Cellular proteome alterations in response to enterovirus 71 and coxsackievirus A16 infections in neuronal and intestinal cell lines

Shie Yien Chan, I-Ching Sam, Jeffrey K.F. Lai, Yoke Fun Chan *

Department of Medical Microbiology, Faculty of Medicine, University Malaya, 50603 Kuala Lumpur, Malaysia

Abstract

Hand, foot and mouth disease is mainly caused by enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16), but EV-A71 is also associated with severe neurological complications. Host factors may contribute to the different clinical outcomes of EV-A71 and CV-A16 infections. A neurovirulent EV-A71 strain (EV-A71/UH1) from a fatal case, a non-neuroviral EV-A71 strain (EV-A71/Shafa66) and a CV-A16 strain (CV-A16/22159) from cases of uncomplicated HFMD were used. Replication of the viruses in SK-N-MC (neuronal) and HT-29 (intestinal) cell lines correlated with the severity of clinical disease associated with each virus. EV-A71/UH1 showed the greatest replication in neuronal cells. In HT-29 cells, both EV-A71 strains replicated well, but CV-A16/22159 showed no effective replication. The proteomes of mock and infected SK-N-MC and HT-29 cell lines were compared by 2D-SDS-PAGE. The differentially expressed proteins were identified by MALDI-TOF/TOF analysis. There were 46 and 44 differentially expressed proteins identified from SK-N-MC and HT-29 cells, respectively, categorized under apoptosis, stress, cytoskeletal, energy metabolism proteins and others. Western blot validation showed that EV-A71/UH1 and CV-A16 also differentially induced proteins involved in viral RNA translation and host cell stress responses in neuronal and intestinal cell lines.

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1. Introduction

Enterovirus 71 (EV-A71) and coxsackievirus A16 (CV-A16) belong to the family of Picornaviridae and the genus Enterovirus. They are the main causes of hand, foot and mouth disease (HFMD) and herpangina, both common childhood infectious diseases. In rare instances, EV-A71 infection causes potentially life-threatening neurological complications such as acute flaccid paralysis, meningitis, meningoencephalitis and encephalomyelitis, and cardiopulmonary complications such as pulmonary edema, cardiac dysfunction and cardiac failure [1–3]. Severe case rates of 0.1–1.1%, and fatality rates of 0.02–0.06% have been reported in HFMD patients [4,5]. Conversely, only three CV-A16 fatal cases have been reported to date, associated with myocarditis [6], pneumonitis [7] and rhabdomyolysis [8]. It is not known why EV-A71 may lead to severe disease and fatalities, while CV-A16 generally does not.

Both EV-A71 and CV-A16 are transmitted via the fecal–oral route and direct contact. Upon virus entry into the host, the primary target organs for viral replication are thought to be the respiratory and gastrointestinal tracts, before the virus enters the circulation. Use of a monkey model showed the importance of the respiratory route in EV-A71 infection with central nervous system involvement [9]. Evidence of replication of EV-A71 in neuronal and intestinal cell lines supports the respiratory tract as a potential route of viral entry [10]. Other studies support the intestine as the first target organ before viremia in the host. In a mouse model, EV-A71 first replicates in the intestine and later spreads to the respiratory system and skeletal muscle through viremia [11,12], before entering the brain by the retrograde axonal pathway [13,14]. Clinical findings support the involvement of motor neurons in spreading the virus to the brainstem. In addition, the virus may enter the brainstem by infecting endothelial cells in the blood brain barrier [15]. In the later stages of EV-A71 infection, cardiovascular complications such as pulmonary edema and cardiopulmonary failure can result from central nervous system involvement [9,16].

Host factors can modulate the outcome of an infection. Much is known about the host factors associated with EV-A71 infection, but not for CV-A16. Receptor usage can be one of the host determinants. To date, six receptors/attachment factors have been identified for EV-A71: SCARB2 [17], PSGL-1 [18], annexin [19], sialic acid [20], heparan sulfate [21] and vimentin [22]. Other important cellular processes include apoptosis and autophagy [15,23–25]. The occurrence of apoptosis is well documented in EV-A71 in both neuronal and non-neuronal cells [23,26]. Various cytokines are expressed in EV-A71-infected hosts, including IL-4, IL-5, IL-6, IL-10, IL-2, TNF-α, IFN-γ, IL-13, IL-1α and macrophage inflammatory protein 1 alpha (MIP-1α) [27]. Pro-inflammatory responses with significant increases of IFN-γ, IL-6, chemokine (C-C motif) ligand 5 (CCL5) and interferon-gamma-induced protein 10 (IP-10) in the EV-A71-infected HT-29 intestinal cell line were also reported [28]. As the gene organization and replication cycle for EV-A71 and CV-A16 are essentially the same, we hypothesize that there may be differentially regulated
proteins induced in a primary target host cell (gastrointestinal) and an end target host cell (neuronal).

2. Materials and methods

2.1. Cells and viruses

SK-N-MC cells (human neuroepithelioma cells; ATCC no. HTB-10) were cultured in Eagle’s minimal essential medium. HT-29 cells (human colorectal adenocarcinoma cells; ATCC no. HTB-37) were cultured in McCoy’s 5A modified medium. RD cells (human rhabdomyosarcoma cells; ATCC no. CCL-136) for the titration assay were cultured in Dulbecco’s modified Eagle’s medium. All cells were grown in media supplemented with 10% v/v heat inactivated FBS, 2 mM l-glutamine, 1 × non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. SK-N-MC cells were also supplemented with 1 mM sodium pyruvate. All cell lines were incubated at 37 °C with 5% CO2.

Virus isolates were provided by the Diagnostic Virology Laboratory, University of Malaya Medical Centre. Two EV-A71 virus isolates, EV-A71/UAH1 (GenBank accession number AM396587) and EV-A71/Sha66 ([GenBank accession number AM396586]) and a CV-A16 virus isolate (CV-A16/22159, GenBank accession number JQ746673) were used in the study. EV-A71/UAH1 is a neurovirulent strain isolated from the brain of a patient who died of neurogenic pulmonary edema [29]. EV-A71/Sha66, isolated from a mild HFMD case, was chosen as a non-neurovirulent EV-A71 strain [30]. CV-A16 was isolated from a patient with mild HFMD. All viruses were initially propagated in RD cells.

2.2. Kinetics of virus replication

The viral kinetics of the EV-A71 and CV-A16 strains were compared in neuronal SK-N-MC and intestinal HT-29 cells. Virus infections were performed in 24-well plates at MOI of 2 TCID50/cell. Negative control mock cells were prepared by replacing the inocula with serum free medium. Briefly, the collected culture media was centrifuged at 10,000 × g, 4 °C for 10 min, and the supernatants were collected as extracellular samples. The cell pellets were resuspended in serum free medium as intracellular samples. The titration assays were performed in RD cells in 96-well plates, and the Reed and Muench method was used to calculate the virus titer. At least two independent experiments were performed.

2.3. Proteomics analysis of EV-A71/UAH1-, EV-A71/Sha66- and CV-A16/22159-infected cell lines

Total protein from SK-N-MC cells was harvested at 3 days post-infection (dpi), while HT-29 cells were harvested at 4 dpi. To harvest the proteins, cells were scraped in the presence of 0.1% protease inhibitor cocktail (Sigma Aldrich, USA). The cells were briefly spun and the pellets were washed twice with homogenization buffer (containing 5 ml of 2 M Tris hydrochloride (pH 7.5) in 11, 150 mM potassium chloride, 2 mM magnesium chloride, 2 mM calcium chloride and protease inhibitor cocktail). Cell pellets were then lysed in ReadyPrep Sequential Extraction Kit Reagent 2 (Bio-Rad, USA) with 18 mM dithiothreitol and agitated at 4 °C overnight. The insoluble components were pelleted by centrifuging the lysates at 10,000 × g, at 4 °C for 20 min. Glycerol (PlusOne, GE Life Science, United Kingdom) was added to a final concentration of 10% v/v and samples were stored in 80 °C until further use. At least two biological replicates were prepared for each sample.

Isoelectric focusing (IEF) separation of SK-N-MC cell proteins was performed with 11 cm IPG strips of pH 3–10 and pH 5–8 (Bio-Rad, USA) using 450 μg and 900 μg protein, respectively. IEF separation of HT-29 cell proteins was performed using IPG strips of pH 3–10, pH 4–7 and pH 7–10 with 500 μg, 1000 μg and 1000 μg protein, respectively. Each IPG strip was rehydrated at 50 V for 12 h. IEF focusing was performed as follows: 200 V for 200 Vhr (linear), 500 V for 500 Vhr (linear), 1000 V for 1000 Vhrs (linear), and finally 8000 V for 36,000 Vhrs (rapid). Second dimension separation of the proteins by molecular weight was performed by SDS-PAGE. The frozen IPG strips after the IEF step were thawed and equilibrated before SDS-PAGE. The IPG strips were incubated 15 min in equilibration buffer 1 (2% w/v dithiothreitol, 6 M urea, 2% w/v SDS, 50 mM Tris hydrochloride, 30% glycerol) followed by 15 min of equilibration buffer 2 (5% iodoacetamide, 6 M urea, 2% w/v SDS, 50 mM Tris hydrochloride, 30% glycerol). The 11 cm IPG strips were separated in a Criterion electrophoresis cell (Bio-Rad, USA) for 70 min with 150 V. Three analytical gels were prepared for each sample. The gels were stained with colloidal Coomassie Blue G-250 staining [31] and gel images were scanned using a GS-800 calibrated densitometer (Bio-Rad, USA).

The gel images were analyzed by PDQuest Basic version 8.01 (Bio-Rad, USA). Briefly, all the gel images were corrected with adaptive filter (suppressing Gaussian noise and salt and/or pepper in images) at a size of 9 × 9 pixels. Spot detection in the images was done by “Spot Detection Parameter Wizard” with options of “Gaussian model during test” and “Reduce over-detection of large spots” and normalized by a local regression model. Protein spots with significant >2.0-fold change among groups (supported by Student’s t-test, 95% significance level) were selected for identification.

2.4. Identification of differentially expressed proteins by MALDI-TOF/TOF mass spectrometry

Protein spots with significant fold changes were excised from colloidal Coomassie Blue G-250 stained gels for identification by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) tandem mass spectrometry (MS). The gel plugs were destained 3 times in 100 mM ammonium bicarbonate solution for 30 min each with constant agitation. In-gel digestion of the proteins was performed by adding 10 μl of 0.02 μg/μl sequencing grade modified trypsin (Promega, USA) in 20 mM ammonium bicarbonate solution into dried gel plugs and incubated at 37 °C for 2 h. Digested peptides were eluted by adding 60 μl of peptide eluent [0.1% v/v TFA, 50% v/v ACN (Merck Millipore, USA)] for 30 min. The peptides were desalted and purified using ZipTip C18 pipette tips (Millipore, USA) following the manufacturer’s instructions. The eluted samples were mixed with 0.5% v/v TFA + 50% v/v ACN supplemented with saturated α-cyano-4-hydroxy cinnamic acid in 1:1 ratio. Then, 0.7 μl of the mixture was spotted on the Opti-TOF 384-well insert plate. The peptide mass spectra were generated in a 4800 Plus MALDI-TOF/TOF analyzer (Applied Biosystems, USA) in the positive ion reflector mode. The 20 most abundant ions were selected for subsequent fragmentation by high-energy collision-induced dissociation for generation of MS/MS spectra. Data were interpreted using Global Protein Server Explorer 3.6 software (Applied Biosystems, USA). Database search was performed using the Mascot program (MatrixScience, United Kingdom) with the following parameter settings: NCBInr database, trypsin, variable modifications of cysteine carbamidomethylation and methionine oxidation, monoisotopic, peptide mass tolerance at ±100 ppm, fragment mass tolerance at ±0.2 Da and maximum missed cleavage of 1.

2.5. Validation of differentially expressed host proteins by Western blot

To validate the regulation of host proteins detected by the 2D-SDS-PAGE and MALDI analysis, selected proteins were confirmed by Western blotting. The following primary monoclonal antibodies were used at the manufacturer’s recommended dilutions: HRP-conjugated antibodies from the loading control antibody sampler kit HRP conjugate (Cell Signaling, USA), antibodies from the heat shock protein (HSP)/chaperone antibody sampler kit (Cell Signaling), heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 (2A2) mouse mAb (Cell Signaling), HSP27 (G31) mouse mAb (Cell Signaling), HnRNP H antibody (N1C1) (GeneTex, USA) and HnRNP C1/C2 antibody (GeneTex). The membranes were finally incubated in Amersham ECL Prime Western Blotting
Detection Reagent (GE Healthcare, USA) for 5 min. Chemiluminescence signals on the nitrocellulose membranes were detected by BioSpectrum AC Imaging System (UVP, USA).

3. Results

3.1. Virus replication kinetics in human cell lines

Both intracellular and extracellular samples for infected SK-N-MC cells were collected at 8-hour intervals until 120 h post-infection (h.p.i) (Fig. 1a and b). EV-A71/UH1 is replicated to higher titers than EV-A71/Sha66 and CV-A16/22159 in SK-N-MC cells. The intracellular titer of EV-A71/UH1 in SK-N-MC cells peaked at 40 hpi and maintained at 10^6 TCID_{50}/ml up to 120 h.p.i. The titer of extracellular EV-A71/UH1 peaked at 48 h.p.i and maintained at 10^5 TCID_{50}/ml up to 72 h.p.i. The intracellular titer of EV-A71/Sha66 only increased to 10^4 TCID_{50}/ml after 72 h.p.i while the extracellular titer gradually increased to 10^6 TCID_{50}/ml at 80 h.p.i. The intracellular CV-A16/22159 virus titer only increased to 10^2 TCID_{50}/ml at 36 h.p.i, and showed no significant increase after this time point. The extracellular titer of CV-A16/22159 peaked at 10^4 TCID_{50}/ml after 64 h.p.i.

The kinetics of replication of the three viruses in the human intestinal cell line HT-29, were also distinctively different (Fig. 1c). The intracellular virus titers of EV-A71/UH1 and EV-A71/Sha66 peaked at 10^7 TCID_{50}/ml at 72 h.p.i and 10^5 TCID_{50}/ml at 120 h.p.i, respectively, EV-A71/UH1 maintained its intracellular titer significantly greater by 1–3 log_{10} TCID_{50}/ml up to 168 h.p.i. The extracellular titer of EV-A71/UH1 was also higher than EV-A71/Sha66 before 96 h.p.i (Fig. 1d). CV-A16/22159 showed a constant intracellular virus titer of 10^5 TCID_{50}/ml with no significant increase in intracellular and extracellular virus titers.

These results show that virus replication was different between EV-A71 and CV-A16 in neuronal and intestinal cells. The neurovirus EV-A71/UH1 showed better replication compared to the EV-A71/Sha66 and CV-A16/22159 in SK-N-MC and HT-29 cells. CV-A16/22159 replicated poorly in SK-N-MC and HT-29 cells. Based on the time points at which peak virus titer was achieved by the three viruses, total protein for the subsequent proteomic experiments was harvested at 72 h.p.i for SK-N-MC cells and 96 h.p.i for HT-29 cells.

3.2. 2DE analysis of EV-A71/UH1-, EV-A71/Sha66- and CV-A16/22159-infected cells

To further understand if host responses to EV-A71 and CV-A16 infection are different, the infected cell proteome for SK-N-MC and HT-29 cells was analyzed. There were 141 spots successfully annotated in the SK-N-MC proteome of pH range of 3–10. Among these, 41 spots showed 2-fold changes between the mock and infected cells with significance level \( p < 0.05 \). The proteome of SK-N-MC pH 5–8 showed only 39 spots, and 11 spots were found to show 2-fold changes with significance level \( p < 0.05 \). More proteins were detected in the HT-29 gel sets. There were 139 spots, 56 spots and 52 spots in 2D-E at pH 3–10, pH 4–7 and pH 7–10, respectively. Of these, 37, 1, and 11 spots, respectively showed at least 2-fold changes with significance level >95%. All the differentially expressed spots were further examined by MALDI TOF/TOF.

3.3. Identification of differentially expressed proteins by MALDI TOF/TOF

From the MALDI TOF/TOF analysis, 46 out of 52 differentially expressed protein spots were successfully identified from different proteomes of mock and infected-SK-N-MC cells (Fig. 2). From the

Fig. 1. Replication kinetics of viruses in infected SK-N-MC and HT-29 cells. Intracellular and extracellular virus titers were determined at selected time points by titration assay. The intracellular titers were determined from the cell pellets while extracellular titers were determined from the cell supernatants. The curves were plotted as mean ± standard deviation of at least two independent experiments. The virus replication efficiency was in the order of EV-A71/UH1 > EV-A71/Sha66 > CV-A16/22159. Symbols indicate significant differences by t-test (**, \( p < 0.01 \); ***, \( p < 0.001 \)).
mock and infected HT-29 proteomes, 44 out of 49 protein spots were successfully identified by MALDI TOF/TOF (Fig. 3). Several proteins such as actin, heat shock protein beta-1 (HSPB1), endoplasmic reticulum resident protein 29 (ERP29), hnRNP A/B, and triosephosphate isomerase 1 (TPI1) were detected in more than one spot, suggesting the presence of different isoforms and/or post-translational modifications.

The minimal scores for proteins identified were 38, and minimal coverage percentages were 3%. The details of these proteins are shown in Supplementary Table 1 and Supplementary Table 2.

The proteins were categorized into five categories based on function: apoptosis, stress, cytoskeletal, energy metabolism and others. Ten apoptosis proteins, six stress proteins, six cytoskeletal proteins,

Fig. 2. Significant protein spots from EV-A71-infected SK-N-MC cells identified by MALDI-TOF/TOF. The spots were categorized into five groups according to their functions: (a) apoptosis, (b) stress, (c) cytoskeletal, (d) energy metabolism and (e) others. The protein spots are indicated by arrows (↑).
nine energy metabolism proteins and eight other proteins were identi-
fied in SK-N-MC cells (Fig. 2, Supplementary Table 1). In HT-29 cells, ten
apoptosis proteins, five stress proteins, three cytoskeletal proteins,
seven energy metabolism proteins and fifteen other proteins were iden-
tified (Fig. 3, Supplementary Table 2).

These differentially expressed proteins were further analyzed by
two-way hierarchical clustering analysis (Fig. 4). More than 30 identi-
fied differentially expressed proteins were down-regulated while only
three were up-regulated in infected-SK-N-MC cells (Fig. 4a). A total of
17 differentially expressed proteins were down-regulated and 11
were up-regulated in infected-HT-29 cells (Fig. 4b).

To visualize the relationships between the identified proteins,
Search Tool for the Retrieval of Interacting Genes (STRING) version
9.05 (http://string-db.org/) [32] was used (Fig. 5). Of the 46 identified
proteins in SK-N-MC cells, 31 showed interactions with other proteins
in the map. TP1, ATP5B, ENO1 and HSPD1 showed the highest connec-
tivity with other proteins, suggesting their importance in biological
networks (Fig. 5a). In HT-29 cells, 34 identified proteins showed

![figure 3](image-url)
interactions with other proteins. PDRX1, HSPD1, TP1 and ENO1 were among the proteins with the highest connectivity reaffirming their major roles in biological networks (Fig. 5b).

3.4. Validation of differentially expressed proteins by Western blotting analysis

The differential expression of selected HSP/chaperones and hnRNPs in infected SK-N-MC cells and HT-29 cells were validated by Western blot using commercially available antibodies. Additional HSPs not identified by the proteomics analysis were also included to further decipher the role of these proteins in enterovirus infection. A few common loading control proteins were used in the Western blot validation. The three loading controls β-actin (ACTB), β-tubulin (TUBB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed significant differences in mock and virus-infected SK-N-MC cells, as seen in the 2DE results. Cytochrome c oxidase subunit IV (COX IV) and histone were found to be better loading controls for mock and virus-infected SK-N-MC cells (Fig. 6). ACTB, COX IV, TUBB and GAPDH showed consistency in band intensity and were appropriate loading controls in HT-29 cells.

Heat shock transcription factor 1 (HSF1), HSP90 and hnRNP A2/B1 were down-regulated in both the EV-A71- and CV-A16-infected SK-N-MC cells. This suggests that these proteins are common cellular factors associated with enterovirus infections. The proteins hnRNP C1/C2, hnRNP H, ACTB and TUBB were down-regulated in EV71/UH1-infected-SK-N-MC cells, while binding immunoglobulin protein (BiP), HSP60 kDa 1 (HSPD1) and GAPDH were up-regulated. The proteins hnRNP H, HSF1, BiP and histone were up-regulated in CV-A16/22159-infected HT-29 cells (Fig. 6). Different protein patterns were observed in these cells suggesting that host responses to the virus could be cell-specific.

4. Discussion

Both EV-A71 and CV-A16 are common causes of HFMD, but only EV-A71 is associated with neurological complications. The present study aims to provide insight into the clinical differences, by comparing neurovirulent EV-A71, non-neurovirulent EV-A71 and CV-A16 strains in terms of replication and induction of host proteins in gastrointestinal and neuronal cells. It is difficult to investigate the impact of viruses on the proteome of primary cells or animals, therefore studies based on cell lines are useful.

We found that the replication trends of the EV-A71 and CV-A16 strains in SK-N-MC neuronal cells reflected the severity of their associated clinical disease. Both EV-A71 strains replicated to a greater degree than the CV-A16 strain, with peak infectivity of 1–4 log higher titers. Furthermore, the neurovirulent strain EV-A71/UH1 showed a significant trend for faster replication than the non-neurovirulent EV-A71/Shag66. EV-A71 viruses also replicated well in HT-29 cells, while CV-A16/22159 showed little effective replication. However, it is possible...
that this could be strain-dependent and more CV-A16 virus isolates should be tested in HT-29 cells. Infectious virions of all three enterovirus strains continued to be produced from infected HT-29 cells throughout the 7 day incubation, which may contribute to the prolonged virus shedding seen in clinical HFMD cases [33].

Data on apoptosis-related proteins regulated in SK-N-MC cells could shed light on the mechanism of virus-induced apoptosis in the central nervous system. Three proteins were found to be differentially expressed in both SK-N-MC and HT-29 cells: B23 nucleophosmin (NPM1), HSPD1 and protein disulfide isomerase A3 (PDIA3). NPM1 was down-regulated in all the infected SK-N-MC cells and the EV-A71-infected HT-29 cells. NPM1 interacts with the capsid protein of porcine circovirus type 1 and correlates with the induction of apoptosis in infected cells [34]. Up-regulation of HSPD1, an apoptosis regulatory protein [35,36] in all infected SK-N-MC and HT-29 cells was observed. PDIA3 was down-regulated in EV-A71-infected neuronal cells and CV-A16/22159-infected HT-29 cells, which may increase the activity of host STAT3 signaling pathway [37]. PDIA3 acts together with calreticulin (CALR) and calnexin as chaperones for folding of glycoproteins [38,39]. These proteins are part of the major MHC class I molecules which are important for antigen presentation during host defense against virus infection [40]. Significant down-regulation of CALR was also observed in the EV-A71/UH1-infected SK-N-MC cells in this study. Although BiP was not detected in the 2DE and MALDI analysis, differential expressions of this protein were detected in Western blot. BiP, also known as 78 kDa glucose-regulated protein or HSP70, protects cells from apoptosis activated by caspase-7 [41,42]. In SK-N-MC cells, there was up-regulation of BiP following infection with the neurovirulent EV-A71/UH1 strain, but not the clinically milder strain EV-A71/Shao66 and CV-A16/22159. This suggests that regulation of host factors is virus-dependent. The apolipoprotein A (APOA1) up-regulated in both EV-A71-infected SK-N-MC cells can stimulate the efflux of cholesterol to cell surface caveolae [43]. The presence of membrane surface cholesterol could be one of the factors that promote the entry of enteroviruses [44,45]. The isoforms of HSPB1 were expressed at higher levels in the EV-A71-infected neuronal cells. In early stages of infection, HSPB1 was down-regulated, while it was up-regulated later in EV-A71 infection [46,47].

In HT-29 colorectal cells, coflin-1 (CFL1), keratin 18 (KRT18), keratin 20 (KRT20) and keratin 8 (KRT8) were among the differentially expressed apoptosis regulatory proteins that affect cytoskeleton arrangement. CFL1 is a member of the actin-depolymerization factor/cofilin family which plays an important role in actin dynamics, and functions in cytoskeleton remodelling in motile cells [48]. The increased expression of CFL1 in neuronal cells may be the key factor for initiation of apoptosis in late EV-A71 infection [47]. Increase of keratins during infection with the neurovirulent EV-A71/UH1 may be an indicator for active remodelling of the cytoskeleton network of the host cells. KRT8 and KRT18 are tightly associated and are important for effective replication of many viruses, including enteroviruses [49,50]. Infection of HT-29 cells by the virulent strain EV-A71/UH1 led to increase of ubiquitin-like protein ISG15. The IFN-stimulated genes (ISGs) activate IFNs as part of the innate immune response, with ISG15 as the predominant antiviral response [51,52].

The stress-related proteins, hnRNP H3 and hnRNP H were down-regulated in EV-A71/UH1-infected cells. However, hnRNP H in CV-A16/22159-infected HT-29 cells was elevated despite lower levels in the 2DE and MALDI analysis. The proteins hnRNP K and hnRNP A1 interact with the 5′ UTR of EV-A71, and are important for viral RNA replication and virion production [53]. Poliovirus RNA also interacts with hnRNP C [54,55], while hnRNP D was also cleaved by polioviral 3CD protein [56]. The differential regulation of hnRNP H3 could affect the pathogenesis and replication efficiency of the viruses. Another protein that was differentially regulated was HSF1 detected in the Western blot. In SK-N-MC cells, depletion of HSF1 was observed with the down-regulation of HSP90. HSF1 activity is negatively regulated by the expression of HSP90 [57]. Induction of the heat shock 70 kDa protein 8 (HSPA8) chaperone was detected in 2DE of EV-A71-infected HT-29

Fig. 5. Interaction networks of differentially expressed proteins from infected (a) SK-N-MC and (b) HT-29 proteomes. All the proteins were identified from the MALDI-TOF/TOF analysis. Proteins which were up-regulated (red triangles) and down-regulated (green triangles) in EV-A71/UH1-infected cells compared to CV-A16/22159-infected cells are shown. Proteins with stronger association are linked by thicker lines.

cells but not in CV-A16. HSPA8 takes part in CV-B3 internalization via clathrin-mediated endocytosis [58]. HSPA8 is a variant of HSP70, and it serves as negative regulatory protein of HSFI activation [59]. Overall, the regulation of host stress-induced proteins in SK-N-MC cells infected by neurovirulent EV-A71 has an anti-apoptotic effect, which could be the host’s survival response during viral replication.

Regulation of cytoskeletal proteins is important for virus entry, replication and release. The cytoskeletal proteins actin and TUBB were differentially expressed in the infected cells. Actin polymerization is associated with EV-A71 internalization via the clathrin pathway [60]. Intracellular trafficking of poliovirus also depends on actin microfilaments [61]. The rearrangement of microtubules is important for extracellular release of the poliovirus [62]. F-actin depolymerization and degradation were also induced in echovirus-infected Caco-2 cells [63].

As actin and TUBB were both altered during viral infections in this study, they are not ideal endogenous loading controls for EV-A71 and CV-A16 infection.

In the SK-N-MC cells, most of the identified proteins involved in energy metabolism were down-regulated in EV-A71/UH1 infection compared to mock cells. ATP synthase subunit beta (ATP5B) is also down-regulated in herpes simplex virus infection for productive replication [64]. The lipid metabolism protein, mitochondrial enoyl-CoA hydratase (ECHS1), suppresses STAT3 signaling in active tumor cells [65]. ENO1 takes part in the glycolysis pathway and was highly suppressed in the virulent EV-A71/UH1 strain [66]. In HT-29 cells, many metabolic proteins were up-regulated, especially during EV-A71 infection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in EV-A71/UH1-infected HT-29 cells, but was absent in other HT-29 samples. However, this difference was not detected by Western blot. Infected SK-N-MC cells showed differential expression of GAPDH in Western blot. It has been shown that poliovirus infection results in influx of proteins including GAPDH into the cytoplasm [67]. GAPDH is one of the common endogenous loading controls for Western blot analysis, and suitability will depend on the type of virus and cells. Our Western blot results suggest that cytochrome c oxidase subunit IV (COX IV) is a better endogenous control than GAPDH for the analysis of late EV-A71 and CV-A16 infection in SK-N-MC and HT-29 cells.

Some proteins identified by MALDI analysis were not categorized into the four function groups above. Several hnRNPNs were detected to be differentially regulated in EV-A71- and CV-A16-infected HT-29 cells, namely hnRNP L, hnRNP A/B and hnRNP A2/B1. In SK-N-MC cells, only the virulent strain EV-A71/UH1 was reported to down-regulate hnRNP C [46], similar to our present findings. There are many studies of interactions of other hnRNPs with viral translational control, such as hnRNP A1, hnRNP A2 and hnRNP K; thus hnRNP C identified in our study may be involved in a similar mechanism [68]. Host protein hnRNP L associates with hnRNP A1 in the regulation of several host exons [69], thus increased expression of these host RNA splicing regulators could favor replication of the virulent EV-A71/UH1 strain. Down-regulation of the splicing regulatory proteins such as hnRNP A/B was observed in the 2DE of EV-A71/UH1-infected HT-29 cells. Other proteins involved in nucleic acid metabolism, eukaryotic translation initiation factor 4H (EIF4H) and eukaryotic initiation factor 4A-II (EIF4A2), were down-regulated in EV-A71/UH1-infected SK-N-MC cells and HT-29 cells, respectively. EIF4A2 plays an important role in miRNA-mediated gene regulation [70]. Reduction of EIF4H could induce the formation of cytoplasmic stress granules which in turn inhibit translation, while depletion of EIF4E and EIF4F has the opposite effect [71].

Regulation of intracellular transport is common in virus infection. We found that endoplasmic reticulum resident protein 29 (ERP29), reticulocalbin-1 (RCN) and multiple coagulation factor deficiency protein 2 (MCFD2), which are ER resident proteins, were differentially regulated. The 2B protein of poliovirus could affect Ca2+ homeostasis in the ER, affecting the protein trafficking mechanism of the host cells [72]. ERP29 is highly expressed in brain cells [73]. RCN regulates calcium-dependent activities in the ER lumen or the post-ER compartment [74]. MCFD2 forms a specific cargo receptor for the ER-to-Golgi transport of selected proteins [75]. Voltage-dependent anion channel (VDAC) and VDAC2 are channel-forming proteins on the outer membrane of mitochondria which maintain membrane potential, and are regulated by elastin and free tubulin [75]. Efflux of Ca2+ from the ER lumen could be transported into mitochondria by VDAC, causing mitochondrial dysfunction and apoptosis in poliovirus infection [76].

5. Conclusion

In vitro replication trends of neurovirulent and non-neurovirulent EV-A71 and CV-A16 in SK-N-MC neuronal cells correlated with their associated clinical disease. The proteomic profiles of the neurovirulent EV-A71/UH1 in SK-N-MC and HT-29 cells showed the differential
expression of many proteins involved in apoptosis, stress, the cytoskeletal network, energy metabolism and RNA translation. Compared to non-neurovirulent EV-A71 and CV-A16, the neurovirulent EV-A71/1H1 differentially down-regulated a unique set of proteins (ATP5B, ERP29, hNRPN A/B, hNRPN H3, NPM1, RCN1 and VCP), and up-regulated GAPDH, HSD17B10, KRT8, PDA13, MTAP and VDAC. This study provides insights into host factors which may be important in the pathogenesis of severe EV-A71 infection. These host proteins are potential targets for future pathogenesis study, diagnostic tools and therapeutic interventions.

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