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Identification of SAA and ACTB as potential biomarker of patients with severe HFMD using iTRAQ quantitative proteomics

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ABSTRACT

Hand, foot and mouth disease (HFMD) is an infectious disease caused by a variety of enterovirus infections, and the most common types of virus infections are the newenterovirus71 (EV71) and coxsackievirus A group 16 (CoxA16). A small fraction of HFMD will cause further severe HFMD. A rapid and accurate diagnosis biomarker of severe HFMD is important for the timely treatment. In the study, we conducted a clinical biomarker discovery study using iTRAQ combined with MS. Serum proteome alterations in severe HFMD group (n = 32) and health control group (n = 32) were analyzed. 47 proteins were upregulated (fold change > 1.5) between the severe HFMD group and HC group. The identified proteins were classified into different groups according to the molecular function, biology processes, cellular component. During the up-regulated proteins, serum amyloid A (SAA) and human β-actin (ACTB), were confirmed in the serum of the severe HFMD and HC by ELISA assay. SAA and ACTB levels were significantly higher in the sever HFMD patients ($P < .01$), consistent with iTRAQ-LC-MS/MS analysis. In summary, Our results showed that SAA and human β-actin (ACTB) may be served as a potential biomarker of the clinical diagnosis of severe HFMD.

1. Introduction

The hand, foot and mouth disease (HFMD) has been circulating in the world since its outbreak in population mainly under the age of five year in last decade, the disease has become one of the major concern for children health care [1,2]. As the main etiological agent of HFMD, coxsackievirus A16 (CoxA16) and enterovirus 71 (EV71), were considered to be the main pathogens of the infection, especially the EV71 infection has been associated with high incidence of mortality and morbidity [3,4]. In 2008–2009, Large outbreaks have occurred in Anhui and Guangdong province, China, in Singapore. Moreover, no effective antiviral agent was currently available for patients with severe HFMD [5,6]. To control and prevent a possible sever HFMD pandemic, it was critical to detect a biomarker to sever HFMD, so to improve the diagnostic accuracy of sever HFMD [7]. Novel biomarkers detectable were needed to support earlier clinical diagnosis of sever HFMD [8].

Proteomics was a postgenomic biotechnology [9]. Isobaric tagging for relative and absolute quantitation (iTRAQ) was a powerful proteomic technology for biomarkers research [10]. The technology can identify proteins differentially expressed between disease group and health group [11]. In addition to, iTRAQ technology offers high throughput and have good repeatability [12]. To couple with liquid chromatography-tandem mass spectrometry (LC-MS/MS), iTRAQ gas proven successful to identify specific biomarkers in disease [13]. Serum is an excellent source of protein biomarkers and can reflect physiological or pathological states in human body [14]. Most abundant secreted factors can be observed in the serum, so serum was recognized as highly believable sample for the disease-related biomarkers [15].

In the study, we performed a protein biomarker discovery study, we used iTRAQ-bsed proteomic technology coupled with LC-MA/MA to screen for candidate biomarkers of sever HFMD. The up-regulation of two identified proteins, serum amyloid A (SAA) and Actin cytoplasmic (ACTB) were detected and confirmed in the serum of sever HFMD patients.

2. Results

2.1. Clinical characteristic of children with severe hand, foot and mouth disease (HFMD)

A total of 32 cases of sever HFMD patients were retrospectively analyzed in the study. The clinical characteristics of those patients were analyzed. There were 20 (62.5%) male cases and 12 (37.5%) female cases, and the number of the male was significantly larger than that of the females ($P < .05$). Pathogens were detected in 32 cases, the positive rate was 100%, and CoxA16 in 11 cases was positive, the positive

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Table 1
Clinical characteristics of the children with severe HFMD and health control.

Clinical parameters	Severe HFMD group		Health control group	
	male (n = 20)	female (n = 12)	male (n = 20)	female (n = 12)
Age	2.9	2.6	2.5	2.8
Rash	+	+	−	−
Fever	+	+	−	−
Lethargy (%)	55.8	46.3	0	0
Eclampsia (%)	20.5	22.3	0	0
Headache (%)	16.8	15.2	0	0
Agitation (%)	17.6	13.5	0	0
CRP (> 8 mg/L,n)	8	5	0	0
CA16 positive rate (%)	35	33	0	0
EV71 positive rate (%)	65	67	0	0

rate of 34.37%, EV71 in 21 cases was positive, the positive rate of 65.63%. All children had a fever, rash on hands or feet, oral ulcer, hip skin, etc. Most children were characterized with poor spirit, 20 male cases were characterized with lethargy (55.8%), eclampsia (20.8%), headache (16.8%), agitation (17.6%). 12 female cases were characterized with lethargy (46.3%), eclampsia (22.3%), headache (15.2%), agitation (13.5%) (Table 1).

2.2. iTRAQ-LC-MS/MS analysis and proteins identification

According to the comparation of the protein expression in sever HFMD and HC groups. A total of 403 proteins were identified, among which 47 proteins were up-regulated and the ration of these proteins were > 1.5 (Table 2). Then from the 47 proteins, we choose the proteins which the CV were < 0.1, so we chose to focus on the 14 proteins as potential sever HFMD biomarkers because of the possibility which their expression level correlated with pathological stage of sever HFMD. Go analysis showed that proteins were located mostly in the extracellular region (66%). The molecular function of the proteins were mainly in ion binding (22%), enzyme regulator activity (16%), peptidase activity (13%) and lipid binding (13%) (Fig. 1). Among the proteins, we used string network analysis to identify various possible interactions (Fig. 2).

2.3. Validation of SAA and ACTB by ELISA

Furthermore, we validated the SAA and ACTB expression levels in sever HFMD and HC serum samples. In the study, we detected the elevation of SAA and ACTB from the serum of sever HFMD group , SAA and ACTB levels were significantly higher in the sever HFMD patients ($P < 0.01$), consistent with iTRAQ-LC-MS/MS analysis. Of the SAA level, the sever HTMD group (30.19 ± 5.863 ng/ml), the HC group (6.799 ± 0.3038 ng/ml). Of the ACTB level, the sever HTMD group (4.203 ± 0.642 ng/ml), the HC group (1.152 ± 0.1773 ng/ml). So the SAA and ACTB level of sever HFMD group were significantly different compared with the HC group ($P < .001$) (Fig. 3).

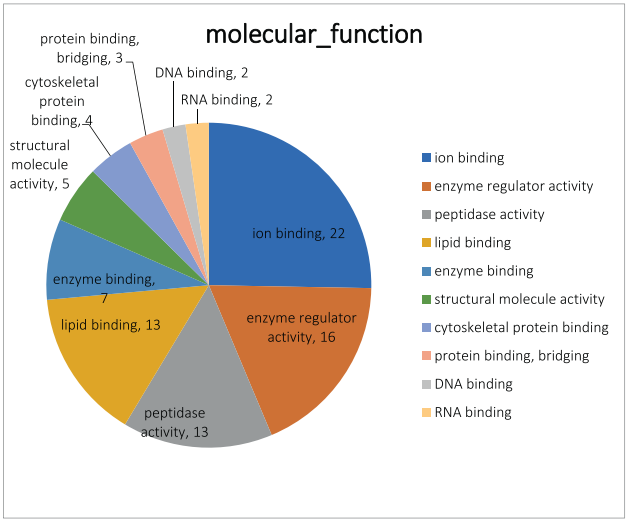
3. Discussion

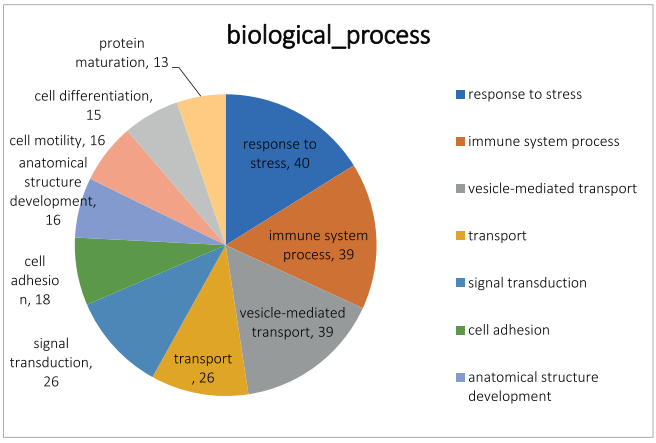
Epidemics of Hand Foot and Mouth Disease (HFMD) is a global infectious disease which caused by multiple enterovirus and commonly affect children who younger than 5 years old [16]. Enterovirus 71 (EV71) and Coxsackieviruses A16 (CoxA 16) are still the main pathogens of HFMD in China [17]. In recent years, a large outbreaks of HFMD, especially caused by EV71, a small proportion of them may suffer from severe complications such as aseptic meningitis, encephalitis, acute flaccid paralysis, pulmonary edema, myocarditis and even death [18]. HFMD had become a major public health issue since 1997 in China, so HFMD was listed as one of the category “C” notifiable diseases [19]. The epidemic may further expand, and how to prevent the disease is still very important in future [20]. Therefore, the clinical characteristics of HFMD and the research of biology, pathogenesis, diagnosis and prevention of the disease are extremely necessary [21].

iTRAQ is short for isobaric tags for relative and absolute quantitation, developed by Applied Biosystems Incorporation of USA [22]. The technology is based on differential isotopic labeling of proteins or peptides which are derived from cell states with either light or heavy

Table 2
List of proteins differentially expressed between groups of severe HFMD and HC.

Accession	Name	Peptides (95%)	Ration > 1.5	C·V
P0C0L4	Complement C4	287	1.52	0.05
P00450	Ceruloplasmin	250	2.67	0.36
B4E1Z4	Uncharacterized protein	131	6.66	0.28
P00738	Haptoglobin	321	17.38	0.26
P01011	Alpha-1-antichymotrypsin	132	5.61	0.49
P10643	Complement component C7	42	3.98	0.23
P04003	C4b-binding protein alpha chain	48	3.04	0.28
P05546	Heparin cofactor 2	56	1.76	0.23
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	40	1.56	0.17
P02748	Complement component C9	35	1.90	0.02
P02763	Alpha-1-acid glycoprotein 1	87	12.00	0.34
P04278	Sex hormone-binding globulin	24	2.14	0.26
O14791	Apolipoprotein L1	26	1.79	0.11
Q08380	Galectin-3-binding protein	22	3.12	0.07
P02671	Fibrinogen alpha chain	20	4.73	0.46
P02750	Leucine-rich alpha-2-glycoprotein	23	2.39	0.06
O75636	Ficolin-3	22	2.02	0.11
P0DJI8	Serum amyloid A-1 protein	49	7.85	0.06
G3XAM2	Complement factor I	17	2.05	0.06
P08571	Monocyte differentiation antigen CD14	12	1.53	0.12
P02743	Serum amyloid P-component	15	2.25	0.32
P19652	Alpha-1-acid glycoprotein 2	48	2.72	0.22
A0A087WUS7	Ig delta chain C region	13	1.59	0.03
P01782	Immunoglobulin heavy variable 3–9	28	2.23	0.11
A0A096LPE2	Protein SAA2-SAA4	43	2.35	0.12
P02675	Fibrinogen beta chain	6	2.12	0.06
P02741	C-reactive protein	6	2.37	0.27
A0A0B4J1Y8	Protein IGLV9–49	4	1.65	0.13
P20742	Pregnancy zone protein	149	5.53	0.15
P01700	Immunoglobulin lambda variable 1–47	11	1.91	0.12
A0A075B6N8	Ig gamma-3 chain C region (Fragment)	43	3.10	0.08
P69891	Hemoglobin subunit gamma-1	9	1.51	0.16
P06681	Complement C2	30	1.80	0.05
A0A0C4DH38	Protein IGHV5–51 (Fragment)	12	2.14	0.29
P18428	Lipopolysaccharide-binding protein	5	1.94	0.19
A0A0B4J231	Immunoglobulin lambda-like polypeptide 5	26	1.76	0.46
C9JEU5	Fibrinogen gamma chain	3	2.12	0.07
P06732	Creatine kinase M-type	3	2.01	0.11
A0A075B6K5	HCG2043239 (Fragment)	10	2.46	0.10
A0A0C4DH29	Immunoglobulin heavy variable 1–3	7	1.57	0.09
P35573	Glycogen debranching enzyme	1	1.60	0.09
J3KT10	Nuclear pore complex protein Nup85	1	1.61	0.37
G5E968	Chromogranin A (Parathyroid secretory protein 1), isoform CRA_b	1	1.53	0.43
A0A0A0MT69	Protein IGKJ4 (Fragment)	1	2.74	0.21
A0A0B4J231	Immunoglobulin lambda-like polypeptide 5	26	1.76	0.46
P02741	C-reactive protein	6	2.37	0.27
P60709	Actin, cytoplasmic 1	11	1.57	0.03





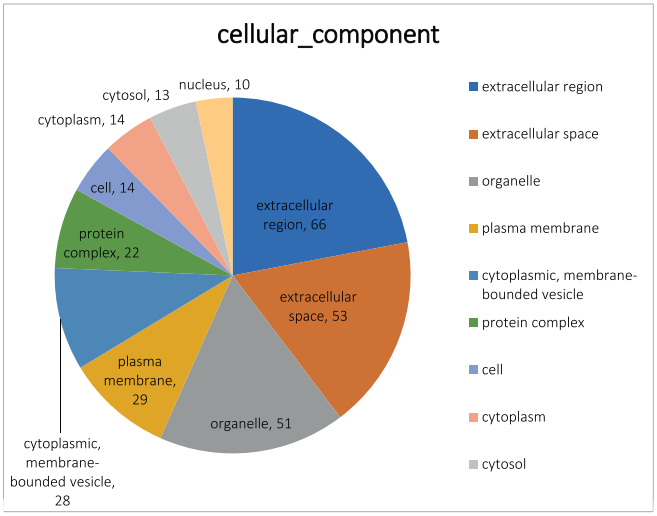


Fig. 1. The analysis of differentially expressed proteins on Gene Ontology terms. According to the comparation of the protein expression in sever HFMD and HC groups. A total of 403 proteins were identified, among which 47 proteins were up-regulated and the ration of these proteins were > 1.5. Go analysis showed that proteins were located mostly in the extracellular region (66%). The molecular function of the proteins were mainly in ion binding (22%), enzyme regulator activity (16%); the biology processes of the proteins were mainly in response to stress (40%), immune system process (39%), vesicle-mediated transport (39%); the cellular component were mainly located in extracellular region (66%), extracellular space (53%). (A) the molecular function. (B) the biological processes. (C) the cellular component.

tags [23]. This technique had shown huge advantages in throughput, sensitivity and quantitative precision to overcome some drawbacks of traditional proteomic techniques and represented a powerful alternative [24].

In the present research, we used iTRAQ to identify 14 protein which were significantly differentially expressed between the severe HFMD group and HC group. Go analysis showed that proteins were located mostly in the extracellular region (66%). The molecular function of the proteins was mainly in ion binding (22%), enzyme regulator activity (16%), peptidase activity (13%) and lipid binding (13%). Among the proteins, we used string network analysis to identify various possible interactions. Then we verified these proteins by ELISA, we detected the elevation of SAA and ACTB from the serum of sever HFMD group , SAA and ACTB levels were significantly higher in the sever HFMD patients ($P < .01$), consistent with iTRAQ-LC-MS/MS analysis. Serum amyloid (SAA) is one of the most sensitive acute phase proteins in verbrates [25]. It is produced principally by the liver in response to acute inflammatory stimuli and its serum concentration may increase by up to 100- to 1000-fold over the basal level [26]. Meanwhile, accumulating evidences also support that under the chronic inflammatory states [27]. The secretion of SAA by human adipocytes, vascular endothelial cells and macrophages can cause a modest increase in the plasma and reflect the inflammatory level exactly [28]. SAA is a family of homologous proteins including 4 isoforms which are encoded by the same gene, SAA1 and SAA2 are the major acute phase reactants [29]. Actin is an abundant and highly conserved protein that found in nearly all eukaryotic cells [30]. Actin had six isoforms which can be distinguished in vertebrates [31]. β-actin had important roles in fundamental cellular functions as a component of the cytoskeleton. In addition to, β-actin was involved in transcription and long-term potentiation of neurons. Cytoplasmic actin was important for cell migration, cell shape maintenance, and was repressed at moderate to high levels in nearly all tissues [32,33].

In summary, Our results showed that SAA and human β-actin (ACTB) may be served as a potential biomarker of the clinical diagnosis of severe HFMD. Although the biomarkers appear to have experiments to verify. Importantly, we might consider SAA and ACTB level when do not assess the development of HFMD.

4. Material and methods

4.1. Participants

The study was conducted in Putian City, Fujian Province, China, from March 2014 through March 2016. All children were inpatients in the wards of the first Hospital of Putian City, a 1200-bed tertiary care government hospital. The patients were recruited consecutively using convenience sampling in the wards. We evaluated prospectively collected data from all of the patients that were admitted to the wards during the 2-year study period. The purpose and methods of the study were explained to all of the participants' parents. The Fujian Medical University Teaching Hospital, First Hospital of Putian ethics committee approved the study, and we obeyed the Declaration of Helsinki informed consent procedures, which were carried out individually. Additionally, written consent was obtained from all of the parents before any procedures were performed.

4.2. Clinical characteristics

To investigate the clinical characteristics of children with hand, foot and mouth disease, the date of 32 children with severe HFMD were collected and retrospectively analyzed. Pathogens were detected in all 32 cases using RT-PCR method. The clinical characteristics included age, rash, fever, lethargy, eclampsia, headache, agitation.

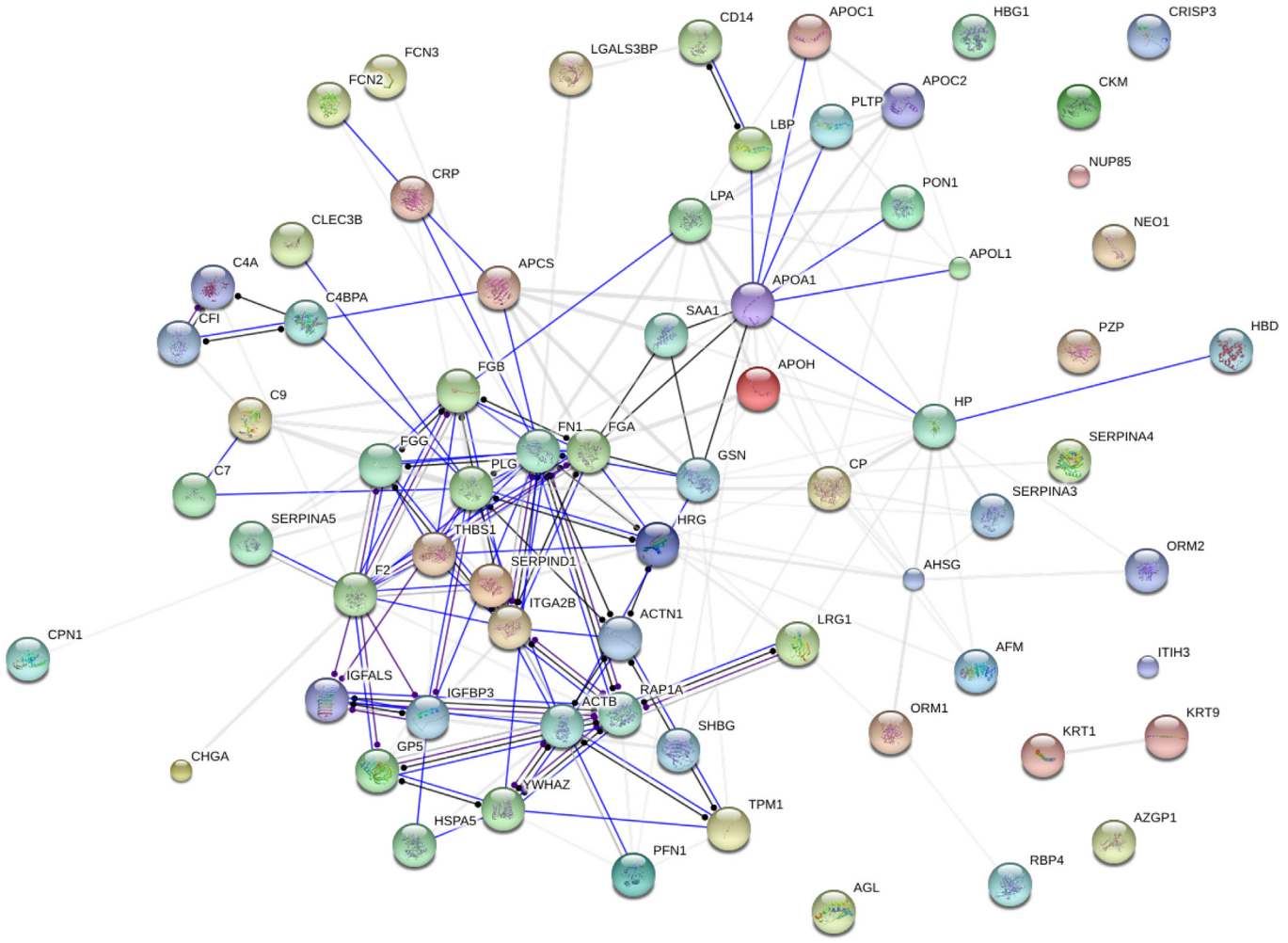


Fig. 2. String network analysis of differentially expressed proteins.

4.3. iTRAQ-LC-MS/MS analysis and identification of serum proteins

Blood samples (5 ml) were drawn from patients. Protein concentration was estimated by BCA Protein Assay Kit (Sangon Biotech, PR China). iTRAQ labeling was performed according to the manufacturer's protocol (Applied Biosystems, Sciex). Each sample was labeled separately with two of the eight available tags. All labeled peptides were pooled together. TheUltimate3000HPLC system (Dionex, USA) equipped with a 2.00-mm-inner diameter *100-mm-long Gemini-NX 3u C18110Acolumns (Phenomenex, USA) was used for High-pH fractionation. Peptides were loaded onto the column and washed isocratically at 95% eluent A (20 mM HCOONH4, 2 M NaOH) (pH 10). Peptide fractionation was performed using a linear binary gradient from 15 to 50% B (20 mM HCOONH4, 2 M NaOH, 80% ACN) (pH 10) at 0.2 ml/min over 45 min. Finally, the column was washed at 90% B for 10 min and returned to 95% A for 10 min.The UV detector was set at 214/280 nm, and fractions were collected every 1 min. In total, 10 fractions were pooled and dried by vacuum centrifuge for subsequent nano-reversed phase liquid chromatography (nano-LC) fractionation. Each fraction was resuspended in 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 MichromBioresources, Auburn, CA). The peptides were separated using the following parameters: 1) mobile phase A:0.1% FA,5%ACN, dissolved in water;2) mobile phase B: 0.1% FA, 95% ACN;3) flow rate: 300 nl/min; 4) gradient: B-phase increased from 5% to 40%,70 min. Then , the LC eluent was subjecttoTripleTOF5600 MS/MS system (AB SCIEX, CA)in an information dependent acquisition mode.MS spectra were acquired across the mass range of 400–1250 m/z in high resolution mode (> 30,000) using 250 ms accumulation time per spectrum. A maximum of 20 precursors per cycle were chosen for fragmentation from each MS spectrum with100 ms minimum accumulation time for each precursor and dynamic exclusion for 20 s. Tandem mass spectra were recorded in high sensitivity mode (resolution > 15,000) with rolling collision energy on and iTRAQ reagent collision energy adjustment on. Relative quantification and protein identificationwere performed with ProteinPilot™ software (version 5.0, Applied Biosystems) using the Paragon™ algorithm 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 cutoff 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 cutoff values.

4.4. ELISA validation

To verify the SAA1 and ACTB results of the ITRAQ analysis, ELISA test for each patient was performed. The ELISA Kits (CUSABIO BIOTECH, Life Sciences Advanced Technologies Inc., USA. Catalog Number.CSB-E08589h, Catalog Number.CDB-E13298h) were used to test the SAA1 and ACTB level in serum samples from sever HFMD and HC groups.

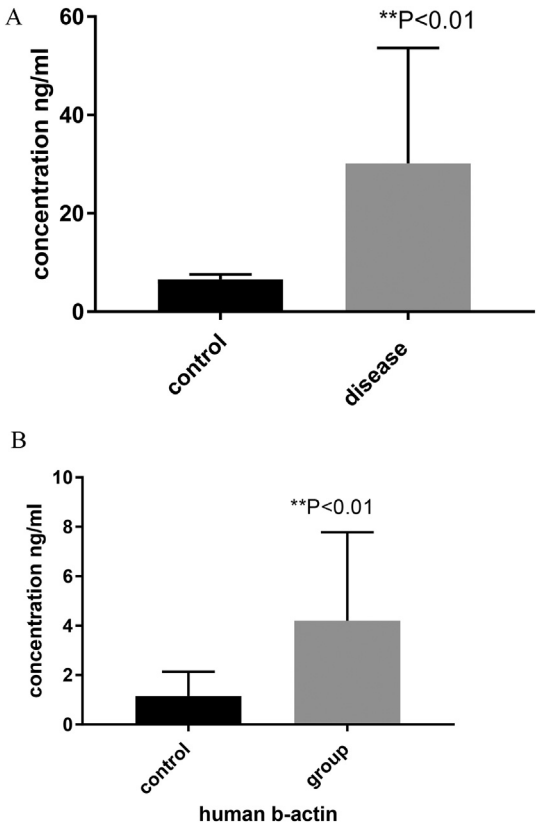


Fig. 3. Validation of human serum amyloid A (A), human β-actin (B) in serum samples from the severe HFMD and the HC by ELISA assay. The protein levels were expressed as the mean ± SD.

4.5. Statistical and bioinformatics analyses

Data was evaluated using GraphPad Prim 7 software. A p value of $p < .05$ was considered significant.

Conflict of interest statement

The authors have no conflict of interest to declare.

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