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 buttocks1. In the past decade, large outbreaks of HFMD in children under five years of age have occurred in some provinces of China2,3, which has become one of the main childhood diseases4. Enterovirus 71 (EV71) is the main pathogenic virus causing HFMD. The infection of EV71 is positively correlated to high mortality and incidence rate5.

Severe HFMD is a major problem worldwide, which causes a heavy burden on children’s health and society6. The clinical manifestations of severe HFMD include aseptic encephalitis, brainstem encephalitis, myelitis, myocarditis, and pulmonary edema, which cause mortality7. 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 diagnosis8,9.

HFMD is caused by an enterovirus, and different proteins are expressed between HFMD and severe HFMD10. Comparative studies between HFMD and severe HFMD may be helpful for further understanding the pathogenesis and biomarkers of HFMD and severe HFMD11. Proteomics is a post-genomic biotechnology12. 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 experiments13. We performed a preliminary study on protein expression differences between HFMD, severe HFMD, and healthy controls by iTRAQ-LC-MS/MS analysis14.

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
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 Bioreources, 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 (x±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). Enterovirus belongs to the small *Reoviridae* enterovirus family, which is a single-stranded positive RNA virus. 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. HFMD is a global infectious disease caused by various enteroviruses, usually occurring in children under 5 years of age. 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. To date, the pathogenesis and molecular mechanism of EV71 and CVB infections remain unclear.

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**Table I. Clinical characteristics of the children.**

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>HFMD group (n=32)</th>
<th>Sever HFMD group (n=32)</th>
<th>Health control group (n=32)</th>
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<tbody>
<tr>
<td>Age</td>
<td>2.69</td>
<td>2.75</td>
<td>2.65</td>
</tr>
<tr>
<td>Rash</td>
<td>±</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lethargy (%)</td>
<td>32.6</td>
<td>51.05</td>
<td>0</td>
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<tr>
<td>Eclemopsia (%)</td>
<td>9.07</td>
<td>21.4</td>
<td>0</td>
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<tr>
<td>Headache (%)</td>
<td>3.2</td>
<td>16.0</td>
<td>0</td>
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<tr>
<td>Agitation (%)</td>
<td>4.82</td>
<td>15.55</td>
<td>0</td>
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<tr>
<td>c-reactive protein (&gt;8 mg/L, n)</td>
<td>2</td>
<td>13</td>
<td>0</td>
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<tr>
<td>Coxsackievirus A group16 positive rate (%)</td>
<td>37</td>
<td>34</td>
<td>0</td>
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<tr>
<td>Enterovirus71 positive rate (%)</td>
<td>63</td>
<td>66</td>
<td>0</td>
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Table II. List of proteins differentially expressed between HFMD and HC groups.

<table>
<thead>
<tr>
<th>Accession</th>
<th>List of proteins</th>
<th>Peptides (95%)</th>
<th>Reaction C.V</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0C0L4</td>
<td>Complement C4</td>
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<td>1.522</td>
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<td>P00450</td>
<td>Ceruloplasmin</td>
<td>250</td>
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<td>B4E1Z4</td>
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<td>P00738</td>
<td>Haptoglobin</td>
<td>321</td>
<td>17.38</td>
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<td>P01011</td>
<td>Alpha-1-antichymotrypsin</td>
<td>132</td>
<td>5.611</td>
<td>0.485</td>
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<td>P04003</td>
<td>C4b-binding protein alpha chain</td>
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<td>0.276</td>
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<td>P05546</td>
<td>Heparin cofactor 2</td>
<td>56</td>
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<td>Q00633</td>
<td>Inter-alpha-trypsin inhibitor heavy chain H3</td>
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<td>11.999</td>
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<td>P02750</td>
<td>Leucine-rich alpha-2-glycoprotein</td>
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<td>O75636</td>
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<td>1.585</td>
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<td>P01782</td>
<td>Immunoglobulin heavy variable 3-9</td>
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<td>A0A096LPE2</td>
<td>Protein SAA2-SAA4</td>
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<td>C-reactive protein</td>
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<td>0.271</td>
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<td>A0A0B4J1Y8</td>
<td>Protein IGLV9-49</td>
<td>4</td>
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<tr>
<td>P20742</td>
<td>Pregnancy zone protein</td>
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<tr>
<td>P01700</td>
<td>Immunoglobulin lambda variable 1-47</td>
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<td>A0A075B6N8</td>
<td>Ig gamma-3 chain C region (Fragment)</td>
<td>43</td>
<td>3.1</td>
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<td>P09891</td>
<td>Hemoglobin subunit gamma-1</td>
<td>9</td>
<td>1.509</td>
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<td>P06681</td>
<td>Complement C2</td>
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<td>5</td>
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<td>26</td>
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<td>Glycogen debranching enzyme</td>
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<td>P02741</td>
<td>C-reactive protein</td>
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<td>2.374</td>
<td>0.271</td>
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Continued
Table II (Continued). List of proteins differentially expressed between HFMD and HC groups.

<table>
<thead>
<tr>
<th>Accession</th>
<th>List of proteins</th>
<th>Peptides (95%)</th>
<th>Ration &gt;1.5</th>
<th>C.V</th>
<th>Expression</th>
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<td>P02751</td>
<td>Fibronectin</td>
<td>153</td>
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<td>Apolipoprotein A-I</td>
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<td>Plasminogen</td>
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<td>0.543</td>
<td>0.155</td>
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<td>P00734</td>
<td>Prothrombin</td>
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<td>P04196</td>
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<td>P15169</td>
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The occurrence of hand-foot-mouth disease has a clear seasonal pattern, the peak period usually occurs from April to July\textsuperscript{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\textsuperscript{23,24}. Isobaric tagging for relative and absolute quantitation (iTRAQ) is a helpful proteomic technology for biomarker identification\textsuperscript{25}. The iTRAQ technology is high throughput and can identify differentially expressed proteins between different groups\textsuperscript{26}. Combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), iTRAQ has been used to identify specific biomarkers in some diseases\textsuperscript{27}. In recent years, deeper insights into the mechanisms underlying the pathogenesis of severe

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Continued
HFMD have been achieved due to the rapid advances in molecular diagnostics. 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.

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

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Table III (Continued). List of proteins differentially expressed between sever HFMD and HC groups.
Serum proteome expression in HFMD

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

---

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

<table>
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<th>C.V</th>
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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.

### Funding

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.

### References

Serum proteome expression in HFMD


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