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Beta-actin variant is necessary for Enterovirus 71 replication

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ABSTRACT

Enterovirus 71 (EV71) is one of the main etiological agents of the Hand, Foot and Mouth Disease (HFMD) and has been known to cause fatal neurological complications such as herpangina, aseptic meningitis, poliomyelitis-like paralysis and encephalitis. EV71 is endemic in the Asia-Pacific region and causes occasional epidemics. In order to better understand EV71 infection, we compared the proteome between EV71-susceptible and EV71-resistant human Rhabdomyosarcoma (RD) cell line. We found significant differences in the β-actin variants between the EV71-susceptible RD cells and EV71-resistant RD cells, suggesting that β-actin, in association with other proteins such as annexin 2 is required in vesicular transport of EV71. This finding further support our previous study that actin potentially plays a role in pathogenesis and the establishment of the disease in HFMD.

1. Introduction

Enterovirus 71 (EV71) is a positive sense, single stranded, non-enveloped RNA virus belonging to Enterovirus genus of the Picornaviridae family [1,2]. It is one of the main etiological agents of the Hand, Foot and Mouth Disease (HFMD) which commonly infects infants and children [1–3]. The virus is usually spread from one person to another through direct contact of vesicular fluid or droplet from the infected or via faecal–oral route [4–7]. Symptoms of EV71 infection include persistent high fever, repeated vomiting, myoclonic jerks, persistent drowsiness or sudden limb weakness [5,6]. In addition, EV71 infection has been known to cause fatal neurological complications like aseptic meningitis, poliomyelitis-like paralysis and encephalitis [3,6,7].

EV71 was first described in 1974 in California (USA) after it was isolated from a patient with central nervous system disease in 1969 [8]. Since then, there have been periodic outbreaks, epidemics and pandemics reported worldwide every two to three years in various countries such as Australia, China, Taiwan, Japan, Korea, Malaysia, Vietnam, Thailand and Singapore [8–12]. Large fatal outbreaks first occurred in Bulgaria in 1975 and subsequently re-emerged in Hungary in 1978, Malaysia in 1997 and Taiwan in 1998 [8,12–16]. In the recent years there has been several large epidemics of HFMD in the Asian-Pacific region, including Singapore, resulting in sudden deaths among young children due to EV71 infections [11,17]. Although EV71 has been widely studied, the exact mechanism of pathogenesis remains unknown with particular debates on the mode of entry and viral shuttling in the host cell.

In order to better understand EV71 infection, we performed proteomic analyses between EV71-susceptible and EV71-resistant human Rhabdomyosarcoma (RD) cell line, using 2-dimensional electrophoresis (2DE).

2. Materials and methods

2.1. Infection of cell line

RD cells (ATCC® catalog No. CCL-136™) was maintained in T75 flask using Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum and 2% penicillin–streptomycin at 37 °C with 5% CO_2 in a humidified incubator. The EV71 resistant cells were obtained by continuous passaging to an estimated 100 times. Cells were confirmed to be resistant by their inability to show cytopathic effect after infection with EV71 for 2 days (data not

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shown). The EV71viral strain used was 5865/sin/000009 (accession number 316321; designated as Strain 41) isolated from a fatal case of HFMD patient during the outbreak in Singapore in October 2000. When seeded cells reach approximately 95% confluency, they were infected with the virus at a MOI of 10 and harvested 2 h post infection.

2.2. Protein extraction and 2-dimensional electrophoresis

Cellular proteins were extracted using ProteoExtract Complete Mammalian Proteome Extraction Kit (Merck, Darmstadt, Germany) according to the manufacturer's instruction. The samples were then used to perform two-dimensional gel electrophoresis (2DE).

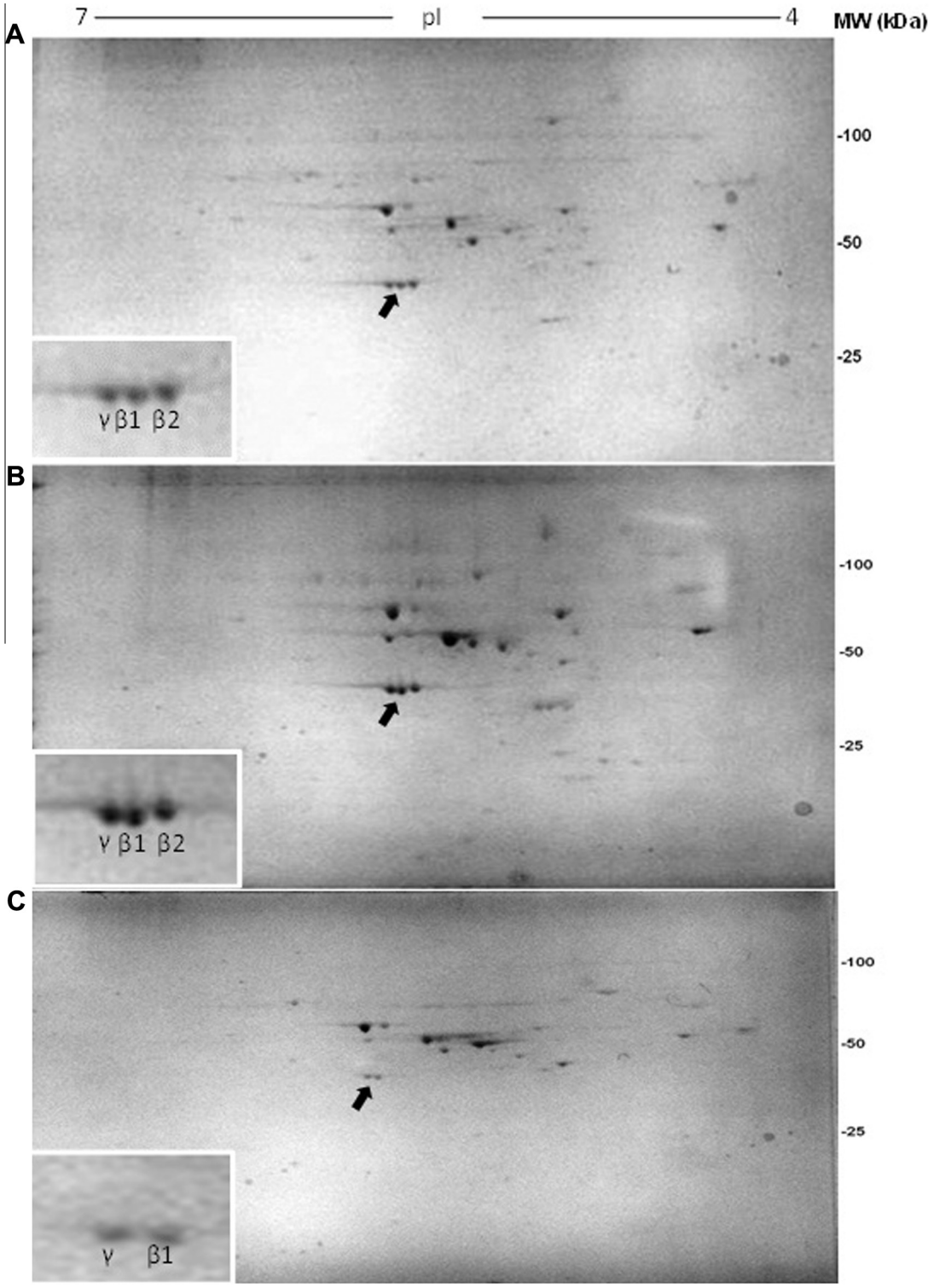

Fig. 1. Proteome of EV71 infection-resistant and susceptible RD cells. (A) Proteome of mock infected RD cells. (B) Proteome of EV71 infected RD cells harvested at 2hpi. (C) Proteome of EV71 infection resistant RD cells harvested at 2hpi. γ denotes γ-actin, β1 and β2 denotes β-actin variants .

Protein concentration were first quantitated using Nanodrop 100 spectrophotometer (ThermoScientific, Waltham, USA) and then diluted to 100 mg/ml with rehydration buffer (Bio-Rad Laboratories, CA, USA) before applying it to the pH 4–7 ReadyStrip IPG Strips (11 cm, Bio-Rad Laboratories, CA, USA). After a 12 h passive rehydration process, isoelectric focusing (IEF) was performed using the Protean IEF cell (Bio-Rad Laboratories, CA, USA) at the following conditions; 250 V for 20 min with linear ramp, 8000 V for 2.5 h with linear ramp and finally 8000 V for 25,000 v-hours with rapid ramping.

After IEF, the IPG strips were equilibrated with equilibration buffers (Bio-Rad Laboratories, CA, USA) before applying it to the second-dimensional SDS–PAGE at 200 V for 65 min using 12.5% Tris–HCl Criterion gel (Bio-Rad Laboratories, CA, USA). After gel electrophoresis, the gels were stained with InstantBlue ultrafast protein stain (Expedeon™, Cambridge, UK) for 2 h before imaging them using VersaDoc™ Imaging System (4000MP, Bio-Rad Laboratories, CA, USA). Protein spots of interest were excised and washed twice with water as previously described by [18]. The gels were first destained in 100 μl of 50 mM ammonium bicarbonate/50% (v/v) acetonitrile for 5 min. The destaining stage was then repeated twice followed by an addition of 50 μl acetonitrile. Reduction was then performed by covering the gel in freshly prepared 100 mM ammonium bicarbonate containing 10 mM DTT after drying down using a vacuum centrifuge. Following incubation at 56 °C for 1 h, alkylation was performed using 55 mM iodoacetamide (Sigma Aldrich, St. Louis, USA) in 100 mM ammonium bicarbonate. The gel was subsequently dehydrated with acetonitrile and dried with a vacuum centrifuge. Digestion was performed with the use of 12.5 ng/μl of sequencing grade modified trypsin (Promega, Wisconsin, USA) in 50 mM ammonium bicarbonate and incubated for 30 min at 4 °C. The peptides were extracted three times using 25 μl of 5% formic acid in 50% aqueous acetonitrile. The extract were incubated for 10 min and concentrated by centrifugation.

The digested peptides were mixed with freshly prepared matrix solution (10 mg of CHCA in 1 ml of 0.5% TFA and 50% acetonitrile) in a 1:1 (v/v) ratio. The peptides were then analysed using the ABI 4700 Proteomics Analyzer with TOF/TOF™ optics (Applied Biosystems, CA, USA). Peptide tolerance was set at 100 ppm with fixed modification of cysteine carbamidomethyl, variable modification of methionine oxidated and permitted missed cleavage of up to 1. Trypsin cleavage of the protein is at the C-terminal side of KR unless next residue is P. The proteins were identified by searching in the National Center for Biotechnology Information nonredundant (NCBInr) database using MASCOT program (http://www.matrixscience.com). The experiment was performed in triplicates to ensure reproducibility.

3. Results and discussion

Comparison of the proteomes of EV71-susceptible and EV71-resistant RD cell lines showed a significant difference (Fig. 1). In particular, two variants of β-actin, labelled as β1 (BAD96752.1, BLAST score 785, identity 100%) and β2 (BAD96645.1, BLAST score 721, identity 93%), were found in the EV71-susceptible RD cells, compared to EV71-resistant RD cells that contains only one variant β1. The variant form of actin, β1 and β2 differ from each other by a domain comprising of ATP, gelsolin and profiling binding sites that spans 24 amino acids. In addition, the intensity of γ-actin (NP_001605.1, BLAST score 750, identity 100%) and β-actin variant β1 were also shown to be lower in the EV71-resistant RD cell.

Although the specific function of the different β-actin variants in response to EV71 infection awaits discovery, it is tempting to propose that EV71 infection is dependent on a specific variant of β-actin. For example, viruses such as adenovirus and HIV were reported to enter host in an actin-dependent manner [19]. Furthermore, an actin-binding protein, annexin 2, was previously reported to bind to the VP1 capsid protein of EV71 and is necessary for the propagation of infection [20].

Annexin 2 binds to cytoplasmic F-actin and is responsible for organization of cytoplasmic actins and intracellular vesicular transport [18,21,22]. Therefore, we proposed that β2 is one of the actin isoforms that annexin 2 binds to and possibly harness for vesicular transport in normal cell process. In the EV71-resistant cells, the loss of β2 variant result in the failure of the virus to be transported into the cytoplasm after binding to annexin 2. The ability of EV71-resistant cells to survive and retain its morphology might also be due to the presence of other cytoplasmic actins such as γ-actin and β-actin variants. This finding further support our previous report in which actin play a role in pathogenesis and the establishment of the disease in an *in vitro* system [18].

In addition, Hussain and colleagues (2011) also demonstrated that the cytoskeletal system comprising of both actin and microtubules were involved endocytic kinetics [23–26]. The knockdown of genes such as ARPC5, ARRB1, and WASF1 that were important in actin polymerisation and the use of actin disrupting drug such as cytochalasin B, have resulted in the decrease in EV71 replication kinetics [19–27].

In conclusion, we demonstrated that RD cells resistant to EV71 infection lack a variant of β-actin as compared to EV71-susceptible RD cells. EV71 infection may be actin dependent and might involve an actin-binding protein, annexin 2. The further characterisation of the identified protein in this study may pave way for the elucidation of the mechanism of pathogenesis of EV71 in HFMD and uncover antiviral therapeutics targets for the treatment of HFMD patients.

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