MICROBIOLOGY

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Etiology, pathogenesis, antivirals and vaccines of hand, foot, and mouth disease

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ABSTRACT

Hand, foot, and mouth disease (HFMD), caused by enteroviruses, is a syndrome characterized by fever with vesicular eruptions mainly on the skin of the hands, feet, and oral cavity. HFMD primarily affects infants and young children. Although infection is usually self-limited, severe neurological complications in the central nervous system can present in some cases, which can lead to death. Widespread infection of HFMD across the Asia-Pacific region over the past two decades has made HFMD a major public health challenge, ranking first among the category C notifiable communicable diseases in China every year since 2008. This review summarizes our understanding of HFMD, focusing on the etiology and pathogenesis of the disease, as well as on progress toward antivirals and vaccines. The review also discusses the implications of these studies as they relate to the control and prevention of the disease.

Keywords: hand, foot and mouth disease, enterovirus, etiology, tropisms, virus–host interaction, pathogenesis, innate immunity, antivirals, vaccine

INTRODUCTION

Hand, foot, and mouth disease (HFMD) primarily affects infants and children younger than 5 years old and displays a wide range of clinical manifestations [1]. The disease is characterized by fever along with vesicular eruptions mainly on the skin of hands, feet, and the oral cavity. HFMD is generally self-limited. However, severe neurological manifestations can present in some cases, ranging from aseptic meningitis to acute flaccid paralysis and brainstem encephalitis (BE), which can be permanently disabled or fatal [2]. The BE caused by HFMD can lead to severe pulmonary edema and shock, which can induce failure of respiratory and circulatory systems [3].

HFMD was first identified in New Zealand and Canada in 1957 [4]. The disease was designated as ‘hand, foot, and mouth disease’ after a similar outbreak occurred in USA in 1959 [5]. HFMD reappeared in New Zealand, the UK, and in the USA in the 1960s [5,6]. Outbreaks of HFMD also occurred in Bulgaria in 1975 and in Hungary in 1978, and 44 and 47 deaths were recorded, respectively [7,8]. HFMD emerged in the 1970s in Japan [9], from which point on numerous outbreaks occurred in the Asia-Pacific region [2,10–16]. After the 1990s, large outbreaks that involved more than 10,000 cases all occurred in this region [2,11,16,17] (Fig. 1). The reasons underlying these outbreaks are still not fully understood.

In 2008 and 2009, large outbreaks of HFMD emerged in mainland China [2]. These outbreaks made HFMD a great public health concern in China, leading to its classification as a category C communicable disease in the Chinese National communicable disease surveillance system in 2008 [18]. From 2008 to 2014, more than 1 million HFMD cases have been reported in China each year, according to data obtained from the Chinese National Notifiable Disease Reporting System.

Great progress has been made in HFMD research over the past two decades, which has helped to pave the way toward prevention and control of HFMD. Here, we summarize the understanding of HFMD etiology and review research progress on the pathogenesis, antivirals, and vaccines of enterovirus 71 (EV71), the main causative agent of severe HFMD.
We also discuss the implications of these studies as they relate to control and prevention of the disease.

ETIOLOGY OF HFMD

HFMD is caused by EVs, which belong to the *Enterovirus* genus of the family *Picornaviridae*. The EV genus contains many well-known viruses, including polioviruses (PV), Coxsackie viruses (CV-A and -B), and ECHO viruses (E). The EV genome is a single positive-stranded RNA molecule, which encodes a 5′-untranslated region (5′-UTR), a polyprotein, and a 3′ UTR. The polyprotein consists of three regions: P1, P2, and P3, which are in turn cleaved into four viral capsid proteins (VP1–VP4) and seven non-structural proteins involved in protein processing and genome replication (2A–2C, 3A–3D) by EV’s proteases 2A and 3C [19] (Fig. 2). The viral capsid is an icosahedron, composed of VP1–VP4. VP1, VP2, and VP3 form a deep canyon that serves as the receptor-binding site at the surface of the capsid. Proteins 2A and 3C are not only essential for EV replication, but also play important roles in virus–host interactions. The protein 3D is an RNA-dependent RNA polymerase (RdRp), which lacks proofreading activity, leading to frequent mutations during EV replication. Based on the phylogenetics of VP1, the major antigenic protein of EVs, the *Enterovirus* genus is proposed to be divided into seven species, including EV-A to D and rhinovirus A–C by the International Committee on Taxonomy of Viruses [19].

Improvement of virus detection methodology and disease surveillance has led to a better understanding of the etiology of HFMD. At least 23 EV serotypes, which belong to two different EV species, have been reported to cause HFMD over the past 50 years (Table 1). Among them, EV71 and CV-A16 are the most prevalent. CV-A16, isolated in 1958, was the first identified HFMD pathogen [4], followed by EV71, which was isolated in the USA in 1969 [20]. Since then, EV71 outbreaks have occurred periodically throughout the world, including in Japan [9], Bulgaria [7], Hungary [8], Australia [21], Malaysia [22], UK [23], and Vietnam [24]. CV-A16 outbreaks also occurred in Australia, England, Singapore, and the mainland China [25]. CV-A16 infection and EV71 infection emerged alternately and these vectors have become the most relevant pathogens of HFMD worldwide to date [2,11,13,14]. In mainland China, HFMD was first reported in Shanghai in 1981 [18]. Outbreaks of CV-A16 infection were identified in Tianjin in 1983 and 1986. EV71 was first isolated in Wuhan in 1995 [18]. The outbreaks of EV71 infection occurred in Linyi, Shandong Province in 2007 and Fuyang,
Anhui Province in 2008 [26,27], initiating the EV71 pandemic in mainland China that has persisted ever since.

The fact that EV71 has been associated with a wide spectrum of acute central nervous system (CNS) syndromes, including aseptic meningitis, poliomyelitis-like paralysis, BE, and acute neurogenic pulmonary edema [28], makes it the predominant serotype in severe (80%) or fatal (93%) laboratory-confirmed cases [2,11]. CV-A16 and most other EV serotypes more intend to cause mild HFMD cases [2,17,29].

Recombination is the major force that drives EV variation [30]. CV-A16 and EV71 often circulate together and their coinfection increases the chance of intertypic recombination [31,32]. A recombinant may be responsible for the large HFMD outbreaks in mainland China [33]. Recombination also contributes to the variation of other EV serotypes [34,35].

Due to using RdRp, the variation rates of EVs are very high. One serotype can be classified into several subtypes based on the phylogeny of VP1. CV-A16 are grouped into subtypes A and B, with the latter further divided into B1a–B1c and B2a–B2c [32]. Although there is a difference in the definition of subgenogroup B1 and B2, the strains that have circulated in China belong to genogroup B [36,37]. Likewise, EV71 is classified into subtypes A, B, C, and D. Subtype A contains only the prototype strain BrCr. Subtypes B and C are each composed of distinct subgenogroups (B1–B5 and C1–C5, respectively) [38]. Subtype D is represented by a single strain which has been isolated from India [39]. The circulating strains of EV71 appear to vary with geographical location and time. For example, in Taiwan China, subgenogroups C2 and B4 were responsible for the 1998 epidemic, while B4 was for the 2002 epidemic, C4 for the 2004–05 epidemic, B5 for the 2008–09 epidemic, C4 for the 2010 epidemic, and B5 for the 2011–12 epidemic [40]. In mainland China in 1996, subgenogroup C2 appeared, followed by C3 in 1997. Since 1998, C4 has been the dominant subgenogroup [28,41,42]. The C4 subtypes underwent three major epidemiologic phases: C4b in 1998–2008, C4a-1 in 2003–09, and C4a-2 in 2007–11 [28]. The constant changing of subgenogroups makes it challenging to design universal vaccines that cover several subgenogroups.

In recent years, the switch of HFMD etiology has been suggested by the increased epidemics of serotypes other than EV71 and CV-A16, including CV-A6, CV-A10, and CV-A12 [43–48]. Disease caused by a new strain of CV-A6 has been found worldwide [49–60] (Fig. 1), causing severe HFMD in children [61] and atypical HFMD in adults [62–64]. In some areas in China, CV-A6 has replaced CV-A16 as a predominant causative agent [59,65]. The increasing trend of CV-A6 spread warrants enhanced precise etiology surveillance of HFMD.

Together, diverse EV serotypes can cause HFMD, and EV71 and CV-A16 are the most common pathogens. EV71 is the major agent causing severe cases. The switch of HFMD etiology requires a precise EV typing in the surveillance for a better HFMD control.
PATHOGENESIS OF EV71

Pathological features

Most pathological research on EVs has been focused on EV71. Studies on EV71 in HFMD patients mainly cause neurological effects by inducing inflammation in the CNS, but not in other organs. The pathological features of EV71-induced viral encephalitis in fatal cases include neuronophagia, perivascular cuffing, focal edema, and neutrophil and macrophage infiltration [66–69]. The inflammation distributes mainly to the hypothalamus, brain stem, spinal cord, and cerebellar dentate nucleus along the motor nerve pathway, indicating that the virus spreads into the CNS through the retrograde peripheral motor nerve [66,67]. Autopsy examinations in EV71 fatal cases revealed brainstem encephalomyelitis, extensive pulmonary edema, and pulmonary hemorrhages [69–71]. However, viral antigens were only detected in the brainstem and in the spinal cord but not in the lung tissue, suggesting that EV71-induced pulmonary edema is neurogenic [69,70].

Although limited, clinical observations provide clues that some inflammatory mediators, including cytokines and chemokines, play an important role in the pathogenesis of EV71-induced BE and other complications. These mediators include interleukin 6 (IL-6), IL-10, IL-13, tumor necrosis factor α (TNF-α), IL-1β, IL-8, and monocyte chemotactic protein 1 [72–75]. Of these factors, elevated IL-6 might be a prognostic parameter for clinical severity [73,74]. Significantly higher levels of IL-10 and IL-8 in the cerebrospinal fluid of the patients with encephalitis and pulmonary edema than in uninfected controls suggests that the increased inflammatory cytokines in the serum of EV71 infected patients may originate in the cerebrospinal fluid [72]. Although there are some studies providing evidence that the vascular cell adhesion molecule 1 and cyclooxygenases-2-induced activation of nuclear factor κB (NF-κB) might represent the pathway for mediating the production of inflammatory cytokines and chemokines, the detailed mechanism of the inflammatory pathogenesis induced by EV71 infection remains unclear [76,77].

As an alternative to clinical and autopsy resources, experimental murine and non-human primate models of EV71 infection have been developed to explore the pathogenesis of EV71 infection. These resources include a monkey model, neonate and adult immunodeficient mice-adapted EV71 models, and an EV71 receptor transgenic mice model [78–80]. However, some limitations exist with these models, as none accurately recapitulates all of the aspects of HFMD pathology in humans. For example, in monkey models, unlike in humans, the pathogenic process, including pulmonary edema and neuron impairment, is dependent upon infection route, only presenting after intracerebral inoculation with EV71 rather than other infection pathways [79,81]. Furthermore, in mice, adaption increases the virulence of EV71 and does not reflect the natural cell and tissue tropism [82]. Pulmonary edema has never been observed in the immunodeficient mouse model infected by the adapted EV71 strains [83–85]. The human scavenger receptor class B, member 2 (SCARB2) transgenic mice display clinical features most similar to humans, exhibiting ataxia, paralysis, and death after infection. These mice are susceptible to EV71 clinical isolates at different ages and with different inoculation routes. However, pulmonary edema symptoms are absent in this model, which limits its application in EV71 pathogenesis exploration [80]. Despite these shortcomings, our knowledge has been greatly potentiated from studies in these animal models.

Receptors and viral factors modulate cell and tissue tropism

Virus–receptor interaction is the first step of a virus life cycle, determining cell and tissue tropism and pathogenicity. Two human receptors for EV71 and CV-A16 were identified in 2009: SCARB2 [86] and P-selection glycoprotein ligand-1 (PSGL-1) [87]. In addition, annexin II and sialylated glycans have also been reported as candidate coreceptors for EV71 infection [88]. SCARB2 is localized in the lysosomal membrane and is ubiquitously expressed in many human tissues and cell types, including in neurons in the CNS. It participates in membrane transportation and the reorganization of endosomal/lysosomal compartments, and shuttles between these compartments and the plasma membrane [86,89,90]. PSGL-1 is expressed exclusively on myeloid and T lymphocytes and plays a role in the early stages of inflammation [87,89,90].

In infected organs of a viral disease, cell-to-cell spread substantially contributes to disease pathogenesis. It has been reported that different cell types use SCARB2 and PSGL-1 to mediate EV71 entry through clathrin- and caveolar-dependent endocytosis, respectively [91,92]. The differential expression of EV71 receptors on different cell types and tissues are considered the primary determinants of tissue tropism [89]. For example, EV71 patients presenting with brain stem encephalitis, autonomic nervous system dysregulation, and pulmonary edema have a high level
of proinflammatory cytokines in serum and cerebrospinal fluid. Therefore, it is speculated that the interaction of EV71 with PSGL-1 on lymphocytes may spread the virus and produce the inflammatory cytokines by trafficking to the CNS to promote brain stem encephalitis and pulmonary edema development. In contrast, it was suggested that EV71 uses the active retrograde axonal transport system to enter the CNS in an orally infected EV71 murine model [93]. Together, these findings suggest that a single receptor interacting with EV71 is not sufficient to elicit EV71 brain stem encephalitis; rather, different receptors may play different roles on different cell types and tissues at distinct stages of EV71 infection. However, several lines of evidence suggest that SCARB2 plays a dominant role in efficient EV71 infection and the development of systemic disease in humans, while other candidates may act as coreceptors or function to help invading the target cells in vivo. First, stable expression of human SCARB2 permitted replication of all tested EV71 and CV-A16 strains in non-susceptible mouse cells, while human PSGL-1 only enables some representative EV71 strains [86,87]. Second, SCARB2 is capable of viral binding, viral internalization, and viral uncoating, while PSGL-1 showed inability to induce viral uncoating, resulting in low infection efficiency in cells expressing PSGL-1 [94]. Last, SCARB2 transgenic mice are susceptible to infection by both EV71 clinical isolates and CV-A16, displaying EV71 neurotropism, neuropathology, and clinical features. PSGL-1 transgenic mice, on the other hand, can only be infected by a mouse muscle-adapted EV71 strain but not the clinical isolates [80, 84, 95, 96].

In addition to receptors of EV71, studies on different murine models have indicated that viral factors, such as viral 5′ and 3′ UTR, genetic modification on the VP1 region, and 3D polymerase, contribute to cell and tissue tropism as well as viral pathogenesis [89,97–100]. However, the detailed mechanism of how these viral factors contribute to the cell or tissue tropism remains unclear.

Evasion of host innate immune responses

During the virus life cycle, EVs use multiple factors from both viral and host for their own benefit. In response, the host develops corresponding immune responses to prevent a viral infection. Innate immunity is the first line to defense against invading pathogens, including type I interferon (IFN) production at the early stages and subsequent activation of downstream events. The type I IFNs promoter is activated by pathogen-associated molecular patterns present on the virus, which are recognized by pathogen recognition receptors (PRRs) [101]. PRRs involved in the recognition of EV71 and in mediated type I IFNs production include melanoma differentiation-associated gene 5 (MDA5), retinoic acid-inducible gene 1 (RIG-I), Toll-like receptor 3 (TLR3), and TLR7 [102,103].

Despite these robust immune responses, several lines of evidence suggest that EV71 has evolved evasion strategies to antagonize the IFN-mediated innate immune responses (Fig. 3). For example, during EV71 infection, type I IFNs are undetectable in cell-based systems and in animal model, while IFNα/β treatment increases the survival rate of mice, and neutralizing antibody to IFNα/β exacerbates EV71-induced disease [104,105]. Additionally, EV71 almost does not stimulate the expression of antiviral genes, such as IFN-stimulated genes 54 (ISG54) and ISG56 [105].

The main IFN antagonists encoded by EV71 have been identified as two viral proteases, 2A and 3C. They directly target diverse key cytosolic molecules of the type I IFN signaling pathways to block host immune responses. EV71 2A protease directly targets MDA5 and induces its cleavage, which is a common event in the infection of EV species [106]. EV71 2A protein, but not 3C, can also target the mitochondria anti-viral signaling protein (MAVS, also named as IPS-1, VISA, Cardif) and cleave it at multiple sites [107]. The resulting EV71 2A-cleaved fragments of MAVS cannot activate type I IFNs production [108]. Although the 3C of CV-B3 was reported to cleave overexpressed MAVS, cleavage by EV71 3C has not been observed [106,107]. In an alternative pathway, EV71 3C protease can inhibit RIG-I-mediated type I IFN responses by impeding the formation of a functional complex between RIG-I and MAVS [105].

A recent study showed that TLR3 signaling activation in macrophages is important for protecting older mice against EV71 infection [103]. Accordingly, our results indicated that EV71 suppresses TLR3-mediated type I IFN responses. In this process, the protease 3C interacts with TIR domain-containing adaptor inducing IFN-β (TRIF), an important adaptor protein of TLR3, and cleaves it upon the proteolytic activity of 3C [109]. As a strong antagonist of IFN, 3C also directly cleaves interferon regulatory factor 7 (IRF7), the downstream molecule of RLRs and TLR3 signaling pathways [110]. Importantly, the cleavage fragments do not limit the replication of EV71, which is consistent with their ability to inhibit IFN production. Coincident with these observations, EV71 3C blocks type I IFN synthesis in a mouse model [111]. These studies suggest that 3C, as an antagonist, may play
critical roles in evading innate immune responses under physiologic conditions.

It is thought that NF-κB signaling is also important for activating IFNs or inflammatory cytokine production in TLR and RLR signaling pathways. We have demonstrated that EV71 3C also inhibits the activation of NF-κB by cleaving the transforming growth factor-β-activated kinase 1 (TAK1) complex [112]. Furthermore, another EV71 non-structural protein, the 2C helicase, has been also demonstrated to inhibit TNF-α-mediated NF-κB activation by suppressing phosphorylation of IkB kinase β (IKKβ) during EV71 infection [113].

IFNs induce the expression of ISGs to inhibit virus replication in an autocrine and paracrine manner [114]. Accordingly, EV71 hinders the IFN-mediated activation of ISGs via interfering with the Janus activated kinase (Jak)-signal transducers and activators of transcription (STAT) signaling pathways. EV71 infection can inhibit the IFN-mediated phosphorylation of STAT1, STAT2, Jak1, and tyrosine kinase 2 through inducing cleavage of type I IFN receptor 1 (IFNAR1) by 2A protease [115]. Besides IFNAR1, EV71 3C protease can cleave IRF9 to block JAK1-STAT signaling [116].

Host factors modulate EV71 replication
EV71 genome contains a type I internal ribosome entry site (IRES) located at the 5′UTR, which requires a number of host IRES-specific transacting factors (ITAFs) to initiate viral protein translation.
It has been reported that four ITAFs, heterogeneous nuclear ribonucleoprotein K (hnRNPK), hnRNPA1, far-upstream element-binding protein 1 (FBP1), and FBP2, interact with the 5′ UTR of EV71 to mediate virus replication [117–120]. hnRNPA1 and FBP1 play positive roles in activating EV71 IRES, whereas FBP2 is a negative regulator. hnRNPK stimulates EV71 replication by interacting with the cloverleaf structure, stem-loop II of the 5′ UTR, or with the stem-loop IV of the IRES [117]. Recently, it has been reported that a serine/threonine kinase, Misschapel NIK-related kinase, is involved in IRES-mediated translation of EV71 by facilitating hnRNPA1 translocation to the cytoplasm [121]. Furthermore, EV71 impairs the processing of host pre-mRNA by cleaving the cleavage stimulation factor 64K subunit (CstF64), which is advantageous for virus replication [122].

Upon RNA virus infection, intracellular host membranes are remodeled to generate a viral RNA replication center. Many host factors, including GTPase ADP-ribosylation factor 1 (ARF1), Golgi-specific Brefeldin A resistance factor 1 (GBF1), acyl-coenzyme A-binding protein domain 3 (ACBD3), and phosphatidylinositol 4-kinase IIIβ (PI4KB), participate in this process to influence virus replication [123–126]. PI4KB can be recruited to the Golgi membranes by ARF1 or ACBD3. Interestingly, T-00127-HEV1, a synthetic inhibitor of PI4KB, can be used to inhibit EV71 replication [124]. This suggests that PI4KB may play pivotal roles in EV71 replication. Further investigation is needed to elucidate the detailed molecular mechanisms of this pathway. Additionally, the endoplasmic reticulum protein reticulon 3 is another important host factor that could affect the EV71-encoded viral proteins synthesis and viral RNA replication by interacting with the 2C protein [127].

The interaction network between EV71 and host cells has been dissected by transcriptomic or proteomic approaches, which are helpful for elucidating the pathogenesis of EV71. These results show that EV71 infection induced changes in many host processes, including in protein translation and modification, protein and ion transport, cell death, autophagy, and cell homeostasis [128–130]. These studies open the door for further highlighting the EV71 and host factor interaction and for examination of how host factors affect the EV71 life cycle.

Interactions between EV71 and cellular miRNAs have also been reported. EV71 infection can induce alteration of miRNA expression and form a unique miRNA profile [131,132]. Conversely, miRNAs can regulate EV71 replication by targeting EV71 genome or proteins [133,134].

miRNAs are also the targets for EV71 to escape innate immune responses [135,136]. For example, EV71-induced miR-146a, a negative-feedback regulator in RLRs signaling, could inhibit IL-1R-associated kinase and TNF receptor-associated factor 6 activation, and further block IFNs production.

In summary, the mechanisms responsible for HFMD pathogenesis have not been fully understood. Studies on EV71 showed that many factors, including receptor binding, viral factors, innate immune evasion, and host factors are involved. A better understanding of the virus–host interactions as well as breakthrough in animal models is needed to provide insights into the mechanisms underlying HFMD pathogenesis.

**STRUCTURE-BASED DESIGN OF ANTIVIRAIALS**

Given the devastating neurological effects that HFMD can have in young children, there is a pressing need to develop anti-EV agents to combat HFMD. Although there are currently no available antivirals to treat HFMD, virological studies have provided critical insights into antiviral development. The available targets for anti-EV compound design can be categorized according to the target type, ranging from the virus capsid structural proteins, the viral encoded non-structural proteins, and the UTR of genomic viral RNA to host proteins implicated in virus infection. Each target, in turn, corresponds to critical steps in virus life cycle, including virus attachment/entry/uncoating, virus protein synthesis and maturation, RNA genome replication, immune evasion, and virus assembly/morphogenesis (Fig. 4).

High-resolution structural information is one of the essential resources for antiviral drug design, optimization, and validation. The structures of viral proteins encoded by EV71 have been studied extensively. Therefore, developing inhibitors against viral proteins can be an efficient pathway to develop HFMD drugs. We here summarize the progress in understanding the role of certain viral components (mainly exemplified by EV71) in antiviral design in a structural biology view.

**Viral capsid proteins**

EV capsid proteins are involved in cell attachment, entry, and uncoating processes, which are among the earliest confirmed targets for antiviral drugs. High-resolution structural analysis of the mature virions
and natural empty particles indicate that the VP1 GH loop can act as an adaptor sensor for cellular receptor attachment and a critical generic mechanism for uncoating, providing novel targets for antiviral design [137]. High-resolution structures of EV71 as empty capsid and inhibitor bound virion allowed for the identification of a large cleft at each icosahedral face of the virus particle, beneath where the hydrophobic floor serves as the docking site for drugs [138,139]. The binding of the drug at the cleft ultimately rigidifies the virus capsid (discussed in detail below), hence hindering the intrinsic conformational dynamics that are essential for the interaction with cellular receptor or subsequent uncoating. Crystal structures of EV71 complexed by four different 3-(4-pyridyl)-2-imidazolidinone derivatives revealed the structure–activity correlates. With the help of quantum mechanics-enhanced ligand docking, the inhibitors were optimized, leading to the synthesis of a compound with an order of magnitude more potent antiviral activity [140]. Rossmann and colleagues crystallized EV71 virion complexed with inhibitor WIN-51711, one of the ‘WIN’ compounds referring to Sterling Winthrop, by whom the inhibitors were developed. Rossmann and colleagues found that

Figure 4. Road map for targeting key events in EV71 life cycle. Key steps in EV71 life cycle provide invaluable insights into drug design. These include virus attachment/entry/uncoating, virus protein synthesis and maturation, RNA genome replication, immune evasion, and virus assembly/morphogenesis.
WIN 51711 replaced the natural pocket factor and occupied the hydrophobic pocket on the VP1 protein. Furthermore, Win-51711 stabilized the EV71 virion and restricted the capsid dynamics that are required for genomic RNA release [139]. The determination of EV71 capsid dynamics provides the basis for capsid-binding inhibitor design and optimization. However, an obvious drawback of a capsid-targeting agent is that most (if not all) of such inhibitors rapidly induce the generation of drug-resistant progeny virus. This is due to the ‘prone-to-error’ nature of RdRp. The fact that the EV structural proteins are in general less conserved than the non-structural proteins also suggests the low genetic barrier to the resistance of capsid-binding inhibitor.

2C helicase

2C helicase is one of the most conserved EV proteins [141], but it remains one of the least characterized to date. Collectively, EV 2C helicase is implicated to possess many key functions within just ∼330 amino acids, including virus uncoating, replication, immune evasion, and morphogenesis, covering nearly every step in the virus life cycle [113,127,142–144]. The N-terminal of EV71 2C is thought to contain an amphipathic helix that can anchor the vesicle membrane, thereby recruiting the virus replication complex. EV71 2C also contains two separate RNA-binding motifs at the N- and C-terminus, which are believed to recognize the clover-leaf structure at the untranslated region of the viral RNA genome. These potential functions allow 2C helicase to be a promising target for the design of a wide spectrum inhibitor. However, the lack of high-resolution structural information for EV71 2C leaves a large knowledge gap for understanding its function on a molecular level, and has hindered inhibitor design.

Virus proteases

Virus proteases are, in general, believed to be the most promising therapeutic targets, as evidenced by the successes of protease inhibitors in treating human hepatitis C virus (HCV) and human immunodeficiency virus infections. The fact that EVs encode two proteases (2A and 3C), which is rare for its limited genomic size, indicates their important role in virus infection. Despite their shared proteolytic activity, the functions of 2A and 3C are not at all redundant. EV71 2A carries out the first cleavage of polyprotein precursor at VP1–2A junction, whereas the subsequent cleavages are taken over by 3C or its precursor 3CD, thus completing viral protein processing. Interestingly, 2A and 3C both also interfere with cellular functions. In addition to antagonizing host innate immunity responses, as discussed above, 2A can also shut down host protein synthesis by targeting eukaryotic initiation factor 4G1 (eIF4G1) to favor IRES-dependent viral protein production [145,146]. The available crystal structures for EV71 proteases show that 2A and 3C maintain the chymotrypsin-like fold and share active site geometry for hydrolysis [147,148]. The structures of 2A and 3C are highly conserved in different EV serotypes, making them ideal targets for wide spectrum inhibitors. Indeed, the human rhinovirus (HRV) inhibitor Rupintrivir, originally designed to dock the substrate-binding pocket of HRV 3C, was found to effectively inhibit EV71 replication with IC50 at nanomolar level. The structural basis for anti-EV71 activity by Rupintrivir was revealed by the crystal structure of the EV71–3C-Rupintrivir and CV-A16–3C-Rupintrivir complexes [149,150]. The structures show that Rupintrivir binds EV71 3C similarly to its binding of HRV 3C (Fig. 5). Although the HRV and EV71 3C proteases only share ∼30% sequence identity, the residues building up the substrate-binding pocket are nearly invariant. EV71 2A crystal structures were determined as apo enzyme and an octopeptide substrate bound form [151] revealed an open, shallow and flexible substrate-binding pocket, which is consistent with the low substrate specificity of 2A. EV71 proteinases play pivotal roles in replication and immune evasion and they are one of the best studied EV proteins structurally. Thus, the current knowledge about EV proteolytic enzyme can readily facilitate rational design of novel antiviral agents. For instance, a recent study showed that peptidyl aldehyde NK-1.8k which targets 3C can effectively suppress EV71 and EV68 infections [152].

RNA-dependent RNA polymerase

RdRp is no doubt an important target for antiviral development, as it is the catalytic unit for virus RNA synthesis. The crystal structures of EV71 RdRp complexed with nucleotide, nucleotide analog, and the viral peptide (Vpg) have been resolved [153,154], providing an essential framework for structure-based inhibitor design. Structural comparison with the RdRps from foot and mouth disease virus and CV-B3 shows significant similarity in overall folding, nucleotide recognition, or Vpg binding, offering evidence that inhibitors targeting other picornavirus RdRp may be also effective against EV71.
Rupintrivir binds EV71 3C active site in a similar mode as the binding to HRV 3C. The active site of EV71 3C is shown as a solvent-accessible surface. The color of the surface is coded according to amino acid conservation among enterovirus 3Cpro. Residues in red are invariant, and increasing amino acid variation is indicated by progressive fading to white, which indicates the least conserved residues. EV71 3C bound Rupintrivir (yellow) and the Rupintrivir bound to HRV 3C (white) are modeled in the active site by superimposition of the HRV and EV71 3C structures. The interaction between Rupintrivir and 3C is primarily mediated by invariant residues. The binding pockets from S2’-S1’ and S1-S4 are indicated. The deviations of conformations of bound inhibitors conformation are most pronounced at the termini close to the S1’ and S2’ pockets, where significant differences were also observed between EV71 3C and HRV 3C.

Few inhibitors target EV RdRp. One such inhibitor is ribavirin, a guanosine analog discovered in 1972. The drug has been used in anti-HCV infection therapy for decades and shows broad spectrum against RNA viruses. Ribavirin was found to be effective in reducing the fatality of EV71 infection by targeting RdRp [155]. The efficacy of Ribavirin could be attributed to its direct inhibition of polymerase activity. The subsequent incorporation of Ribavirin into the virus genome induces mutagenesis, which eventually leads to virus extinction. DTriP-22 is another RdRp inhibitor that is not a nucleoside analog. DTriP-22 is able to inhibit EV71 replication by reducing virus RNA accumulation in cells [156]. DTriP-22-resistant analysis mapped to a K163R mutation within RdRp, suggesting a direct drug-RdRp contact.

In sum, the viral proteins encoded by EV71 have been extensively studied structurally, providing accurate structural information for rational drug design. As there are no homologs present in mammals, inhibitors targeting enteroviral proteins could be a prime choice concerning the safety issue. The unprecedented success in treating HCV infection by direct-acting antivirals opens a new era for combating RNA virus infection.

VACCINE DEVELOPMENT

Protective neutralizing antibody (NAb) response is one of the most critical host defense mechanisms against viral infection [157]. Seroepidemiological data indicate that NAb against both EV71 and CV-A16 are very low in children aged >6 months to ≤1 years and gradually increase in individuals between 1- and 4-year old age [158–160]. These data suggest that infants aged 6–14 months should receive priority vaccination against EV71.

HFMD-associated EVs invade the human body through mucosal surfaces and infect neural system after viremia to cause clinical symptoms. A vaccine against the main causing agents of HFMD should be capable of inducing mucosal immune reaction to prevent virus infection at the first step and/or of eliciting a system IgG reaction afterwards to block the viremia, a crucial step for neural system infection.

The protecting role of system antibodies in EV71 vaccine development has been exemplified by inactivated vaccines. In the last two years, three inactivated EV71 vaccines have been developed for system immunization, and their safety and efficiency have been verified in phase 3 clinical trials [161–163]. These three vaccines share similar formulations and immunization protocols. Each is comprised of formalin-inactivated EV71 virions (genotype C4) from cell culture with alum adjuvant. They are administered two times within a 28-day interval via intramuscular injection. All of the three phase 3 clinical trials are multicenter, randomized, double-blind, placebo-controlled studies, and include more than 10 000 infants and children (6–72 months or 6–35 months) were recruited for the trial. These vaccines induce more than 99% seropositive rates after boosting once and protect 90–97% infants from EV71-associated HFMD in 11–14 months after two intramuscular doses of EV71 vaccines. Most adverse events were mild and the total adverse events and serious adverse rate were similar between the vaccination and the placebo group.

Although the inactivated EV71 vaccines are safe and efficacious, there are some properties that remain to be addressed. First, as anti-EV71 antibody titers decreased 6 months after vaccination in the
serum of immunized patients, the long-term protection of these vaccines should be further explored. A clinical trial showed a booster vaccination at around one year after priming EV71 immunization significantly increased the anti-EV71 antibody titers (more than 10-fold) [164]. However, it should be determined whether this booster vaccination is sufficient and/or necessary for long-term protection. Secondly, HFMD can be caused by a variety of EV serotypes. However, all of the vaccines tested by clinical trials used only the EV71 C4 subgenogroup virus, which is dominant in mainland China, as the immunogen. Although strong cross-subgenogroup immune reaction with various EV71 strains and multiple subgenogroups (B4, B5, C2, and C5) was observed, their cross-protection with other EV71 subgenogroups also needs to be further evaluated [165].

In addition, several studies showed that inactivated intramuscular EV71 vaccines cannot cross-protect against CV-A16 [161–163,165,166]. These findings are consistent with most pre-clinical data, which show the antibody cross-reaction between EV71 and CV-A16 is weak [167–169]. Similar to EV71, inactivated CV-A16 virus can induce a strong systemic immune reaction and protect animals from CV-A16-associated disease [169–171]. A bivalent vaccine including both EV71 and CV-A16 is being considered for development. Experiments in mice indicated that inactivated EV71 and CV-A16 bivalent vaccine [168,172] or virus-like particles [173] could elicit protective immune responses against both EV71 and CV-A16 attack. In these studies, the immunogenicity and protective efficiency of the bivalent vaccine was similar to that of the monovalent vaccine, indicating that it may be advantageous to develop a bivalent vaccine of EV71 and CV-A16 in the future.

All these three EV71 vaccines use the intramuscular injection route for immunization [161–163]. Typically, intramuscular injection does not induce a strong mucosal IgA immune response, which is crucial for preventing virus infection and promoting viral clearance. Therefore, a mucosal vaccination route, which would induce an IgA response may provide an ideal alternative immunization route. Multiple mouse studies indicate that oral immunization with EV71 VP1 protein can induce both systemic anti-VP1 IgG and mucosal IgA reactions. However, the systemic IgG antibody reaction was weaker with oral immunization than with subcutaneous immunization [174–178]. Oral vaccines have also been shown to protect mice from a lethal EV71 attack by adopting different virus/bacteria vector and adjuvant to improve its efficiency [175,177–179]. Furthermore, oral immunization in mice with a murine virulent EV71 strain induced a strong cross-antibody reaction with CV-A16 [175], which was not seen in the performed clinical trial of inactivated EV71 vaccine [161–163]. To stimulate strong immune responses, attenuated live virus is often used in oral immunization strategies. To this end, diverse strategies, including temperature sensitivity [180,181], mutation of the UTR region [99,182], and RNA polymerase [154], have been attempted to make safe live EV71 vaccine strains. Data from these studies show that the live-attenuated vaccine strains of EV71 can induce broad-spectrum NAb responses in animal experiments. However, the virulence of the virus is only partly attenuated [180]. Given the adverse effects of oral poliovirus vaccine, safety observations of the live vaccines should be carefully examined before its application in humans.

Yet another vaccination strategy—subunit vaccines using EV71 capsid proteins—has also been investigated by several groups. VP1 is the most widely used antigen [174–179]. However, several studies using VP2–4 as the immunogen showed cross-protection reaction against multiple subgenogroups of EV71 [183–189], suggesting that the conserved epitopes it contains may make VP2–4 a good candidate for the development of a universal vaccine against multiple EV71 subgenogroup infections.

The availability of EV71 vaccines is very important in lowering the disease burden of HFMD. However, their long-term effectiveness, cross-protection against other EV serotypes, and controlling effectiveness on the epidemic of HFMD will require further observation in the future. Therefore, new vaccine types and vaccination strategies should continue to be explored.

**PERSPECTIVES**

Although great progress has been made in combating HFMD, we need to gain a better understanding of the disease, its etiology, and its pathology. First, changing of HFMD etiology is a great challenge for disease control. Sustained investigation on etiology and strengthened surveillance is important for the prevention, awareness, and control of HFMD outbreaks. To this end, the roles of different EV serotypes in HFMD and the pattern of etiological switch should be further addressed. Second, the pathogenic mechanisms of HFMD-associated EVs are not fully understood, which largely hinders disease treatment and antiviral development. The pathogenesis of EVs depends both on viral and host
factors, the interactions of which have not been fully elucidated. In the future, high-throughput screenings of the host factors that are critical for virus infection and replication as well as the dissection of the viral components that affect EV virulence will help shed light on these processes. These studies stand to uncover new targets for diagnosis, prognosis, and treatment. Simultaneously, further insights into the immune response will also be pivotal to clarify the pathology of HFMD and to improve current vaccine formulations and protocols. Moving these studies forward will require the continuation of efforts to develop animal models that simulate EV infections in humans. Last, due to the complex etiology of HFMD, the cross-protective efficiency of the inactivated EV71 vaccines against multiple EV71 subgenogroups and EV serotypes warrants further evaluation. Multivalent or universal vaccines should also be explored as a strategy to provide broader protection against diverse EV strains. Together, the studies and research we discuss here hold the potential to provide a comprehensive understanding of HFMD and thus, could pave the way to the development of more strategies for the effective control, prevention, and treatment of this important disease.

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