 MS/MS system (AB SCIEX, CA, USA) in information-dependent collection mode. In the high-resolution mode (>30,000), the MS spectrum was collected in the mass range of 400-1250 m/z using the cumulative time of 250 ms for each spectrum. Each cycle selected up to 20 precursors for fragmentation from each MS spectrum. The minimum accumulation time of each precursor was 100 ms, and the dynamic elimination time was 20 s.

Relative quantification and protein identification were performed with ProteinPilotTM software (version 5.0, Applied Biosystems, CA, USA) using the ParagonTM algorithm (Applied Biosystems, CA, USA) as the search engine. Specify processing included quantitate, bias correction, and background correction. All proteins identified must have ≥95% confidence and the protein confidence threshold cut-off was set to 1.3 (unused) with at least more than one peptide above the 95% confidence level. To designate significant changes in protein expression, fold-changes <1.5 were set as cut-off values.

Candidate Protein Verification by ELISA

We quantitatively detected the expression levels of candidate proteins in the serum samples of the three groups by ELISA using Human-LRG1/SAA1 ELISA kit (Catalog No.: ab260066, ab100635, Abcam, Cambridge, UK) and Human Fibronectin ELISA kit (Catalog No.: ab219046, Cambridge, UK). HITACHI 7100 was used for the c-reactive protein(CRP) and Apolipoprotein tests.

Statistical Analysis

Normal distribution data was expressed as the mean±standard deviation (χ±SD). Data were evaluated by GraphPad Prism 9.0 software (GraphPad Prism Software, CA, USA). Analysis of variance (ANOVA) was used for comparisons between groups. Two-way ANOVA was used for compar-

isons between the three groups. A p-value <0.05 was considered statistically significant.

Results

Clinical Characteristics of Individuals in Each Group

The clinical characteristics of patients in each group are summarized in Table I.

iTRAQ-LC-MS/MS Analysis

The protein expressions of the HFMD group, severe HFMD group, and healthy controls (HC) group were compared and analyzed. A total of 507 proteins were identified, among which we selected those with ratios of >1.5. The results showed that 46 proteins were up-regulated and 43 proteins were down-regulated between the HFMD and HC groups (Table II). Moreover, 36 proteins were up-regulated, and 39 proteins were down-regulated between the severe HFMD and HC groups (Table III). Furthermore, 13 proteins were up-regulated, and 23 proteins were down-regulated between the severe HFMD and HFMD groups (Table IV).

Candidate Protein Selection

Gene Ontology (GO) analysis showed that the proteins were mainly located in the extracellular region, and their primary molecular functions were ion binding, enzyme regulation activity, peptidase activity, and lipid binding. Comparing the protein expression between the severe HFMD, HFMD, and control groups, we screened six proteins (Leucine-rich alpha-2-glycoprotein, Serum amyloid A-1 protein, C-reactive protein, Fibronectin, Plasminogen, and Apolipoprotein A) as candidate proteins, which passed the ELISA validation test.

ELISA for Candidate Protein Verification

The ELISA results (Figure 1) showed that the levels of SAA1 and CRP were higher in the severe HFMD group compared to the HFMD group (p<0.05) and the HC group (p<0.05). The levels of Fn and Apo A proteins were lower in the severe HFMD group compared to the HFMD group (p<0.05) and the HC group (p<0.05). Therefore, serum amyloid A (SAA) and CRP proteins were increased, while Fn and Apo proteins were decreased in the severe HFMD group.

Discussion

Enterovirus is the main pathogen of hand-foot-mouth disease (HFMD)[15]. Enterovirus belongs to the small *Reoviridae* enterovirus family, which is a single-stranded positive RNA virus[16]. The major serotypes that cause this disease include enteroviruses of the Coxsackie virus (CV) group A and B types, as well as some echovirus serotypes, and enterovirus A71[17]. HFMD is a global infectious disease caused by various enteroviruses, usually occurring in children under 5 years of age[18]. In recent years, several major outbreaks of HFMD have occurred in China, a small proportion of which exhibited serious symptoms, such as aseptic meningitis, encephalitis, acute flaccid paralysis, pulmonary edema, myocarditis, and even death[19]. To date, the pathogenesis and molecular mechanism of EV71 and CVB infections remain unclear[20].

Table I. Clinical characteristics of the children.

Clinical parameters	HFMD group (n=32)	Sever HFMD group (n=32)	Health control group (n=32)
Age	2.69	2.75	2.65
Rash	±	+	-
Fever	±	+	-
Lethargy (%)	32.6	51.05	0
Eclampsia (%)	9.07	21.4	0
Headache (%)	3.2	16.0	0
Agitation (%)	4.82	15.55	0
c-reactive protein (>8 mg/L, n)	2	13	0
Coxsackievirus A group16 positive rate (%)	37	34	0
Enterovirus71 positive rate (%)	63	66	0

Table II. List of proteins differentially expressed between HFMD and HC groups.

Accession	List of proteins	Peptides (95%)	Ration >1.5	C.V	Expression
P0C0L4	Complement C4	287	1.522	0.051	up
P00450	Ceruloplasmin	250	2.674	0.36	
B4E1Z4	Uncharacterized protein	131	6.661	0.278	
P00738	Haptoglobin	321	17.38	0.258	
P01011	Alpha-1-antichymotrypsin	132	5.611	0.485	
P10643	Complement component C7	42	3.981	0.232	
P04003	C4b-binding protein alpha chain	48	3.035	0.276	
P05546	Heparin cofactor 2	56	1.762	0.225	
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	40	1.56	0.169	
P02748	Complement component C9	35	1.901	0.017	
P02763	Alpha-1-acid glycoprotein 1	87	11.999	0.342	
P04278	Sex hormone-binding globulin	24	2.141	0.258	
O14791	Apolipoprotein L1	26	1.785	0.11	
Q08380	Galectin-3-binding protein	22	3.119	0.068	
P02671	Fibrinogen alpha chain	20	4.734	0.461	
P02750	Leucine-rich alpha-2-glycoprotein	23	2.392	0.06	
O75636	Ficolin-3	22	2.02	0.108	
P0DJI8	**Serum amyloid A-1 protein**	**49**	**7.846**	**0.056**	
G3XAM2	Complement factor	17	2.0457	0.062	
P08571	Monocyte differentiation antigen CD14	12	1.531	0.119	
P02743	Serum amyloid P-component	15	2.252	0.318	
P19652	Alpha-1-acid glycoprotein 2	48	2.717	0.224	
A0A087WUS7	Ig delta chain C region	13	1.585	0.027	
P01782	Immunoglobulin heavy variable 3-9	28	2.227	0.113	
A0A096LPE2	Protein SAA2-SAA4	43	2.35	0.12	
P02675	Fibrinogen beta chain	6	2.117	0.058	
P02741	**C-reactive protein**	**6**	**2.374**	**0.271**	
A0A0B4J1Y8	Protein IGLV9-49	4	1.647	0.132	
P20742	Pregnancy zone protein	149	5.532	0.149	
P01700	Immunoglobulin lambda variable 1-47	11	1.905	0.12	
A0A075B6N8	Ig gamma-3 chain C region (Fragment)	43	3.1	0.08	
P69891	Hemoglobin subunit gamma-1	9	1.509	0.163	
P06681	Complement C2	30	1.797	0.052	
A0A0C4DH38	Protein IGHV5-51 (Fragment)	12	2.138	0.289	
P18428	Lipopolysaccharide-binding protein	5	1.936	0.19	
A0A0B4J231	Immunoglobulin lambda-like polypeptide 5	26	1.763	0.457	
C9JEU5	Fibrinogen gamma chain	3	2.123	0.07	
P06732	Creatine kinase M-type	3	2.008	0.113	
A0A075B6K5	HCG2043239 (Fragment)	10	2.461	0.102	
A0A0C4DH29	Immunoglobulin heavy variable 1-3	7	1.572	0.086	
P35573	Glycogen debranching enzyme	1	1.598	0.085	
J3KT10	Nuclear pore complex protein Nup85	1	1.606	0.37	
G5E968	Chromogranin A (Parathyroid secretory protein 1)	1	1.531	0.428	
A0A0A0MT69	Protein IGKJ4 (Fragment)	1	2.742	0.213	
A0A0B4J231	Immunoglobulin lambda-like polypeptide 5	26	1.763	0.457	
P02741	C-reactive protein	6	2.374	0.271	

Continued

Table II *(Continued)*. List of proteins differentially expressed between HFMD and HC groups.

Accession	List of proteins	Peptides (95%)	Ration >1.5	C.V	Expression
P02751	**Fibronectin**	**153**	**0.611**	**0.215**	down
P02647	Apolipoprotein A-I	160	0.303	0.278	
P00747	**Plasminogen**	**82**	**0.543**	**0.155**	
P00734	Prothrombin	77	0.635	0.025	
P06396	Gelsolin	54	0.591	0.231	
P43652	Afamin	44	0.5	0.069	
P07996	Thrombospondin-1	29	0.554	0.117	
P02765	Alpha-2-HS-glycoprotein	91	0.543	0.368	
P35858	Insulin-like growth factor-binding protein complex acid labile subunit	25	0.522	0.093	
P25311	Zinc-alpha-2-glycoprotein	31	0.654	0.248	
V9GYM3	Apolipoprotein A-II	80	0.418	0.275	
P27169	Serum paraoxonase/arylesterase 1	36	0.523	0.155	
P04196	Histidine-rich glycoprotein	24	0.474	0.102	
P02749	Beta-2-glycoprotein 1	20	0.645	0.29	
P04264	Keratin, type II cytoskeletal 1	13	0.653	0.417	
P15169	Carboxypeptidase N catalytic chain	16	0.608	0.029	
P29622	Kallistatin	15	0.658	0.13	
Q5VY30	Retinol binding protein 4, plasma, isoform CRA_b	20	0.601	0.103	
P60709	Actin, cytoplasmic 1	11	0.496	0.028	
P08519	**Apolipoprotein(a)**	**16**	**0.539**	**0.123**	
P55058	Phospholipid transfer protein	6	0.612	0.065	
P05452	Tetranectin	17	0.581	0.079	
P35527	Keratin, type I cytoskeletal 9	10	0.627	0.053	
K7ER74	Protein APOC4-APOC2	18	0.477	0.13	
P05154	Plasma serine protease inhibitor	7	0.48	0.167	
P01861	Ig gamma-4 chain C region	61	0.459	0.208	
P11021	78 kDa glucose-regulated protein	6	0.59	0.095	
Q15485	Ficolin-2	10	0.568	0.07	
K7ERI9	Apolipoprotein C-I (Fragment)	12	0.504	0.196	
P17936	Insulin-like growth factor-binding protein 3	6	0.371	0.18	
P02042	Hemoglobin subunit delta	24	0.623	0.095	
P40197	Platelet glycoprotein V	3	0.623	0.069	
A0A0C4DGZ8	Glycoprotein Ib (Platelet), alpha polypeptide	4	0.635	0.068	
A0A075B6N7	Ig alpha-2 chain C region (Fragment)	29	0.534	0.252	
P07737	Profilin-1	2	0.585	0.091	
J3KPA1	Cysteine-rich secretory protein 3	4	0.65	0.075	
P62834	Ras-related protein Rap-1A	3	0.506	0.079	
A0A075B7D0	Protein IGHV1OR15-1 (Fragment)	5	0.617	0.055	
Q92859	Neogenin	1	0.5	0.338	
H9KV75	Alpha-actinin-1	1	0.291	0.05	
P08514	Integrin alpha-IIb	1	0.65	0.155	
E7EX29	14-3-3 protein zeta/delta (Fragment)	1	0.479	0.019	
F5H7S3	Tropomyosin alpha-1 chain	1	0.517	0.03	

Table III. List of proteins differentially expressed between sever HFMD and HC groups.

Accession	List of proteins	Peptides (95%)	Ration >1.5	C.V	Expression
P00450	Ceruloplasmin	250	1.976	0.417	up
B4E1Z4	Uncharacterized protein	131	8.523	0.254	
P00738	Haptoglobin	321	15.233	0.279	
P01011	Alpha-1-antichymotrypsin	132	7.413	0.465	
P10643	Complement component C7	42	4.151	0.245	
P04003	C4b-binding protein alpha chain	48	3.265	0.293	
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	40	1.729	0.15	
P02748	Complement component C9	35	2.094	0.026	
P02763	Alpha-1-acid glycoprotein 1	87	14.232	0.298	
B4DPQ0	Complement C1r subcomponent	33	1.556	0.02	
P04278	Sex hormone-binding globulin	24	2.057	0.126	
Q08380	Galectin-3-binding protein	22	2.824	0.093	
P02671	Fibrinogen alpha chain	20	2.173	0.494	
P00748	Coagulation factor XII	21	2.023	0.205	
P02750	Leucine-rich alpha-2-glycoprotein	23	3.87	0.13	
O75636	Ficolin-3	22	1.562	0.115	
P0DJI8	**Serum amyloid A-1 protein**	**49**	**13.95**	**0.085**	
G3XAM2	Complement factor I	17	2.192	0.064	
P00742	Coagulation factor X	15	1.603	0.181	
P02743	Serum amyloid P-component	15	3.264	0.296	
P19652	Alpha-1-acid glycoprotein 2	48	3.029	0.273	
P01782	Immunoglobulin heavy variable 3-9	28	1.717	0.198	
G3V2W1	Protein Z-dependent protease inhibitor	10	1.73	0.191	
A0A096LPE2	Protein SAA2-SAA4	43	2.343	0.15	
P02741	**C-reactive protein**	**6**	**5.601**	**0.246**	
A0A0B4J1Y8	Protein IGLV9-49	4	1.968	0.181	
P20742	Pregnancy zone protein	149	2.225	0.171	
P01700	Immunoglobulin lambda variable 1-47	11	1.673	0.142	
A0A075B6N8	Ig gamma-3 chain C region (Fragment)	43	2.412	0.142	
P30447	HLA class I histocompatibility antigen, A-23 alpha chain	3	3.053	0.148	
P06681	Complement C2	30	1.507	0.096	
P18428	Lipopolysaccharide-binding protein	5	2.646	0.187	
P06732	Creatine kinase M-type	3	2.64	0.057	
P35573	Glycogen debranching enzyme	1	1.507	0.029	
A0A0A0MT69	Protein IGKJ4 (Fragment)	1	2.925	0.101	
P02741	C-reactive protein	6	5.601	0.246	

Continued

The occurrence of hand-foot-mouth disease has a clear seasonal pattern, the peak period usually occurs from April to July[21,22]. Hence, it is meaningful to monitor the incidence of HFMD during the epidemic period, especially for distinguishing critically ill patients as soon as possible. In order to better control and prevent possible severe HFMD, the use of a biomarker to diagnose severe hand, foot, and mouth disease can improve the diagnostic accuracy of hand, foot, and mouth disease[23,24].

Isobaric tagging for relative and absolute quantitation (iTRAQ) is a helpful proteomic technology for biomarker identification[25]. The iTRAQ technology is high throughput and can identify differentially expressed proteins between different groups[26]. Combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), iTRAQ has been used to identify specific biomarkers in some diseases[27].

In recent years, deeper insights into the mechanisms underlying the pathogenesis of severe

Table III *(Continued)*. List of proteins differentially expressed between sever HFMD and HC groups.

Accession	List of proteins	Peptides (95%)	Ration >1.5	C.V	Expression
P02751	**Fibronectin**	**153**	**0.307**	**0.24**	down
P02647	Apolipoprotein A-I	160	0.38	0.25	
P00747	**Plasminogen**	**82**	**0.322**	**0.419**	
P01042	Kininogen-1	111	0.651	0.259	
P43652	Afamin	44	0.616	0.215	
P07996	Thrombospondin-1	29	0.404	0.089	
P35858	Insulin-like growth factor-binding protein complex acid labile subunit	25	0.528	0.08	
P68871	Hemoglobin subunit beta	44	0.497	0.032	
P04196	Histidine-rich glycoprotein	24	0.563	0.134	
P02749	Beta-2-glycoprotein 1	20	0.658	0.287	
P36955	Pigment epithelium-derived factor	19	0.631	0.057	
P29622	Kallistatin	15	0.542	0.142	
Q5VY30	Retinol binding protein 4, plasma, isoform CRA_b	20	0.459	0.116	
P00739	Haptoglobin-related protein	125	0.386	0.28	
P60709	Actin, cytoplasmic 1	11	0.353	0.011	
A0A0C4DFP6	Cartilage acidic protein 1	10	0.555	0.131	
P08519	**Apolipoprotein(a)**	**16**	**0.165**	**0.171**	
K7ER74	Protein APOC4-APOC2	18	0.434	0.225	
P05154	Plasma serine protease inhibi-tor	7	0.358	0.194	
P11021	78 kDa glucose-regulated pro-tein	6	0.619	0.122	
Q15485	Ficolin-2	10	0.657	0.071	
K7ERI9	Apolipoprotein C-I (Fragment)	12	0.472	0.149	
P17936	Insulin-like growth factor-binding protein 3	6	0.321	0.102	
P06312	Immunoglobulin kappa varia-ble 4-1	15	0.585	0.231	
P69891	Hemoglobin subunit gamma-1	9	0.624	0.145	
P02042	Hemoglobin subunit delta	24	0.581	0.162	
Q6UXB8	Peptidase inhibitor 16	3	0.649	0.043	
P40197	Platelet glycoprotein V	3	0.622	0.01	
A0A0C4DGZ8	Glycoprotein Ib (Platelet), al-pha polypeptide	4	0.615	0.105	
P07737	Profilin-1	2	0.467	0.092	
J3KPA1	Cysteine-rich secretory protein 3	4	0.637	0.075	
P62834	Ras-related protein Rap-1A	3	0.518	0.09	
X6RJP6	Transgelin-2 (Fragment)	1	0.616	0.14	
H9KV75	Alpha-actinin-1	1	0.358	0.004	
P08514	Integrin alpha-Iib	1	0.621	0.159	
E7EX29	14-3-3 protein zeta/delta (Fragment)	1	0.442	0.049	
Q9HCU0	Endosialin	1	0.594	0.121	
Q12907	Vesicular integral-membrane protein VIP36	1	0.578	0.499	
F5H7S3	Tropomyosin alpha-1 chain	1	0.579	0.133	

HFMD have been achieved due to the rapid advances in molecular diagnostics[28,29]. Although prompt treatment is important, successful outcome and improvement in overall survival is often impeded by a delay in diagnosis because of the heterogeneity of the syndrome, the variable clinical manifestations, and the lack of specificity of clinical and laboratory results. The serum is a good source of protein biomarkers and can reflect the physiological or pathological state of the human body. Rich secretion factors can be observed in serum, making it a highly reliable sample for disease-related biomarkers[30-32].

In this study, we used iTRAQ to identify four serum proteins that were significantly differentially expressed between the HFMD group, se-

Table IV. List of proteins differentially expressed between sever HFMD and HFMD groups.

Accession	List of proteins	Peptides (95%)	Ration >1.5	C.V	Expression
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	149	1.621	0.062	up
P00734	Prothrombin	77	1.617	0.146	
P01876	Ig alpha-1 chain C region	78	1.931	0.091	
P27169	Serum paraoxonase/arylesterase 1	36	2.089	0.094	
P02750	Leucine-rich alpha-2-glycoprotein	23	1.62	0.131	
P0DJI8	**Serum amyloid A-1 protein**	**49**	**1.999**	**0.116**	
Q96KN2	Beta-Ala-His dipeptidase	9	1.779	0.207	
P01861	Ig gamma-4 chain C region	61	2.685	0.192	
P02741	**C-reactive protein**	**6**	**2.515**	**0.198**	
P30447	HLA class I histocompatibility antigen, A-23 alpha chain	3	2.434	0.079	
P14780	Matrix metalloproteinase-9	1	1.678	0.259	
Q99592	Zinc finger and BTB domain-containing protein 18	1	3.232	0.254	
P02741	C-reactive protein	6	2.515	0.198	
P02751	**Fibronectin**	**153**	**0.499**	**0.181**	down
P00747	**Plasminogen**	**82**	**0.582**	**0.425**	
P05546	Heparin cofactor 2	56	0.502	0.111	
P01857	Ig gamma-1 chain C region	82	0.382	0.134	
O14791	Apolipoprotein L1	26	0.526	0.058	
P68871	Hemoglobin subunit beta	44	0.578	0.043	
P02671	Fibrinogen alpha chain	20	0.437	0.14	
Q16610	Extracellular matrix protein 1	14	0.496	0.297	
P00739	Haptoglobin-related protein	125	0.5	0.276	
P08519	**Apolipoprotein(a)**	**16**	**0.294**	**0.14**	
A0A087WUS7	Ig delta chain C region	13	0.489	0.048	
P02675	Fibrinogen beta chain	6	0.511	0.074	
A0A075B6K4	HCG2043238 (Fragment)	6	0.626	0.065	
P20742	Pregnancy zone protein	149	0.37	0.046	
P06312	Immunoglobulin kappa varia-ble 4-1	15	0.558	0.099	
P69891	Hemoglobin subunit gamma-1	9	0.423	0.121	
A0A0C4DH38	Protein IGHV5-51 (Fragment)	12	0.64	0.049	
A0A0B4J231	Immunoglobulin lambda-like polypeptide 5	26	0.605	0.101	
C9JEU5	Fibrinogen gamma chain	3	0.429	0.078	
A0A075B6K5	HCG2043239 (Fragment)	10	0.57	0.075	
A0A0J9YVT0	Immunoglobulin heavy varia-ble 3-33 (Fragment)	23	0.591	0.193	
Q12907	Vesicular integral-membrane protein VIP36	1	0.517	0.098	
A0A0B4J231	Immunoglobulin lambda-like polypeptide 5	26	0.605	0.101	

vere HFMD group, and healthy control group. We used String network analysis to determine their various possible interactions. Then, we used ELISA to quantitatively detect these proteins. We compared the levels of SAA and CRP proteins in three groups and found that the levels of SAA and CRP in the serum of patients with severe HFMD were significantly higher ($p<0.05$), consistent with iTRAQ-LC-MS/MS analysis. We found that the levels of Fn and Apo A proteins were significantly lower in the severe HFMD patients ($p<0.05$), also consistent with iTRAQ-LC-MS/MS analysis. This study showed that serum SAA, CRP, Fn, and Apo might be biomarkers for the diagnosis of severe HFMD.

Conclusions

In summary, the changes in serum proteins are related to the severity of HFMD in Chinese children. This study provides important clues for

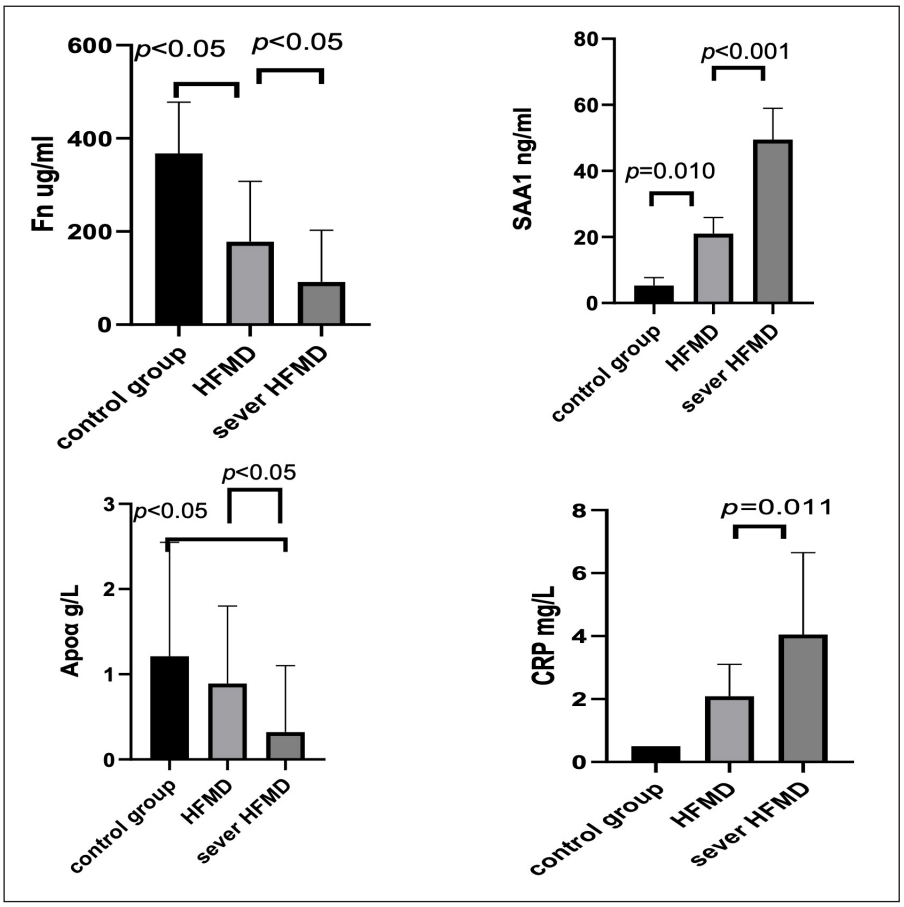

Figure 1. Validation of human serum amyloid A (SAA1), c-reactive protein(CRP), fibronectin(Fn), apolipoprotein(a) (Apoa) in serum samples from the Hand-foot-mouth disease (HFMD), severe Hand-foot-mouth disease (sever HFMD) and the health control group(HC) by ELISA assay. Figure 1 showed that the levels of SAA1 and CRP were higher in the severe HFMD group compared to the HFMD group ($p<0.05$) and the HC group ($p<0.05$). The levels of Fn and Apo A proteins were lower in the severe HFMD group compared to the HFMD group ($p<0.05$) and the HC group ($p<0.05$).

further elucidating the pathogenesis of HFMD and identifying potential biomarkers. However, it is necessary to further explore the interactions between serum proteins in HFMD and its mechanism in disease diagnosis and progression.

Ethics Approval
The study was approved by the Ethics Committee of Chengdu Fifth People's Hospital (No. 2022011), Sichuan, China, and conducted according to the principles in the Declaration of Helsinki.

Informed Consent
Written consent was obtained from all the patients' guardians before any procedure was performed.

Availability of Data and Materials
Data is available upon request from the corresponding author.

Conflict of Interest
The authors have no conflicts of interest to declare.

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None.

Authors' Contributions
Zhou Fangye: Conceived and analyzed data and drafted the manuscript; and Yupeng Luo: designed the experiments; Zhang Guangjie Collected the data and helped in data analysis; Huang Min: collected the data.

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