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