Analysis of differentially expressed proteins involved in hand, foot and mouth disease and normal sera

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Abstract

We implemented 2-D DIGE technology on proteins prepared from serum obtained from children with hand, foot and mouth disease (HFMD) and controls, to study the differentially expressed proteins in control and HFMD serum samples. Proteins found to be differentially expressed were identified with matrix-assisted laser desorption/ionization time-of-flight/ time-of-flight mass spectrometry (MALDI-TOF/TOF MS) analysis. We identified 30 proteins from mild HFMD samples and 39 proteins from severe HFMD samples, compared with the normal controls. 25 proteins among them (14 up-regulated and 11 down-regulated proteins) are found in both HFMD groups. Classification analysis and protein–protein interaction map showed that they associate with multiple functional groups, including transporter activity and atalytic activity. These findings build up a comprehensive profile of the HFMD proteome and provide a useful basis for further analysis of the pathogenic mechanism and the regulatory network of HFMD.

Keywords: Hand, foot and mouth disease, dimensional fluorescence difference gel electrophoresis, proteomics
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Introduction

Hand, foot and mouth disease (HFMD), a highly infectious disease that mainly affects children <5 years old, is characterized by fever, papules on the hands and feet, and mouth ulcers. It can be caused by viruses that belong to the enterovirus genus (group), including polioviruses, coxsackieviruses, echoviruses and enteroviruses [1]. Among them, Coxsackieviruses 16 (CA16) and enterovirus 71 (EV71) have been identified as the most frequent pathogens of HFMD [2]. The virus transmits through a direct contact with mucous, oral or nasal secretions or faeces of an infected person. HFMD patients with or without symptoms are the major infectious sources [3]. The disease causes fever, tetter on the hands and feet and ulceration in the mouth, and may further develop into myocarditis, pulmonary oedema, aseptic meningoencephalitis and other complications [4,5].

Since the first HFMD case was reported in New Zealand in 1957, subsequent outbreaks have been reported worldwide. Historically, the outbreaks of HFMD were reported spontaneously and at a small scale in European countries and the United States [6]. Since 1997, large epidemic outbreaks of HFMD associated with severe neurological complications

and high mortality rates have occurred in Malaysia, Taiwan, Singapore, Japan and other Asian-Pacific areas. Large outbreaks of HFMD have been reported since 2008 in mainland China, resulting in millions of infections and hundreds of deaths in children [2]. Noticeably, both the number of severe cases and mortality rate for HFMD have significantly increased in recent years and the epidemics of HFMD pose serious public health threats to children throughout the world [7,8]. Although treatment has been improved with the application of new technologies, there is currently no specific antiviral therapy to cure HFMD and no vaccine to prevent severe HFMD infection [2].

Usually the enterovirus exhibits strong transitivity; moreover, the recent infection rate is high and the transmission paths are complicated, causing large epidemics in a short period of time. Screening and identification of HFMD play important roles in preventing its occurrence and development. Therefore rapid and accurate diagnosis is very important. Serum is rich in protein composition which changes in abundance during different stages. Discovering molecular markers from serum may be valuable in the early diagnosis of HFMD.

Proteomics analysis is currently considered to be a powerful tool for global evaluation of protein expression, and has been widely applied in studying biomarkers for diseases [9]. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), a new proteomics technology, has been developed in recent years. It shows significantly improved sensitivity and repeatability when compared with the traditional two-dimensional electrophoresis. It is a new means to provide an overall view of the proteome of the disease.

A recent study carried out a comparative proteome analysis between EV71 and CA16, the two most causative agents of HFMD in cultured human rhabdomyosarcoma (RD) host cells [10]. The study showed the effect of the viral infection (e.g. active shutting down of the protein synthesis of the host cell), as well as cellular response to the viral infection, such as the intracellular immune response. The study also provided interesting molecular explanations of the disease phenotype, such as loss of muscle tone caused by down-regulated HSP27 and desmin. However, the study focused on the intracellular differentially expressed proteins using cancer cell lines, which deviates from the *in vivo* situation. Moreover, the infection may cause a series of responses by multiple cell types in the human body, which is far more complex than the cell line model. Also, there is no analysis of the extracellular proteins in infected cells. In this study, we aim to identify potential proteins as biomarkers in HFMD serum samples using 2D-DIGE and matrix-assisted laser desorption/ionization time-of-flight/ mass spectrometry (MALDI-TOF MS) methodologies to identify differentially expressed proteins in HFMD compared with normal serum samples and further to determine the possible molecular markers for HFMD. Classification analysis of the proteins identified based on the PANTHER and protein–protein interaction analysis indicated that these proteins might have important roles in a variety of cellular processes and structures, including spermatogenesis, cell signalling, cell skeleton and metabolism.

Materials and Methods

Preparation of serum samples

Blood samples from 16 HFMD children (eight mild and eight severe) according to the 'foot and mouth disease prevention control guide' (2008 Edition) issued by the Ministry of Health, China (http://www.moh.gov.cn/publicfiles/business/htmlfiles/mohbgt/s9511/200805/34775.htm.), were randomly selected to be analysed. These children were proven to have no other disease after a series of checks in the hospital. Clinical symptoms and laboratory test (Enterovirus 71 (EV71) nucleic acid detection kit) confirmed that the EV71 infections caused all these HFMD cases. Another eight blood samples from normal children were collected as controls. A pooled sample consisting of equal amounts of each of 16 experimental samples (mild HFMD compared with normal control; severe HFMD compared with normal control) was used as a pooled internal standard. Serum was separated by centrifugation at 800 *g* for 30 min. Aliquots of serum were collected and stored at −80°C until ready for use. Serum samples were processed using the ProteoPrep Blue Albumin Depletion Kit (Sigma, St. Louis, MO, USA), which selectively removes albumin and IgG from the serum sample according to the manufacturer's instructions. To depurate the protein extraction and determine the final protein concentration, the 2-D Clean-up Kit (GE Healthcare, UK) and 2-D Quant Kit (GE Healthcare, London, UK) were used sequentially, following the manufacturer's instructions.

Protein labelling with CyDye DIGE fluor

Protein extracts were labelled with three CyDye DIGE fluors, Cy2, Cy3 and Cy5, for 2-D DIGE technology according to the manufacturer's recommended protocols. A total of 50 μg of protein from a normal sample was labelled with 400 pmol of Cy3, and 50 μg of protein from a mild HFMD sample or severe HFMD sample was labeled with 400 pmol of Cy5. An internal reference standard, consisting of two mixed samples used in the experiment, was labelled with

Cy2. The labelling reaction was incubated on ice for 30 min under dark conditions. The reactions were then quenched with the addition of 10 mM lysine for 10 min on ice in the dark. The labelled samples were pooled and prepared for the subsequent steps of the experiment.

2-D electrophoresis

2-D electrophoresis was performed as described earlier with a few minor modifications [11]. Immobilized pH gradient (IPG) strips (24 cm, pH 4–7) were hydrated in hydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM DTT, 1% IPG buffer, pH 4–7 and 0.002% w/v Bromophenol blue) and isoelectric focusing (IEF) was performed according to the manufacturer's instructions using pH 4–7 IPG strips with an Ettan IPGphor II System (GE Healthcare). After the isoelectric focusing (IEF), the proteins were reduced and alkylated by successive treatment for 15 min with equilibration buffer containing 2% w/v DTT followed by 2.5% w/v IAA. Second-dimension separation was performed on a 12.5% SDS-PAGE gel using an Ettan DALT six instrument (GE Healthcare).

Gel image acquisition and analysis

Labelled proteins were visualized using the Typhoon 9400 imager (GE Healthcare) and analysed using DeCyder Software (V6.0; GE Healthcare) as described previously [12,13]. Briefly, the Cy2, Cy3 and Cy5 labelled images for each gel were scanned with excitation/emission wavelengths of 488/520, 532/580 and 633/670 nm, respectively. After the CyDye labelling, signals were imaged, and the gels were stained using Deep Purple total protein stain (GE Healthcare) according to the standard protocol and scanned with excitation/emission wavelengths of 532/560 nm. Then, 2-D DIGE gel images were analysed with DeCyder software. The protein expression patterns of mild HFMD or severe HFMD serum samples were compared with the control (control vs. mild HFMD or control vs. severe HFMD). Protein spots with significant differences in abundance (more than 1.5-fold) [14] were selected in the stained gels for spot picking.

Spot picking and enzymatic digestion

Protein spot-features that were significantly ($p < 0.05$) increased or decreased in all HFMD samples compared with control samples were chosen for further analysis. Three preparative gels were performed to enable recovery of sufficient protein within the individual spot feature to allow protein identification. In the automated procedure, the selected protein spots were picked up, washed with 25 mM NH_4HCO_3 and 50% v/v methanol and then digested with 12.5 ng/mL trypsin (Sequencing-grade; Promega, Madison, WI, USA) in 25 mM NH_4HCO_3 for 12 h at 37°C. The tryptic peptides were extracted with 60%v/v ACN and 0.1% v/v TFA, then dissolved in 4 mg/mL CHCA matrix in 70% (v/v) ACN and 0.1% (v/v) TFA and spotted on the mass spectrometry (MS) sample plate.

MALDI-TOF/TOF MS analysis

MS analysis was performed on an Utraflex III MALDI-TOF/TOF MS (Bruker, Karlsruhe, Baden-Württemberg, Germany) operating in positive ion reflector mode. Monoisotopic peak masses were acquired in a mass range of 700–4000 Da with a signal-to-noise ratio (S/N) >200. Four of the most intense ion signals, excluding common trypsin autolysis peaks and matrix ion signals, were automatically selected as precursors for MS/MS acquisition. The peptide mass fingerprint combined MS/MS spectra were searched against the NCBInr database using the Biotools software (Version 3.2; Bruker) and MASCOT Version 2.2 (Matrix Science, Boston, MA, USA) with the following parameters: Homo sapiens, trypsin cleavage, one missed cleavage allowed, carbamidomethylation as fixed modification, oxidation of methionines allowed as variable modification, peptide mass tolerance at 100 ppm, fragment tolerance at 0.7 Da. The criteria for successfully identified proteins include: ion score confidence interval (CI %) for PMF, MS/MS data ≥95%; a minimum of four peptides hits required in PMF data-based identification; and at least two peptides of distinct sequence identified.

Database searching

The MS/MS spectra were searched using MASCOT to search human and EV71 protein databases to identify proteins. The following peptide search criteria were employed: species: Homo sapiens and EV71 virus, trypsin digest (one missed cleavage allowed), peptide tolerance: 100 ppm, mass value: monoisotropic, protein mass ranged from 1 to 100 kDa. NCBI databases were searched. Protein identification by PMF was performed using the MASCOT search engine.

Protein categorization

The identified proteins were classified according to the PANTHER (Protein ANalysis THrough Evolutionary Relationships) system (http://www.pantherdb.org), which classifies genes and proteins by their functions. The PANTHER ontology, a highly controlled vocabulary (ontology terms) by molecular function, biological process and molecular pathway, was employed to categorize proteins into families and subfamilies with shared functions.

Predication of protein–protein interaction

The identified proteins were preceded by the STRING (functional Protein Association Networks) system (http://

www.string-db.org). STRING is a database of known and predicted protein interactions, including direct (physical) and indirect (functional) associations.

Results

2-D DIGE analysis of differential protein expression

The goal of this study was to look for some proteins that were considered potential biomarkers for early diagnosis of HFMD. Characterization of HFMD children's samples from which serum was obtained for DIGE analysis is presented in Fig. 1. Samples were processed with the ProteoPrep Blue Albumin Depletion kit to remove the two most abundant serum proteins from serum for higher sensitivity to visualize less abundant proteins. As previously described [15], differential protein analysis was then performed using 2-D DIGE. After 2-D DIGE analysis, differential protein spots were detected using DeCyder analysis software. Protein profiles were compared between the HFMD samples and the control samples. We selected the numbered spots that were at least 1.5-fold differentially expressed proteins.

MALDI-TOF PMF analysis

A total of 30 differentially expressed proteins (18 up-regulated and 12 down-regulated) were identified in the mild HFMD samples compared with the level in control samples. Thirty-nine differentially expressed proteins (22 up-regulated and 17 down-regulated) were identified in the severe HFMD samples compared with the level in control samples (Table 1). Table 2 summarizes the 25 proteins that all appeared in both the mild and severe HFMD samples compared with the level in control samples. Among these, 14 proteins had increased and 11 proteins had decreased in the HFMD samples compared with the level in control samples.

Classification of identified proteins

According to cellular functions and processes, the proteins were distributed into categories based on molecular function, biological processes involved and protein classes using the PANTHER classification system. The results are shown in Fig. 2. The most dominant function that the identified protein was involved in was transporter activity (28.0%) followed by binding (24.0%), enzyme regulator activity (16.0%), catalytic activity (16.0%) and receptor activity (12.0%). Most of the identified proteins played roles in the metabolic process (17.3%) and immune system process (15.4%). Other biological processes that the proteins were involved in included response to stimulus (11.5%), cellular process (11.5%), transport (11.5%), etc. As for protein class, most of the proteins belonged to transporter (40.0%), transfer/carrier protein (13.3%) or signalling molecule (13.3%); others belonged to receptor (6.7%), defence/immunity protein

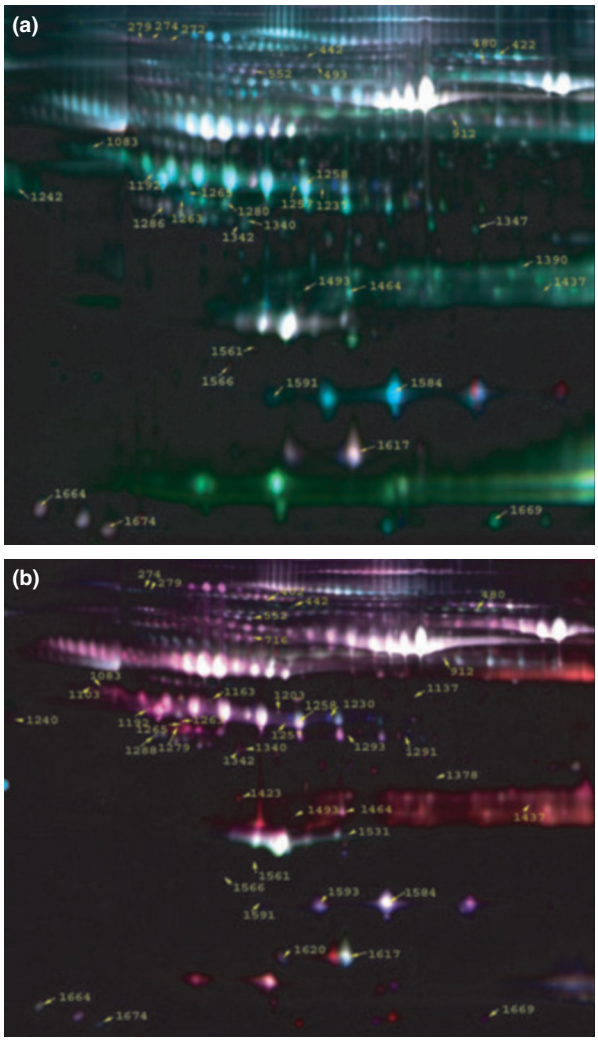

FIG. 1. Analysis of hand, foot and mouth disease (HFMD) samples as revealed by 2-D DIGE analysis. Proteins extracted from HFMD and control samples were labelled with Cy3 and Cy5, respectively. An internal standard protein sample (a mixture of HFMD and control samples) was labelled with the Cy2 dye. The Cy3-labelled HFMD samples and the Cy5-labelled control samples were mixed and separated on a 2-D DIGE gel (IEF on pH 4–7 strips followed by 12.5% second dimension SDS-PAGE). The green spots represent down-regulated proteins, while the red spots represent up-regulated proteins in HFMD samples compared with the control samples. The yellow arrow represents the identified proteins that showed significantly differential expression in the HFMD and control samples. The number in the figure corresponds to that presented in Tables 1 and 2. (a) Mild HFMD vs control samples. (b) Severe HFMD vs control samples.

TABLE 1. Differential proteins identified by mass spectrometry (MS) after 2-D DIGE of the mild and severe hand, foot and mouth disease (HFMD) samples vs. control samples

Master No.	Accession No.	Mr	pI	MASCOT score	Coverage (%)	Av. ratio		*t*-test		Mw	Proteins
						Mild HFMD	Severe HFMD	Mild HFMD	Severe HFMD		
272	gi|896277	11776	4.9	37	28	−1.51	—	0.0036	—	130	This CDS feature is included to show the translation of the cor responding V_region. Presently translation qualifiers on the V_region features are illegal
274	gi|55958063	105606	4.8	134	9	−1.96	−1.66	0.00021	0.0064	130	Inter-alpha (globulin) inhibitor H2
279	gi|119581148	57754	4.82	344	20	−1.89	−1.55	0.00076	0.0085	130	Keratin 9 (epidermolytic palmoplantar keratoderma)
422	gi|134105218	85583	6.32	339	20	1.63	—	0.0067	—	111	Chain A, human complement factor B
442	gi|224053	162072	5.45	234	5	−2.24	−2.25	0.0023	0.0023	109	Macroglobulin alpha2
480	gi|177870	164600	6.26	582	13	−2.1	−1.7	0.00047	0.0028	107	Alpha-2-macroglobulin precursor
552	gi|4501987	70963	5.3	332	27	−1.62	−1.65	0.0083	0.012	97	Afamin precursor
716	gi|2258128	61728	5.26	304	20	—	1.7	—	0.0057	68	Complement 9
912	gi|6573461	37499	6.12	122	17	−1.7	−1.52	0.0075	0.0034	62	Chain A, crystal structure of human beta-2-glycoprotein-I (apolipoprotein-H)
1083	gi|16418467	38382	4.54	324	14	1.76	1.59	0.01	0.012	51	Leucine-rich alpha-2-glycoprotein 1 precursor
1103	gi|113197968	48171	4.49	115	6	—	1.69	—	0.013	49	KRT9 protein
1137	gi|27692693	48641	5.99	104	24	—	−1.64	—	0.0049	46	ALB protein
1163	gi|169214179	45642	5.09	304	31	—	1.75	—	0.038	42	Predicted: similar to complement component 3
1192	gi|78101271	40204	4.82	220	37	2.02	2.41	0.014	0.012	39	Chain C, human complement component C3c
1203	gi|15277503	40536	5.38	467	46	—	1.53	—	0.033	38	ACTB protein
1230	gi|47124562	31647	5.61	551	34	—	1.9	—	0.028	37	HP protein
1237	gi|21961605	59020	5.54	346	24	2.08	—	0.032	—	36	Keratin 10
1242	gi|38305346	17032	4.22	37	22	2.64	2.74	0.03	0.0041	36	Antigen MLAA-42
1257	gi|119579599	51388	5.41	265	14	2.77	2.75	0.0062	0.0081	35	Haptoglobin, isoform CRA_b
1258	gi|47124562	31647	5.48	386	23	2.07	2.61	0.014	0.029	36	HP protein
1265	gi|118138017	104913	4.99	628	26	2.16	2.98	0.038	0.046	34	Chain B, human complement component C3b
1279	gi|118137965	104912	4.97	468	15	1.56	3.4	0.044	0.033	33	Chain B, structure of complement C3b: insights into complement activation and regulation
1288	gi|338305	36997	4.85	432	26	—	−1.53	—	0.0075	33	'SP40,40'
1291	gi|157831596	44280	5.94	136	26	—	−1.59	—	0.025	33	Chain A, Alpha1-Antitrypsin
1293	gi|7770217	33466	5.71	130	39	—	2.03	—	0.015	33	PRO2675
1340	gi|27574252	15928	5.23	39	29	1.6	2.02	0.0085	0.0023	31	Chain B, deoxy haemoglobin (A-Gly-C:v1m; B,D:v1m,C93a,N108k)
1342	gi|26665859	16267	5.21	310	44	1.75	1.64	0.0089	0.00084	31	CLU
1347	gi|27754776	33395	6.21	400	50	1.51	—	0.038	—	31	Ficolin 3 isoform 1 precursor
1378	gi|122920512	68408	6.06	194	15	—	−1.95	—	0.0087	30	Chain A, human serum albumin complexed with myristate and aspirin
1390	gi|119390093	23689	6.41	539	52	2.17	—	0.049	—	30	Chain B, Crig bound to C3c
1423	gi|40737308	48037	5.21	213	7	—	1.82	—	0.029	29	C4B1
1437	gi|170684576	24426	6.49	226	45	2.82	1.95	0.048	0.03	29	Immunoglobulin kappa 4 light chain
1493	gi|576259	23358	5.44	344	26	1.5	1.58	0.016	0.039	28	Chain A, the structure of pentameric human serum amyloid P component
1531	gi|55961583	13327	5.68	112	31	—	−1.6	—	0.025	26	Apolipoprotein M
1561	gi|178779	43358	5.25	111	16	−2.37	−2.92	0.0091	0.0056	23	Apolipoprotein A-IV precursor
1566	gi|563320	28141	5.14	264	23	−2.64	−2.96	0.011	0.004	21	Apolipoprotein A-IV
1584	gi|296653	42126	5.86	106	20	2.63	3.89	0.044	0.037	19	hp2-alpha
1591	gi|223092	18672	5.31	53	14	2.9	4.31	0.031	0.029	19	Ceruloplasmin CpF5 fragment
1593	gi|296653	42126	5.57	126	20	—	3.91	—	0.042	18	hp2-alpha
1617	gi|224510585	12996	5.68	461	96	−1.75	−1.97	0.0063	0.00082	16	Chain A, crystal structure of the apo form of human wild-type transthyretin
1620	gi|189339646	12740	5.4	246	56	—	−1.61	—	0.011	16	Chain A, crystal structure of the F87mL110M mutant of human Transthyretin At Ph 4.6.
1664	gi|186972736	8759	4.33	99	24	−2.2	−2.32	0.0013	0.0028	12	Chain A, structure and dynamics of human apolipoprotein C-Iii
1669	gi|225986	11675	6.29	94	53	24.26	29.28	0.016	0.012	10	Amyloid-related serum protein SAA
1674	gi|14277770	8909	4.6	86	73	−2.15	−2.76	0.014	0.0038	10	Chain A, Nmr structure of human apolipoprotein C-Ii in the presence of SDS

'—' not detected.

TABLE 2. Differential proteins identified all appeared by mass spectrometry (MS) after 2-D DIGE in both the mild and severe hand, foot and mouth disease samples vs. control samples

Master no	Accession No.	Mr	pI	Mw	Proteins
274	gi|55958063	105606	4.8	130	Inter-alpha (globulin) inhibitor H2
279	gi|119581148	57754	4.82	130	Keratin 9 (epidermolytic palmoplantar keratoderma)
442	gi|224053	162072	5.45	109	Macroglobulin alpha2
480	gi|177870	164600	6.26	107	Alpha-2-macroglobulin precursor
552	gi|4501987	70963	5.3	97	Afamin precursor
912	gi|6573461	37499	6.12	62	Chain A, crystal structure of human beta-2-glycoprotein-I (Apolipoprotein-H)
1083	gi|16418467	38382	4.54	51	Leucine-rich alpha-2-glycoprotein 1 precursor
1192	gi|78101271	40204	4.82	39	Chain C, human complement component C3c
1242	gi|38305346	17032	4.22	36	Antigen MLAA-42
1257	gi|119579599	51388	5.41	35	Haptoglobin, isoform CRA_b
1258	gi|47124562	31647	5.48	36	HP protein
1265	gi|118138017	104913	4.99	34	Chain B, human complement component C3b
1279	gi|118137965	104912	4.97	33	Chain B, structure of complement C3b: insights into complement activation and regulation
1340	gi|27574252	15928	5.23	31	Chain B, deoxy haemoglobin (A-Gly-C:v1m; B,D:v1m,C93a,N108k)
1342	gi|26665859	16267	5.21	31	CLU
1437	gi|170684576	24426	6.49	29	Immunoglobulin kappa 4 light chain
1493	gi|576259	23358	5.44	28	Chain A, the structure of pentameric human serum amyloid P component
1561	gi|178779	43358	5.25	23	Apolipoprotein A-IV precursor
1566	gi|563320	28141	5.14	21	Apolipoprotein A-IV
1591	gi|223092	18672	5.31	19	Ceruloplasmin CpF5 fragment
1593	gi|296653	42126	5.57	18	hp2-alpha
1617	gi|224510585	12996	5.68	16	Chain A, crystal structure of the apo form of human wild-type transthyretin
1664	gi|186972736	8759	4.33	12	Chain A, structure and dynamics of human apolipoprotein C-Iii
1669	gi|225986	11675	6.29	10	Amyloid related serum protein SAA
1674	gi|14277770	8909	4.6	10	Chain A, Nmr structure of human apolipoprotein C-Ii in the presence of SDS

(6.7%), enzyme modulator (6.7%), protease (6.7%) or structural protein (6.7%) (Fig. 2a–c).

Protein–protein interaction network

The protein interaction network for the identified proteins was constructed by STRING (Fig. 3) in order to better understand the pathogenic mechanisms in HFMD. Biological systems can be modelled as complex network systems with many interactions among the components in different pathways. These interactions provide us with essential information about the function and behaviour of the analysed system.

Discussion

Treatment of HFMD is currently symptomatic, and mild HFMD resolves spontaneously without complications within 7–10 days. However, severe HFMD can cause severe neurological symptoms and complications, such as acute flaccid paralysis, aseptic meningitis and neurogenic pulmonary oedema. In recent years, although treatment has been updated with the application of new technologies, there is currently no specific antiviral therapy to cure and no vaccine to prevent severe HFMD infection. Therefore, early detection of severe HFMD is important and precise staging is necessary for assessing the risk of progression and planning the appropriate treatment.

Due to the limited practicability of clinical markers for early diagnosis, the focus of HFMD investigation has shifted to molecular markers. Protein profiles of specific stages can be more accurate in reflecting the status of disease progression. In the present study, we took advantage of DIGE-based proteomics to identify stage-specific markers that reflect the status of HFMD. DIGE-based proteomics has the advantages of adequate sensitivity, high reproducibility, wide linear dynamic range and minimized experimental variation over traditional proteomics because of internal standards and fluorescence labelling. Although no protein identified was reported to have direct connection to HFMD the majority of the differentially expressed proteins are involved in the host immune response system, inflammation and protein aggregation.

ITIH2 (inter-alpha (globulin) inhibitor H2), A2M (macroglobulin alpha2), etc. (protease inhibitors), were suggested to be involved in the viral infection. ITIH2 belongs to the inter-alpha-trypsin inhibitors (ITI) family, which is involved in extracellular matrix stabilization and the host-virus interaction process. A2M, a multifunctional protease inhibitor and cytokine transporter, inhibits various proteases, including trypsin, thrombin and collagenase, and is an essential component in salivary innate immunity against haemaglutination mediated by S-OIV [16]. LRG1 (leucine-rich alpha-2-glycoprotein 1 precursor) belongs to the leucine-rich repeat (LRR) family of proteins, which is expressed during granulocyte differentiation [17], indicating that LRG1 might play a role in the immune response when the host clears infections. The up-regulation of LRG1 and down-regulation of ITIH2 and A2M suggests the immune response to infection with HFMD.

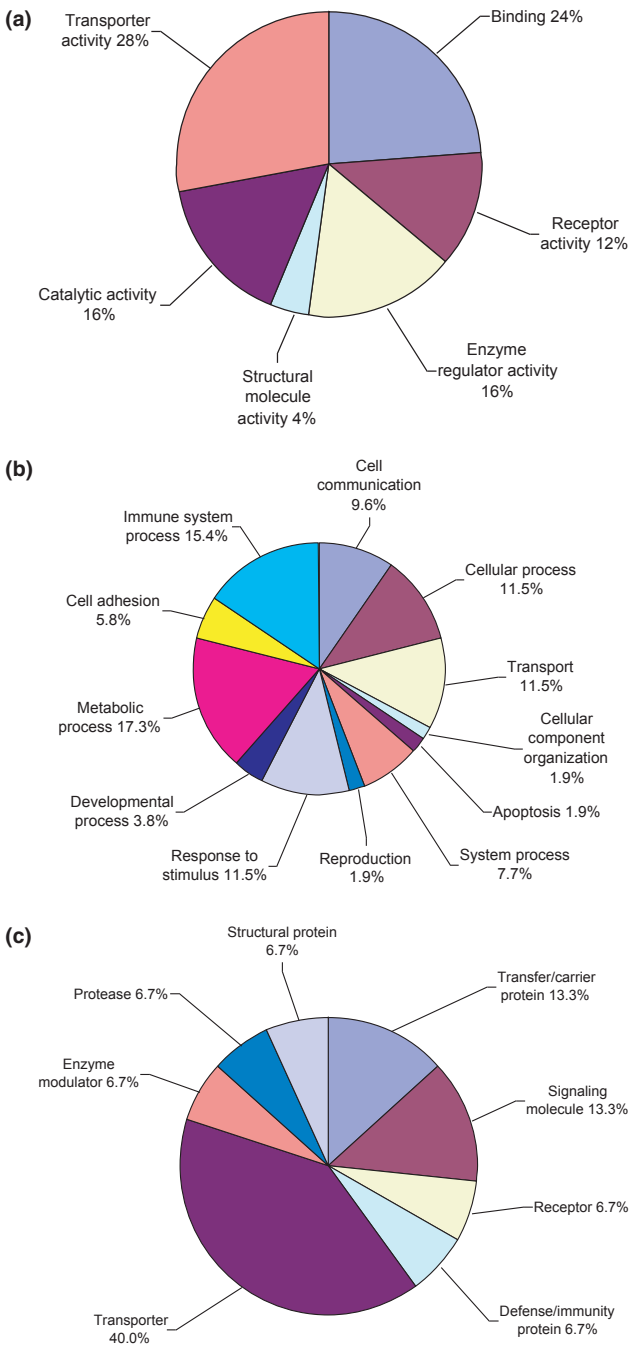

FIG. 2. Classification analysis of proteins found in hand, foot and mouth disease (HFMD) samples. Categorization was based on information that was obtained from the online PANTHER classification system. (a) Classification of the identified proteins of HFMD samples according to the molecular function. (b) Classification of the identified proteins of HFMD samples according to the biological processes. (c) Classification of the identified proteins of HFMD samples according to the protein class.

Interestingly, A2M is also involved in the amyloid response in Alzheimer's disease together with apolipoproteins [18]. These proteins bind the misfolded Aβ and the complex is then cleared by LRP1 (low density lipoprotein receptor-related protein 1). Down-regulation of A2M in HFMD decreases the amyloid clearance. APCS (chain A, the structure of pentameric human serum amyloid P component) and TTR (chain A, crystal structure of the apo form of human wild-type transthyretin), which were found to be up-regulated in HFMD patients, are also related to protein aggrega-

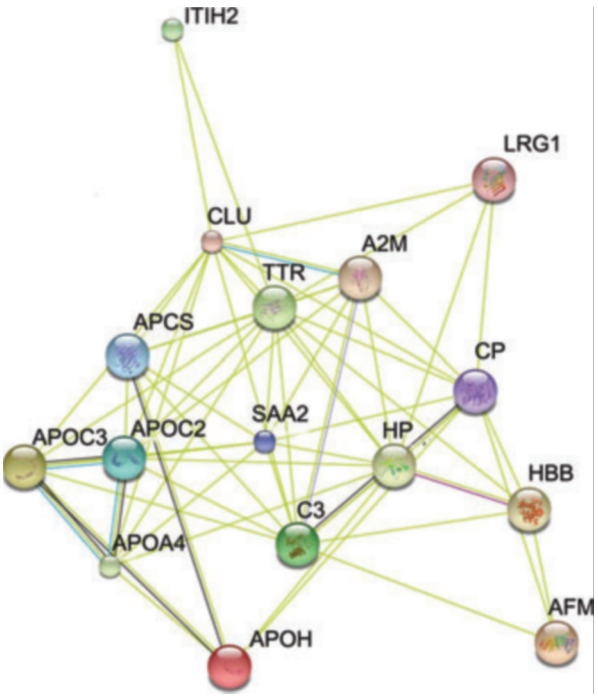

FIG. 3. The network of protein–protein interactions of HFMD.

tion. APCS is a universal constituent of the abnormal tissue deposits in amyloidosis, and its glycosylated form is resistant to degradation, causing the amyloid deposits to remain [19]. Extracellular deposition of wild-type TTR caused by transthyretin amyloidosis leads to amyloid fibril formation in various tissues and organs, and may further result in failure of organs (e.g. heart failure) [20,21]. SAA, which was found to be the most up-regulated (more than 20-fold) in both mild and severe HFMD, was reported to be closely related to tissue damage or inflammation (may increase 1000-fold) [22]. Interestingly, SAA is also found to be elevated in Alzheimer's disease and Down's syndrome, especially in the prolonged stage [23]. As an acute phase protein, SAA is found in only some patients with early-stage AD; however, the inflammation may impair Aβ clearance due to the loss of apoE from CSF-HDL [23,24]. These findings strongly suggested that the amyloidosis, including increasing of the amyloidgenic material (TTR), increasing of the amyloid-stabilizing factor (APCS) and decreasing of the amyloid clearance (A2M and SAA), may be an important molecular event in HFMD.

When comparing with the previous study using a cultured cancer cell line [10], β-actin is the only protein among their findings which appears in our study. In our study, we found that β-actin was up-regulated in the serum of the severe HFMD patients. β-Actin is an intracellular protein. Its increase in serum may indicate cell death caused by serious infection.

In the current study, although none of the above-mentioned differentially expressed proteins is specifically correlated with HFMD, the combination of their variations may still serve as a useful hint for diagnosis of early-stage HFMD prior to the appearance of severe symptoms.

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Transparency Declaration

The authors declare that no conflicting of interests exist.

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