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Histopathological features and distribution of EV71 antigens and SCARB2 in human fatal cases and a mouse model of enterovirus 71 infection

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ABSTRACT

Enterovirus 71 (EV71) is a neurotropic pathogen that causes hand, foot, and mouth disease. While infection is usually self-limiting, a minority of patients infected with EV71 develop severe neurological complications. In humans, EV71 has been reported to utilize the scavenger receptor class B, member 2 (SCARB2) as a receptor for infectious cellular entry. In this study, we define the pathological features of EV71-associated disease as well as the distribution of EV71 antigen and SCARB2 in human fatal cases and a mouse model. Histopathologically, human fatal cases showed severe central nervous system (CNS) changes, mainly in the brainstems, spinal cords, and thalamus. These patient further exhibited pulmonary edema and necrotic enteritis. Immunohistochemical analysis of human fatal cases demonstrated that EV71 antigen and SCARB2 were observed mainly in neurons, microglia cells and inflammatory cells in the CNS, and epithelial cells in the intestines. However, skeletal muscle tissue was negative for EV71 antigen. In a mouse model of EV71 infection, we observed massive necrotic myositis, different degrees of viral diseases in CNS, and extensive interstitial pneumonia. In mice, EV71 exhibits strong myotropism compared to the neurotropism seen in humans. EV71 antigen was detected in the spinal cord and brainstem of mice. However, there was no clear correlation between mouse SCARB2 and EV71 antigen distribution in the mouse model, consistent with previous results that SCARB2 functions as a receptor for EV71 in humans but not mice. The EV71-induced lesions seen in the mouse model resembled the pathological changes seen in human samples. These results increase our understanding of EV71 pathogenesis and will inform further work developing a mouse model for EV71 infection.

1. Introduction

Enterovirus 71 (EV71) is a member of the *Picornaviridae* family and is the major etiological agent of hand, foot, and mouth disease (HFMD). HFMD is a common self-limiting childhood disease and is characterized by rapidly ulcerating vesicles in the mouth, and vesicular lesions on the hand and foot. In children under the age of five however, EV71 infection can result in severe neurological disease. Clinical symptoms of EV71 infection in young children include aseptic meningitis, brainstem encephalitis, pulmonary edema, acute flaccid paralysis, and death (Brown et al., 1999; McMinn, 2002; Tee et al., 2010).

EV71 was first isolated from a child with encephalitis in California in 1969 (Schmidt et al., 1974). Since that time, outbreaks,

Abbreviations: EV71, enterovirus 71; HFMD, hand, foot, and mouth disease; SCARB2, scavenger receptor class B, member 2; CNS, central nervous system; i.p, intraperitoneal; RD, rhabdomyosarcoma.

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epidemics, and pandemics of EV71 have been reported (Alexander et al., 1994; Melnick et al., 1980; Nagy et al., 1982). In the Asia-Pacific region, frequent outbreaks of EV71 have been reported, and these outbreaks are associated with a high incidence of neurovirulence and fatalities (Cardosa et al., 2003; Chan et al., 2003; Komatsu et al., 1999). In Mainland China, EV71 was first isolated from a patient suffering from HFMD without neurological symptoms in 1987. Large-scale outbreaks of HFMD have subsequently been reported (Mao et al., 2010; Yang et al., 2009a; Zhang et al., 2009, 2010; Zheng et al., 1995). From March 2008 to June 2009, more than 600,000 HFMD cases and 126 deaths were reported in Fuyang City of Anhui province. Of these deaths, 23 were confirmed as positive for EV71 infection (Zhang et al., 2010). Clearly, HFMD outbreaks caused by EV71 or related enteroviruses are a major global public health issue. Increased surveillance, especially in the Asia-Pacific region will be needed to combat these diseases (Solomon et al., 2010; Wong et al., 2010; Yip et al., 2013b). EV71 epidemics in Hong Kong in recent years were attributed to the novel double-recombinant strains of subgenotype C4, which the authors propose designating as genotype D (Yip et al., 2013a,b). Furthermore, it is likely that an increased understanding of EV71 pathology will greatly inform the generation of new public health measures aimed at controlling EV71 outbreaks.

Fatalities associated with EV71 infection are the result of neurogenic inflammation in the spinal cord and brain regions leading to pulmonary edema or hemorrhage (Huang et al., 1999; Lum et al., 1998; Shieh et al., 2001; Yang et al., 2009b). In patients with EV71-associated neurogenic pulmonary edema or cardiopulmonary, recovery from infection is often associated with sequelae including limb weakness and atrophy (Chang et al., 2007).

Due to the difficulty of studying EV71 pathogenesis in humans, development of a suitable animal model would greatly assist in the generation of therapeutic interventions and establishing safe and effective vaccines against EV71. Previous studies have reported successful EV71 infection in neonatal mice (Khong et al., 2012; Wang et al., 2004; Yu et al., 2000), monkeys (Hashimoto and Hagiwara, 1982) and gerbils (Yao et al., 2012) following intraperitoneal (i.p.) and oral routes of inoculation. Infection of neonatal mice with mouse-adapted EV71 strains resulted in early and transient viral replication in the intestines, followed by replication in the spinal cord, brain and muscle at later time points. This later replication resulted in massive necrosis in limb muscles, neuronal loss and apoptosis in the spinal cord and brainstems (Wang et al., 2004). Infection of mice with a non-mouse-adapted EV71 strain also exhibits neurotropism, resulting in massive lesions in limb muscles, brainstems, and anterior horn areas (Khong et al., 2012).

In humans, EV71 utilizes the scavenger receptor class B, member 2 (SCARB2) as a receptor for productive infection (Yamayoshi and Koike, 2011; Yamayoshi et al., 2009). SCARB2 is ubiquitously expressed in numerous tissues and participates in membrane transport, reorganization of endosomal/lysosomal compartments (Kuronita et al., 2002), and facilitates high density lipoprotein endocytosis (Eckhardt et al., 2006). In vitro experiments have demonstrated that expression of SCARB2 in mouse L929 cells confers susceptibility to EV71 infection and infection efficiencies are similar to that of RD (rhabdomyosarcoma) cells, a cell type that is highly susceptible to EV71 infection (Yamayoshi and Koike, 2011). However, there are likely important differences in the receptors used by EV71 to infect human and mice cells. For example, EV71 infection in suckling mice results in myotropism as well as neurotropism, and this increased tropism is not seen in humans. A specific receptor for EV71 in mice has yet to be identified.

Although several previous autopsy studies have examined HFMD-associated EV71 (Jiang et al., 2012; Yang et al., 2009b), most studies have examined the histopathology of EV71 infection in mouse models (Chen et al., 2004; Wang et al., 2011). However, comparative pathology of EV71 infection in patients with HFMD or mice is not well characterized. In addition, the distribution of SCARB2 in human and mice is not well understood. The lack of comparative pathological reports characterizing the association of EV71 antigen and SCARB2, as well as histopathological changes in EV71 human fatal cases and mouse models severely limits our understanding of EV71 pathology. Here, we comprehensively describe the histopathological features and distribution of EV71 antigen and SCARB2 in fatal human cases and a mouse model of EV71 infection. We characterized the histopathology of EV71 infection in human tissues and our mouse model and characterized EV71 antigen distribution and its relationship with SCARB2. This allowed us to draw important conclusions regarding the suitability and efficacy of mouse models to study EV71 infection. Our findings may increase our understanding of EV71 pathology in both human fatal cases and mouse models, thereby improving our ability to develop effective medications and prophylactic treatments.

2. Materials and methods

2.1. Human tissue samples

Formalin-fixed, paraffin-embedded (FFPE) tissues were obtained from 10 patients who suffered fatalities and were confirmed to be positive for EV71 infection. Samples from seven patients were obtained from the Department of Pathology, Peking University Health Science Center, and the three samples were obtained from the Department of Pathology, Shandong Provincial Hospital. Mean age of the 10 cases was 24.6 months, ranging 13–36 months, and gender ratio (male/female) was 6/4. All experimental protocols involving human samples were approved by the Human Ethical Commission at Peking University Health Science Center and Shandong Provincial Hospital.

2.2. Cells and viruses

RD cells were maintained in complete media (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum plus 2 mM L-glutamine, 100 IU of penicillin, and 100 μg of streptomycin per ml) and incubated at 37 °C and 5% CO_2. A non-mouse-adapted EV71 strain, EV71/Queenmary/HongKong/2012 (GenBank accession number KF444809) was grown in RD cells, as previously described (Jia et al., 2010). This virus was originally isolated from a fatal human case with encephalitis during an HFMD outbreak in Hong Kong. To prepare virus stocks, EV71 was propagated in RD cells as reported previously (Lin et al., 2009). Working virus stocks contained $10^{5.0}$ $TCID_{50}$ per ml.

2.3. Reverse transcription polymerase chain reaction (PCR)

Viral RNA of EV71/Queenmary/HongKong/2012 was extracted from cell supernatant using an RNeasy Mini Kit (Qiagen, Netherlands). RNA was reverse transcribed into cDNA using the Superscript III First Strand Synthesis Kit (Invitrogen, USA) according to the manufacturer's instructions. The genomic sequences of multiple EV71 strains were aligned to identify conserved regions and nine pairs of overlapping primers were designed to amplify the viral genome (Table S1). PCR amplifications were performed using KOD-plus-DNA Polymerase (Toyobo, Japan), and were carried out at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 45 s, and 68 °C for 1 min, then extended at 68 °C for 10 min. The reactions were analyzed by electrophoresis on 1.0% agarose gels.

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