Xingli Xu · Yanchun Che ·
Qihan Li *Editors*

Molecular Biology of Hand-Foot-Mouth Diseases

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Preface

Hand-foot-mouth disease (HFMD) is an infectious disease that threatens the health and even the lives of children worldwide. Although there have been a number of outbreaks of HFMD worldwide in the past few decades, we are impressed by the large-scale HFMD outbreak in March 2008 in Fuyang, Anhui Province, China. The outbreak was mainly caused by enterovirus 71 (EVA71) infection. In May 2008, the Ministry of Health of China listed HFMD as a Class C infectious disease in the Prevention and Control of Infectious Diseases law in the People's Republic of China. Since then, Chinese doctors, disease control and prevention experts, virologists, and vaccinologists have attempted to understand, diagnose, treat, and prevent HFMD. Fortunately, we have achieved much regarding many areas of HFMD, including the isolation and identification of HFMD-related pathogens, increased molecular epidemiology data, increased clinical case experience, the standardization of clinical diagnosis and treatment methods, and the establishment of animal models. Furthermore, we have a better understanding of the epidemiological characteristics, clinical symptoms, interactions between the virus and immune system, and pathogenesis of HFMD. This knowledge has also promoted the successful development of a Chinese EVA71-related HFMD prophylactic vaccine.

The authors are proud to be from the Institute of Medical Biology, Chinese Academy of Medical Sciences, and were involved in the development of the EVA71 inactivated vaccine, the world's first HFMD preventive vaccine. Since the outbreak of HFMD in Fuyang in 2008, we carried out isolation and culture, established an infectious animal model, prepared an inactivated vaccine, and evaluated the safety and immune protection efficiency of the EVA71 inactivated vaccine. After eight years of innovative vaccine research and development, a breakthrough was achieved from concept to practice, and the China Food and Drug Administration approved the world’s first Enterovirus 71 inactivated vaccine (human diploid cell) registration application on 3 December 2015. With the application of the vaccine, the incidence of HFMD in China has been greatly reduced. More significantly, the death rate of HFMD has also been reduced.

Although the application of the EVA71 inactivated vaccine has effectively controlled HFMD in China, the long-term prevalence of HFMD has led to an increasing

number of pathogens and more complex species, mainly including enterovirus (EVA71), coxsackie virus (CAV4, CAV6, and CAV10), and echo virus. The same enterovirus can cause more than one clinical symptom, and the same clinical symptom can be caused by different viruses. Therefore, the clinical symptoms of HFMD are increasingly diverse, and the pathogenic mechanism is increasingly complex. Although the pathogenesis of HFMD is still not completely clear, we summarized our experience in the research and development of the EVA71 inactivated vaccine, analyzed many studies, and overviewed the epidemiological data of HFMD, which was collated into this book. We hope to provide more comprehensive and practical information on the molecular epidemiology of HFMD, the classification and development of HFMD pathogens, the clinical symptoms and pathogenesis of HFMD, the interaction between HFMD virus and human host and its related molecular mechanism, and the development direction of HFMD vaccines.

It is hoped that this book will provide a reference and enlightenment for the research on HFMD and lead more clinicians, disease control and prevention experts, and researchers to devote themselves to the related research work of HFMD. We hope this book will provide more knowledge for the prevention and treatment of HFMD and make more contributions to the development of the vaccine industry.

Kunming, China Xingli Xu
Kunming, China Yanchun Che
Kunming, China Qihan Li

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Editors and Contributors

About the Editors

Xingli Xu graduated with a Ph.D. from the Peking Union Medical College in China. She has worked at the Institute of Medical Biology, Chinese Academy of Medical Sciences, and has been engaged in research on viruses and immunity for about 10 years. She has participated in the development of the inactivated EV-A71 vaccine, F genotype mumps live attenuated vaccine, inactivated COVID-19 vaccine, and inactivated EV-A71/CV-A16 bivalent vaccine. In addition, Xu received support from a number of state-level foundation projects on the interactions between herpes simplex virus 1 and hosts and their mechanisms, as well as for the development of a live attenuated HSV1 vaccine. Her studies have been published in many journals, such as *PLoS Pathogens*, *Clinical and Translational Medicine*, and *Frontiers in Microbiology*.

Yanchun Che received her education in epidemiology and health service management from the Kunming Medical College and earned her master's degree in 2006. She has been working at the Institute of Medical Biology, Chinese Academy of Medical Sciences, for over 30 years. She was involved in the implementation of the China Vaccine Project loaned by the World Bank for innovating the poliomyelitis vaccine production line according to the European GMP during the period from 1992 to 1996. Following this, she became the head of the General Office, and Research and Development Department, responsible for the management of government-funded research projects and international collaborations between the institute and foreign universities and academic institutions. She joined the research team on regulatory affairs and clinical trials of new vaccines such as the inactivated EV-A71 vaccine and F genotype mumps live attenuated vaccine in 2013. Now she is the head of the Vaccine Clinical Trial Center, responsible for conducting clinical trials of newly developed vaccines and post-marketing surveillance of safety and effectiveness for new vaccines licensed by the institute. She has published over 20 papers in academic journals as the first author.

Qihan Li is a famous virologist, immunologist, and vaccinologist in China. He has worked at the Institute of Medical Biology, CAMS, for over 30 years. And he served as director of the Institute of Medical Biology, CAMS, for more than 20 years. As chief scientist, he led the team to explore and develop several novel vaccines, including inactivated EV-A71 vaccine, F genotype mumps live attenuated vaccine, inactivated EV-A71/CV-A16 bivalent vaccine, and inactivated COVID-19 vaccine. Among that, inactivated EV-A71 vaccine, as the first vaccine for the prevention of hand, foot and mouth disease in children, has been used in China for 8 years. Both phase III clinical and real-world vaccination data showed that the protective efficacy of the vaccine was over 90% and the vaccine is safe for children. The results have been published in the *New England Journal of Medicine*. His main research focuses on virus vaccines, virus molecular biology, virus immunology, and host-virus interactions. He has been committed to the analysis of the molecular biological functions of important virus-encoded molecules and the study of host cell-specific biological responses to viral infections, including research on the interaction of poliovirus and HSV1 with the human host. He has published over 260 papers in academic journals as the first author or corresponding author.

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Chapter 1
Epidemics of Hand, Foot, and Mouth Disease

Ying Zhang

Abstract Since the mid-twentieth century, hand, foot, and mouth disease (HFMD) was seen as a common cause of viral rash, typically self-limited syndrome in children and adults with classic skin findings. Till the past two decades, HFMD has received new attention because it has led to millions of attacks and several outbreaks across the world. This disease may have completely different clinical epidemiological and etiological characteristics from what was initially believed. Especially, HFMD can be associated with severe complications, such as brainstem encephalitis, meningitis, acute flaccid paralysis (AFP), pulmonary edema (NPE), severe neurological sequelae, and high case-fatality rates. In recent years, it has become a serious health threat and economic burden across the Asia–Pacific region. Historically, outbreaks of HFMD were mainly caused by various enteroviruses. Different pathogens are prevalent in different countries. Vaccines have been developed to provide protection against the most common pathogens in specific countries (e.g., vaccine against enterovirus 71 in China). However, the epidemic of HFMD is complex, such as simultaneous circulation of more than one causative virus and modification of the molecular epidemiology of infectious agents. Awareness of the epidemiological situation and patterns may lead providers to appropriate diagnosis and management.

Keywords Epidemiology · Circulating viruses · Epidemic cycle

1 Introduction

Hand, foot, and mouth disease (HFMD) is an acute infectious disease caused by multiple viral pathogens, such as coxsackievirus A and enteric cytopathogenic human orphan (ECHO) viruses, and commonly occurs in children under five years

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old [1]. Typical clinical manifestations include fever, skin eruptions on the hands and feet, and vesicles in the mouth. Most cases usually recover within one week. Some cases involve the central nervous system (CNS), and progress systemic fatal complications, including aseptic meningitis, brainstem encephalitis, neurogenic pulmonary edema (NPE), and acute flaccid paralysis (AFP). Occasionally, cases with rapid progression can result in death [2–4]. Outbreaks of severe neurological disease, mimicking polio but associated with non-polio enteroviruses, have highlighted the public health impact of enteroviruses [5–7].

2 HFMD Epidemiology

HFMD was first reported and confirmed in Europe since the twentieth century [8–11] and has gradually spread to Asia–Pacific countries [12–16], where large epidemics and numerous and severe cases have been reported [17–21], posing a great threat to public health (Fig. 1.1). In recent years, the epidemiological pattern has changed from sporadic cases to large epidemic clusters (Fig. 1.1).

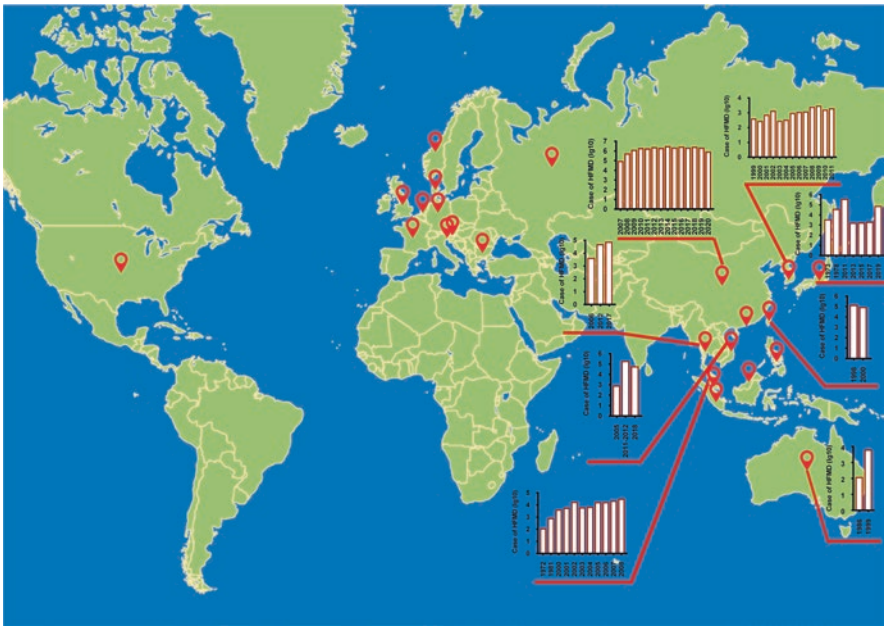

Fig. 1.1 HFMD epidemics worldwide. (Red dots indicate countries with large outbreaks; bars indicate HFMD case numbers in key regions of Pacific Rim countries)

The 1970s–1980s

Sporadic cases or regional outbreaks were reported in Europe, Asia, and America and were primarily characterized by typical CNS symptoms.

Europe

As the research on pathobiology in the twentieth century was in its infancy and a disease surveillance system had not yet been established, the manifestations of HFMD were identified to be mild and related pathogens were not well understood, only small outbreaks of cases were reported in Hungary, Sweden, and Bulgaria [9–11, 22]. The 1st HFMD case in New Zealand was confirmed in 1957 [8]. Then, there are some national reports one after another [9–11, 22]. The clinical manifestations of these HFMD cases were primarily CNS symptoms, including severe encephalitis and AFP, and typical mild skin eruptions and vesicles were rarely observed. Severe encephalitis and AFP that were observed during the outbreaks in Bulgaria in 1975 [10] and Hungary in 1978 [9] were caused by pathogens associated with HFMD. A total of 705 cases of feverish illnesses with no typical HFMD symptoms caused by enterovirus 71 (EV-A71) infection were reported in children under 5 years old in Bulgaria between May and September 1975. Of these patients, 545 (77.3%) were diagnosed with aseptic encephalitis, 149 (21.1%) were diagnosed with AFP, 68 (9.6%) were diagnosed with spinal and bulbar muscular atrophy (SBMA), and 44 (6.2%) died [10]. It is worth noting that many cases of SBMA progressed rapidly, with death occurring within 10–30 hours after illness. A large epidemic of EV-A71 infection occurred in Hungary between May and September 1978 [9]; this epidemic was characterized by 826 cases of aseptic meningitis and 724 cases of encephalitis with cerebellar ataxia and AFP. Those cases with severe neurological complications included 13 deaths, from which only 4 were reported to have typical HFMD symptoms [9].

Asia

Similar to Europe, small HFMD outbreaks were described in many areas of Asia–Pacific countries.

Japan: HFMD outbreaks caused by EV-A71 infection were reported in 1973 [23–25] and 1978 [26–28], with clinical manifestations of acute central nervous system disease. Using the national HFMD sentinel surveillance network, covering approximately 3000 pediatric clinics, which was established by the National Institute of Infectious Diseases (NIID) in 1981 [29], HFMD outbreaks caused by coxsackievirus A10 (CV-A10) infection emerged in Japan between July 1981 and January 1982 [30].

China: Multiple small outbreaks of HFMD associated with EV-A71 infection were reported in Taiwan (China) in 1980, 1981, and 1986 [15, 31, 32]. A small outbreak of HFMD with several AFP cases caused by EV-A71 infection was reported in Hong Kong (China) in 1985 [33]. In mainland China, the first outbreak of HFMD caused by EV-A71 infection was described in Hubei Province in 1987; in this outbreak, no patients developed AFP or aseptic meningitis [34].

Singapore: HFMD epidemics occurred in Singapore in 1972 and 1981 [35]. Thereafter, HFMD and aseptic meningitis associated with EV-A71 infection occurred in 1987 [36].

Oceania

Australia: Small HFMD epidemics have been continuously reported in the eastern region of Australia since 1972 [36, 37]. HFMD and acute neurological disorders caused by EV-A71 infection were reported in outbreaks in Melbourne and Victoria, Australia, in 1973 and 1986 [38, 39].

The Americas

The United States of America (USA): In the USA, several HFMD outbreaks have been reported, which associated with multiple pathogens, such as EV-A71 and EVD68, first isolated and identified from patient samples collected during these outbreaks [37, 40–43].

Brazil: In Brazil, there have been several reports of HFMD outbreaks caused by EV-A71 infection [44, 45].

1990–1999

In this decade, major HFMD epidemics occurred in Asian countries, with multiple large outbreaks. Due to the development of pathogen isolation technology, EV-A71 and CV-A16 infections were identified as common causes of outbreaks in some regions. The clinical manifestations of HFMD were diverse, but it was confirmed that neurological disorder complications remain the major causes of death.

Europe

Very few HFMD epidemics have been reported in European countries. The Britain's largest outbreak on record caused by CV-A16 virus infection occurred in Wales in the fourth quarter of 1994. In this outbreak, 952 HFMD patients aged 1–4 years, most of which had mild symptoms, were observed in surveillance clinics [46].

Asia

In contrast to Europe, regions with large populations in Asia–Pacific countries have experienced multiple large HFMD outbreaks, which were caused by EV-A71 infection, since 1997. Many cases of these outbreaks have been associated with HFMD and vesicle angina pectoris, and some cases were reported with neurological disorder complications, such as aseptic meningitis, encephalitis with cerebellar ataxia and AFP. Of these neurological complications, NPE is by far the most worrying form, which is associated with severe brainstem encephalitis and has a high fatality rate [47, 48].

Malaysia: A total of 2628 HFMD cases were reported in Sarawak, Malaysia, in April 1997 [12], and 34 patients died due to NPE syndrome [48, 49]. Thereafter, several HFMD outbreaks occurred in the Malay Peninsula [50–52]. As a result of these HFMD outbreaks, a HFMD sentinel surveillance program was established in March 1998 by the Sarawak State Department of Health, and the system has played an essential role in the monitoring of HFMD outbreaks [53].

Japan: HFMD outbreaks were reported in Japan in 1997 [13].

Republic of Korea: The National Enterovirus Sentinel Surveillance System, which covers 35 primary clinics, 105 secondary hospitals, and 40 tertiary hospitals, was established by the Korean Centers for Disease Control and Prevention since 1993. However, there was no report of a HFMD outbreak until 1999 [18].

Singapore: Small HFMD outbreak was reported in Singapore between 1997 and 1998 [14].

China: In Taiwan (China), the largest EV-A71-associated HFMD outbreak ever recorded was reported in 1998 [15, 54–56]. There were two waves that year. The first wave occurred from March to July with 15,758 cases at peak period, affecting the whole island of Taiwan; the second wave occurred from September to November with 3177 cases at peak period, focusing on the southern region of Taiwan Island [15, 57]. It was estimated that approximately 1.5 million HFMD cases occurred during the outbreak [36]. EV-A71 and CV-A16 were identified as the major pathogens causing this outbreak, and EV-A71 was isolated from approximately 2/3 of HFMD patients [15, 58]. Additionally, in this outbreak, 405 severe neurological complications resulting from EV-A71 infection were reported and 78 patients died of NPE [15, 56, 59].

Oceania

Australia: There was a community-wide EV-A71-associated HFMD outbreak in Perth, Western Australia, in 1999 [16]; approximately 6000 HFMD cases were reported, and 29 patients associated with severe neurological complications. In this outbreak, the incidence of neurological disease was approximately 1‰ among EV-A71-infected patients [16]. Similar to those pathogens in HFMD epidemics in Taiwan (China) in 1998, the major pathogens were EV-A71 and CV-A16, with equal distribution. However, in this outbreak, neurological diseases, including aseptic meningitis, acute cerebellar ataxia, brainstem encephalitis, and AFP, were

attributed to only EV-A71 infection. Patients with neurological disease were followed-up until complete recovery. Importantly, no NPE cases were reported during this epidemic [16].

The Americas

Similar to Europe, in the Americas there were a few reports of HFMD cases [43].

2000–2015

Since entering the twenty-first century, HFMD epidemics were reported in all Asia–Pacific countries. Large-scale HFMD outbreaks have emerged in European and Asian countries. Many Asian countries have even experienced the worst HFMD outbreak on record.

Europe

Multiple HFMD epidemics have been reported in European countries since 2000.

United Kingdom: In UK, approximately 20221 EV-positive cases were reported between 2006 and 2017. A HFMD outbreak caused by CV-A6 infection occurred in Scotland in 2014 [1].

Finland: HFMD cases were identified throughout all countries of Finland in autumn 2008 [60, 61].

France: HFMD outbreak was reported in France in 2010 [62].

Asia

Asian countries experienced the most severe HFMD outbreaks during 2000–2015. Very large and severe outbreaks emerged in many regions of many countries at the same time.

China: Among all Asia–Pacific countries, China has experienced the highest number of EV-A71-associated HFMD outbreaks since 2007. In 2007, more than 80000 HFMD cases and 17 deaths were reported in mainland China [63]. Thereafter, HFMD was designated as a class "C" notifiable disease for reporting according to the "Infectious Disease Prevention and Treatment Law of the People's Republic of China," established by the Ministry of Public Health in May 2008 and implemented in July 2009 [17]. According to data published by the China Center for Disease Prevention and Control, approximately 13.7 million HFMD cases were reported between 2008 and 2015, of which 123,261 cases were severe and 3322 resulted in death [64, 65]. The HFMD incidence rate ranged from 1221.3/million to 1616.4/

million per year between 2010 and 2012, and the mortality rate reached its highest value in 2010 [17].

Taiwan (China): HFMD outbreaks caused by EV-A71 infection emerged in Taiwan, China, in 2000, 2001, 2005, 2008, and 2012. More than 600 severe cases and 51 deaths were reported by the Taiwan Centers for Disease Prevention and Control in 2000 and 2001, respectively [66]. A total of 142 severe cases and 16 deaths were reported in 2005 [67, 68]. A total of 373 severe cases and 14 deaths were reported in 2008 [18]. Another HFMD outbreak was reported in Taiwan in 2012 [18, 69].

Japan: In Japan, the largest HFMD outbreak recorded occurred in the summer of 2011 [18]. A total of 347362 confirmed cases were reported, and the vast majority of cases occurred in children under 3 years old. There were 1515 and 1590 HFMD cases in 2013 and 2015, respectively [18]. It is worth noting that the majority of HFMD cases have been caused by CV-A6 infection since 2011 [18]. EV-A71 infection was less reported in the HFMD epidemics in 2010 and 2012 [18], and EV-A71 has been rarely detected since October 2014; however, the rate of EV-A71 detection started to increase at the end of 2017 (see the following description).

Singapore: A large HFMD outbreak occurred in 2000, with a peak number of 3790 cases in October [70]. A total of 76 EV-A71 cases confirmed by laboratory testing, as well as 4 deaths, were reported [70]. The total numbers of HFMD cases ranged from 5187 to 20,003 per year between 2001 and 2007 [71]. The largest HFMD outbreak of Singapore's recorded, comprising 30000 cases was reported in 2008 [19]. The dominant pathogens in this outbreak were CV-A6, EV-A71 and CV-A10 [19].

Republic of Korea: The first HFMD outbreak associated with EV-A71 was reported in 2000, with 12 patients requiring hospitalization [20, 72]. Thereafter, HFMD cases have been reported every year. The total number of HFMD cases has surged since the spring of 2009, with 2427 reported cases [20]. Of these, 94 patients with HFMD confirmed by laboratory tests resulted in CNS complications, and 2 patients died [73]. EV-A71, CV-A5, and CV-A6 are the main viral pathogens of this outbreak [73].

Malaysia: Since 2000, large HFMD epidemics with peaks between February and April have occurred almost every 3 years [53, 74–76]. In 2006, a total of 250 cases with CNS complications were reported in Sarawak state, and 2 of these patients died [75].

Kingdom of Cambodia: Epidemiological data is limited, but the largest recorded EV-A71-associated HFMD outbreak occurred in the first half of 2012. In this outbreak, 56 deaths attributed to severe encephalitis were reported [21].

Thailand: Since 2001, the Thai Epidemiology Department, Ministry of Public Health, established a HFMD surveillance system based on hospital. A total of 502,329 HFMD cases were reported between 2001 and 2018 (769–79,910 per year) [18]. The peak incidence occurred in 2016, and the trough occurred between 2001 and 2011. Incidence rates were approximately 1.2–28.4/100,000 [18]. It is worth noting that the number of deaths decreased from 7 in 2006 to 2 in 2012; in contrast, the number of HFMD cases increased from 3961 in 2006 to 45,464 in 2012 [18],

which might be attributed to mainly CV-A6 infection rather than EV-A71, as a pathogen with a high mortality [18, 77].

Vietnam: The first HFMD case associated with EV-A71 was officially reported in 2003 [78]. In the 2nd half of 2005, an EV-A71-related HFMD outbreak occurred, with reported 700+ cases, 51 severe cases, and 3 deaths [78]. In 2011, Vietnam experienced its most severe outbreak of hand, foot, and mouth disease (HFMD) caused by the EV-A71 virus. This outbreak resulted in 200,000 hospitalizations and 200 deaths, with a mortality rate of 0.5% [79].

India: CV-A6-associated HFMD outbreaks were reported between 2009 and 2010 in India [80].

Oceania

Australia: There was HFMD outbreak caused predominantly by EV-A71 infection in Sydney from 2000 to the summers of 2001, leading to 200 hospitalizations, with 14 patients with severe cases [81, 82]. In the 1st half of 2013, another EV-A71-infected HFMD epidemic occurred in Sydney [83, 84]. The epidemic started in the community of North Sydney Seashore and gradually expanded to encompass the whole area of Sydney. Many severe neurological system disorders were observed in mid-November of 2012, and the number of cases increased rapidly until reaching a peak in March 2013. As a result, approximately 120 severe cases caused by EV-A71 infection and 4 deaths caused by EV-A71-associated neurological disorders were reported [85].

America

USA: From November 2011 to February 2012, the US Centers for Disease Control and Prevention received reports of 63 possible HFMD cases [86, 87]. During 2014–2016, a total of 2967 cases were reported [88], with the largest number of EVD68 cases occurring in 2014 [89].

Brazil: Between 2009 and 2016, HFMD outbreaks associated with CV-A16 and CV-A6 infection occurred in Brazil [90, 91].

2016 to the Present

Major HFMD outbreaks have still occurred in Asia, sporadic cases have been reported in Europe, and few cases have been reported in the Americas. In 2018, a relatively concentrated and large-scale HFMD outbreak occurred worldwide. Fortunately, two inactivated EV-A71 vaccines were licensed in China at the end of 2015, and its administration greatly helped eliminate severe and fatal HFMD cases.

Following, the pathogenic spectrum of HFMD has changed in China, and the morbidity rate of other pathogens has increased.

Europe

There were several sporadic HFMD outbreaks associated with EV-A71 in Spain, Germany, and other European countries in 2016 [4, 92, 93]. In 2018, HFMD cases caused by enterovirus D68 (EVD68) clade D1 emerged in France [94] and Italy [95].

Asia

China: The numbers of severe and fatal HFMD cases have been significantly reduced, and the HFMD-associated pathogenic spectrum has varied as a result of the administration of the inactivated EV-A71 vaccine. According to data published by the China Center for Disease Prevention and Control, the total annual numbers of severe HFMD cases were significantly reduced by 87.4% (in 2019), 96.1% (in 2020), and 89.4% (in 2021) compared to the average cases during 2010–2015, and those of fatal cases were sharply reduced by 95.8% (in 2019), 99.2% (in 2020), and 95.9% (in 2021) compared to the average cases during 2010–2015, respectively (Fig. 1.2). As an example, in Guangxi Province, among infants aged 0–12 months,

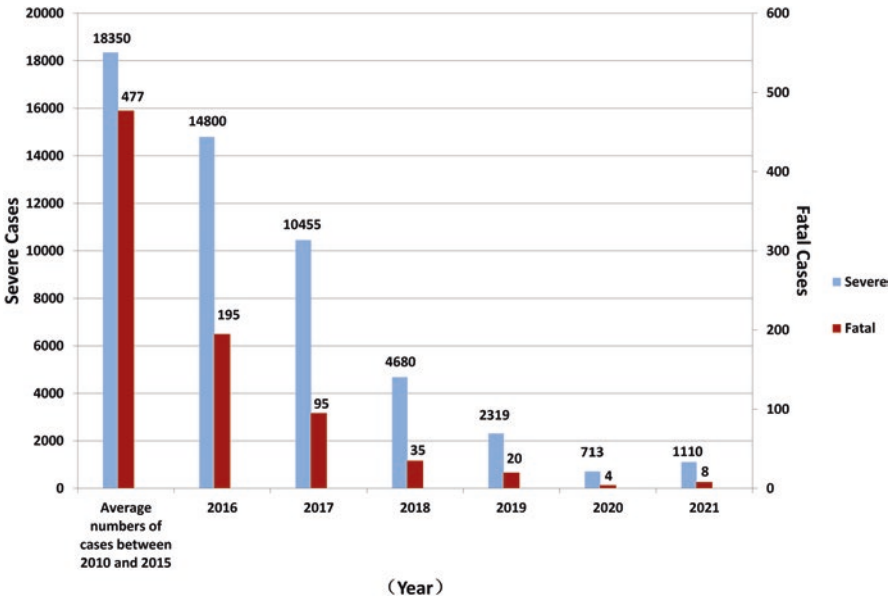

Fig. 1.2 Severe and fatal HFMD cases between 2010 and 2020 in mainland China (data source: http://www.nhc.gov.cn/jkj/new_index.shtml)

the prevalence of HFMD decreased from 23.0% in 2013–2015 (before vaccination) to 15.3% in 2017–2019 (after vaccination). Especially, the severe fatality rate dropped sharply from 37.4% in 2014 (before vaccination) to 2.5% in 2019 (after vaccination) [96].

Japan: A total of 1900 HFMD cases were reported in 2017 [18], which was similar to the number reported in 2013. However, unlike previous HFMD outbreaks, which were attributed to CV-A6 or CV-A16 and EV-A71 infection, the major pathogen in the 2017 outbreak was only EV-A71. In 2018, the epidemic expanded, and the number of hand, foot, and mouth disease (HFMD) cases peaked at approximately 70,000 [18].

Thailand: In 2016, regional EV-A71-related HFMD outbreaks were reported in Chiang Rai and Phayao states in northern Thailand; in these outbreaks, 55% of cases occurred in children under 2 years old [97]. In 2017, HFMD outbreak was reported throughout Thailand, with approximately 70,000 cases and 3 deaths [98].

Vietnam: In 2018, more than 53000 HFMD cases and 6 deaths were reported in Vietnam. In this outbreak, the main pathogen is EV-A71 [99].

Singapore: In 2018, an EV-A71-related HFMD outbreak with high transmission was reported. A total of 1249 cases were identified within one week [100].

The Americas

In 2016, a small HFMD outbreak emerged in Brazil [90]. In 2018, HFMD cases associated with CV-A16 were reported in Uruguay [101].

3 Variety of Circulating Viruses

To date, more than 20 EVs have been associated with HFMD. Circulating viruses vary among different regions worldwide. Considering previous epidemiological and molecular etiology data, we describe variations in circulating viruses.

EV-A71 is a Major Pathogen Leading to Severe and Fatal Cases

Previous molecular etiology studies demonstrated that EV-A71 was the most common pathogen causing multiple severe HFMD outbreaks and HFMD-related fatalities. Representative severe HFMD events included the following: an outbreaks resulting in severe encephalitis and AFP cases in Bulgaria in 1975 [10] and Hungary in 1978 [9]; an outbreak characterized by aseptic meningitis in Singapore in 1987 [36]; outbreaks associated with acute neurological disorder development in Melbourne in 1973 [38] and in Victoria, Australia in 1986 [39], respectively; an outbreak resulting in 250 cases of neurological disorder complications and 6 deaths

in Sarawak state, Malaysia in 2006 [75]; outbreaks resulting in multiple cases of neurological disorders in Taiwan (China) and mainland China in 2008 [102–104]; and an outbreak attributed to EV-A71 infection with a high mortality rate (>60%) in the Kingdom of Cambodia in 2012 [21]. The above events were all severe outbreaks caused by EV-A71 and resulted in higher mortality rates. In addition, the clinical manifestations of severe HFMD cases tended to diversification. At early stages, severe encephalitis and AFP were characterized in Europe, and NPE and acute cerebellar ataxia appeared one after another in Asian countries in recent years. We suspect that such variation might be attributed to the following: first, the continuous development of medical treatment technology and medical systems, which allow the recording of more detailed observations and clinical manifestations; and second, the evolution or mutation of viral strains in the transmission process, leading to large variations in clinical manifestations.

Different genotypes of EV-A71 have different distributions in different countries around the world.

All over the world, the distribution of EV-A71 of different genotypes in Asian countries is the most diverse and complex. Besides the HFMD outbreaks associated with EV-A71 mentioned above, multiple HFMD outbreaks have emerged in many other countries and regions in the world, including Japan (1973, 1978, 2017) [13, 18, 23–28, 30]; Taiwan, China (1980, 1981, 1986, 1998, 2000, 2001, 2005, 2008, 2012) [15, 18, 31, 32, 54–56, 66–69, 105]; Hong Kong, China (1985) [33]; mainland China (1987, 2007–2015) [17, 34, 63–65]; Australia (1973, 1986, 1999, 2000–2001, 2013) [16, 38, 39, 81–84]; Malaysia (1997, 2000, 2003, 2005, 2006, 2008–2009) [106, 107]; and Thailand (2016) [97].

EV-A71 consists of four genotypes, A, B, C and D, based upon gene sequence differences in the capsid protein VP1 [108, 109]. Genotype A includes only one strain (BrCr-CA-70), which was isolated in California in 1970 [37]. Genotypes B and C are reported more commonly and consist of sub-genotypes B1–B5 and C1–C5, respectively [108, 110]. Genotype D includes also only one strain (R-13223-IND-01), which was isolated in India in 2002 [109]. A systemic analysis of the EV-A71 pathogens causing HFMD worldwide revealed that the EV-A71 genotypes reported in Asian countries included almost all the B (B1-B5) and C (C1-C5) sub-genotypes, as well as the D genotype. In European and American countries, before 1990 it was principal genotype B (mainly B1 and B2) and thereafter was genotype C (mainly C1 and C2) (Figs. 1.3 and 1.4) [2, 22, 111, 112].

In detail, sub-genotypes B3 and B4 have been reported to circulate alongside other genotypes in Asian countries, including Japan, Malaysia, Singapore, and Taiwan (China) [113]. Sub-genotype B5 was initially reported during the HFMD outbreak in Singapore in 2000 and has since been frequently reported in various Asian countries, including Japan [114], Malaysia [53], Taiwan China [115], Brunei [116] and Vietnam [117] since 2003 [2]. Sub-genotypes C1 and C2 have frequently circulated with other genotypes in multiple HFMD outbreaks in Asian countries since the 1990s [118–123]. Sub-genotype C4 was first described in mainland China and Taiwan (China) [105, 118, 121] and was subsequently found in other Asian countries, including Japan [122], the Republic of Korea [120], Vietnam [124], and

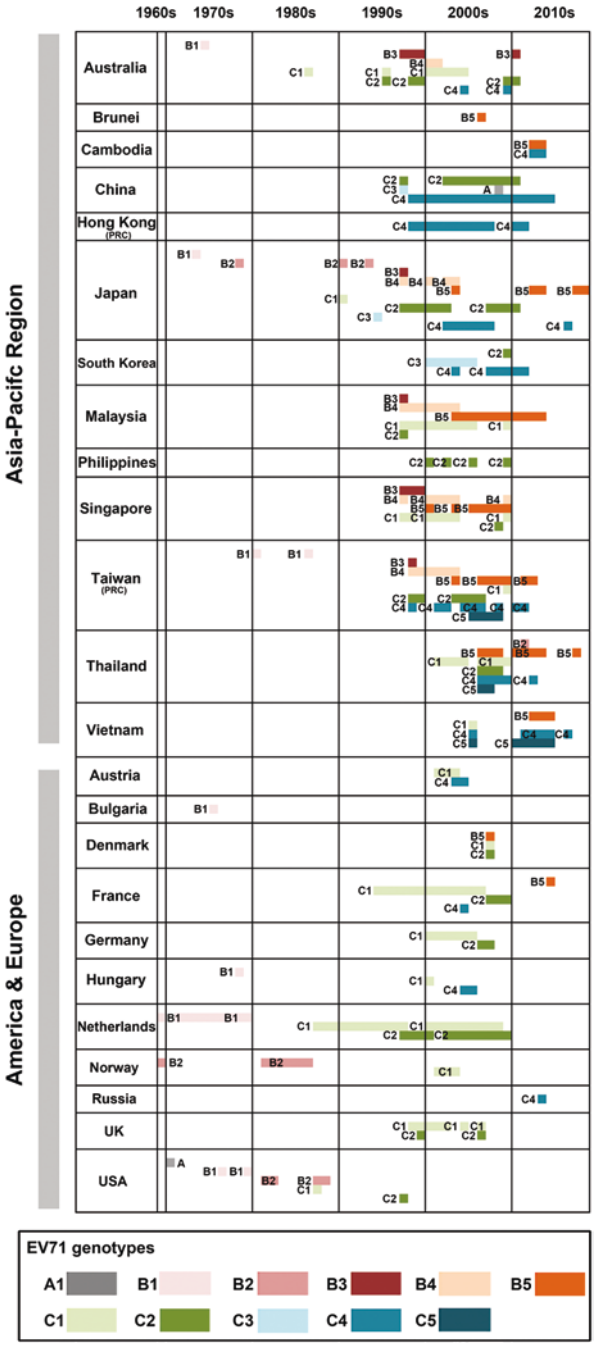

Fig. 1.3 EV-A71 genotypes associated with HFMD outbreaks worldwide

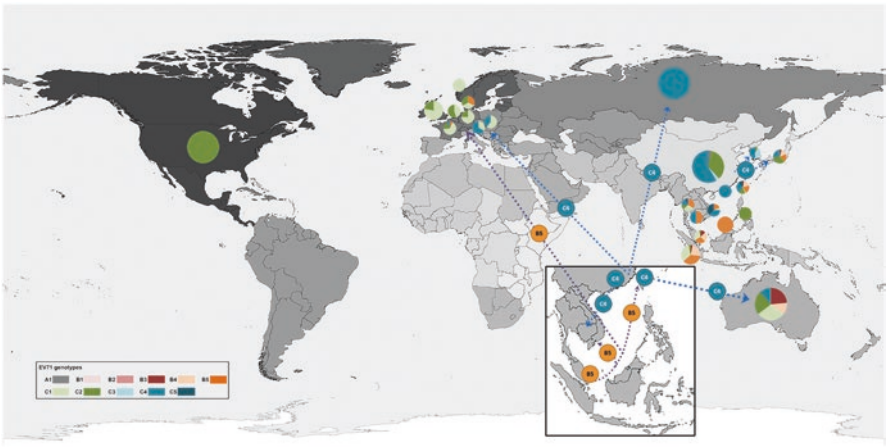

Fig. 1.4 Distribution and transmission of EV-A71 with different genotypes in HFMD outbreaks between 1997 and 2018 worldwide

Thailand [125]. Sub-genotype C5 was reported in Taiwan (China) and Vietnam in 2005 [2, 78] and Thailand in 2006 [111], but this sub-genotype has not been reported in other countries. Among European and American countries, sub-genotypes B1 and B2 were consistently detected in the Netherlands, Norway, and USA before 1990 [37, 126]; sub-genotypes C1 and C2 were reported in the USA, the Netherlands, Norway, and Russia from the 1980s to 2009 [22, 37, 126, 127]; and sub-genotype C4 has not been reported in Austria, France, Hungary, or Russia until 2000 [22, 108, 112, 127].

HFMD Pathogens Tend to Exhibit Great Variability

In recent years, the proportion of EV-A71 among the total pathogens leading to HFMD has gradually decreased, while in parallel, the proportions of other EVs have gradually increased. HFMD pathogens tended to exhibit great variability after the initiation of inactivated EV-A71 vaccination in mainland China. As an example, in the Guangxi Region, the most common pathogen causing HFMD shifted from EV-A71 (62.8%, 1684/2682) during 2013–2015 to other EVs (67.2%, 2307/3432) during 2017–2019 [96]. And the EV-A71 infection rate decreased from 46.7% during 2013–2015 to 23.6% during 2017–2019 [96]. At present, although the dominant pathogens causing HFMD are CV-A16 and EV-A71 [17, 86, 128], which circulate alternatively or together in epidemic areas, the numbers of HFMD cases caused by CV-A6 and CV-A10 infections have started to increase [62, 128, 129].

CV-A6-related HFMD cases have been reported in multiple countries, such as Finland (2008) [60], Singapore (2009) [19], France (2010) [62], mainland China

(2010, 2013) [129], Taiwan of China (2009–2010) [130], Japan (2007, 2009, 2010–2011) [131], Spain (2008, 2011) [132], the Republic of Korea (2009) [133], Thailand (2012, 2017) [98, 134], Vietnam (2013–2015) [135], and Brazil (2009–2016). As an example, in the United Kingdom, there was a rapid increase in the number of HFMD cases caused by CV-A6 infection between 2006 and 2017, with an increase from 1% in 2007–2008 to 10% in 2016–2017 [1]. Additionally, the rate of EV-A71 infection gradually decreased from 10% in 2006–2010 to 3% in 2012 [1].

CV-A10-associated HFMD has emerged in multiple countries, including USA (1950) [136, 137], Japan (1981–1982) [30], Singapore (2008) [19], Thailand (2008–2013 and 2016) [97, 138, 139], India (2009–2010) [140], France (2010) [62], and Vietnam (2013–2015) [141].

Other EV-associated HFMD outbreaks have been reported in some regions of the world [6]. For example, CV-A24-associated HFMD outbreaks have been reported in mainland China (2010–2011, 2013) [142–144], India (2010) [145], Brazil (2003–2005, 2009) [146], West Africa (2011) [147], and French Guiana (2017) [148]. Echovirus 30-associated HFMD outbreaks have been reported in European, American, and Asia–Pacific countries [6, 149–151]. Small EVD68-associated HFMD outbreaks have been reported in some regions of North America, Europe, and Asia [152–155].

Importantly, HFMD epidemics can be attributed to a complex combination of pathogens. In Singapore, the predominating viruses were CV-A16 (40%) and EV-A71 (30%) together with other EVs between 2001 and 2007 epidemics [71]. In the HFMD outbreak in Finland in autumn of 2008, CV-A10 and CV-A6 infections accounted for 28% [33] and 71% [83] of the HFMD cases, respectively [61]. Among the 222 HFMD patients in France in 2010, CV-A10 (39.9%) was reported to be the major pathogen (39.9%), followed by CV-A6 (28%) [62]. In the HFMD outbreak in the Republic of Korea in 2009, the most common pathogens were EV-A71, CV-A5, and CV-A6 [73].

4 HFMD Epidemic Cycle

Epidemiological data from the 1960s to the present demonstrate that the HFMD epidemic cycle is approximately every 2–3 years. For example, in mainland China, HFMD outbreaks occur approximately every 2 years (an interval of about one year). Before 2016, peak numbers of HFMD cases were observed in 2010, 2012, and 2014, during which over 1.7 million cases and over 190 deaths were reported. The total number of HFMD cases decreased at 1 year post-outbreak, with approximately 1.61–1.99 million cases and 129–509 deaths being reported (Fig. 1.5; Data source: http://www.nhc.gov.cn). However, after 2016, when the inactivated EV-A71 vaccines were available, the total number of deaths was sharply reduced, though the

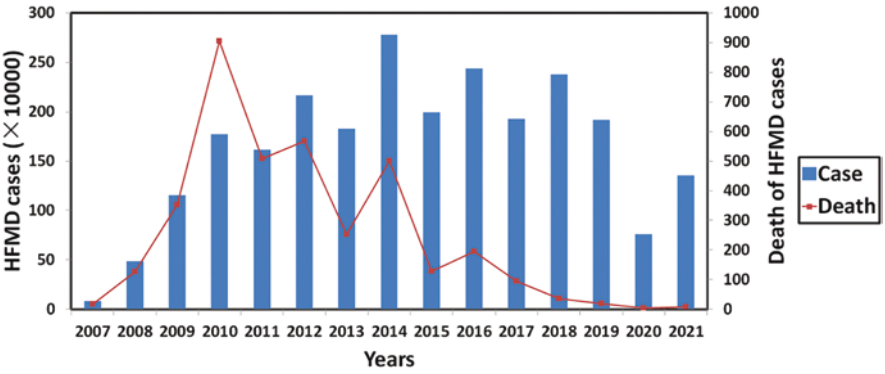

Fig. 1.5 Numbers of HFMD cases and deaths reported between 2007 and 2021 in China

HFMD epidemic cycle remained at an approximately 1-year interval (Fig. 1.5). Similar with mainland China, the HFMD outbreaks also occurred every 2 years in Japan and the USA. There were HFMD outbreaks in Japan in 2013, 2015, and 2017 [18], as well as in the USA in 2014, 2016, and 2018 [88, 89, 156]. HFMD epidemics in Malaysia and other Asia–Pacific countries have generally occurred every 3 years since 2000 [18, 53, 74, 76]. These outbreaks are typically characterized by rapid decreases in the numbers of HFMD cases in the 2nd and 3rd years post-outbreak [53]. Cyclical patterns of HFMD have been reported in other Asia–Pacific countries and regions [6, 18, 112].

Some scholars thought that similar pathogens lead to the occurrence of similar epidemic cycle. For example, EV-A71-associated HFMD epidemics in Asia–Pacific countries tend to occur with a cycle of every 2–3 years [17, 53, 65]. Nevertheless, the epidemic cycle of CV-B4 infection is every 1 year, and that of CV-B3 infection is every 4 years, while that of CV-E30 infection fluctuates between 5 and 3 years, that of CV-A4 infection has fluctuated between 1 and 2 years, and that of E25 infection shows less regular patterns since 2004 [157–162]. However, it is still difficult to accurately predict future HFMD outbreaks, due to changes in viral properties, local viral diversity, and cross-reactive infection.

In addition to regular annual or multi-annual cycles, HFMD epidemics show seasonality and temporal trends [163]. HFMD epidemics in temperate regions frequently occur in the summer and beginning of autumn [164]. In mainland China, HFMD epidemics in most of the regions have occurred during the summer period from March to June, but some have occurred in September in some regions [17, 164]. Moreover, the peak time in southern China is slightly earlier than that in northern China. The HFMD outbreak cycles were similar in Japan and Vietnam [78]. Hong Kong and Singapore have observed changes over time in the periodicity of the peaks [165, 166]. In the USA, the average peak time periods are July in the state of Texas and September in the state of Colorado [167]. The peak of HFMD in

Malaysia is from February to April [18]. In contrast, HFMD epidemics in tropical regions have failed to show an association with season, which might be attributed to the minor seasonal changes in these areas. For example, in Florida (USA), it experiences less pronounced seasonal peaks in transmission [167]. In Thailand, the number of HFMD cases reached high levels during the rainy season [77, 97, 139], while the cases decreased significantly during the dry season [98, 139].

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Chapter 2
Etiology of HFMD

Dandan Li

Abstract Hand, foot, and mouth disease (HFMD) is caused by multiple viruses in the genus Enterovirus, which include the well-known polioviruses (PVs), coxsackieviruses A and B (CV-A and CV-B), and enteric cytopathic human orphan (ECHO) viruses. The genome of EVs is single-stranded, positive-sense RNA. The open reading frame (ORF) region in EVs genome encodes structural proteins that further formed EV capsids. EVs are among the fastest evolving viruses with high genetic variability. Thus, the classification of EVs is initially based on clinical manifestations of their infection, subsequent serological characteristics, and differences in the nucleotide sequences up to the modern era. Currently, the genus Enterovirus consists of 3 human RV species (RV-A, -B and -C), 4 human EV species (HEV-A, -B, -C, and -D), and 6 animal EVs. The major pathogens (EV-A71, CV-A16, CV-A6, CV-A10, etc.) causing HFMD are included in the species of EV-A. The HFMD outbreaks were alternatively caused by infections with CV-A16 and EV-A71 ever since 1990s; however, the pathogenicity of HFMD changed greatly in the past decade, with new pathogens being identified continuously. Rapid diagnosis and genotyping of EV infection are through virus isolation and culture, serological assays, PCR identification or supplementary analysis, such as microarray.

Keywords Genome · Classification · Major pathogens · EV-A71 · CV-A16 · Diagnosis methods

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1 Enteroviruses (EVs)

Discovery of EVs

Hand, foot, and mouth disease (HFMD) is caused by multiple viruses in the genus Enterovirus, family Picornaviridae, including the well-known polioviruses (PVs), coxsackieviruses A and B (CV-A and CV-B), and enteric cytopathic human orphan (ECHO) viruses.

CV-A

In 1948, Dalldor and Sickles conducted a study on poliomyelitis in a suckling mouse model, which had been successfully established for the study of yellow fever virus, to replace the monkey model that was difficult to obtain [1]. Excrements collected from 2 suspected poliomyelitis patients residing in the town of Coxsackie, New York State, were suspended and inoculated into suckling mice, and the suckling mice subsequently developed paralysis symptoms. However, this pathogen was identified to be different from polioviruses and a number of other neurotropic viruses because it caused paralysis development in the muscles rather than the central nervous system and even death in suckling mice; in contrast, no such illness was observed in mature mice, hamsters, or rhesus macaques. Later, it was understood that this isolated virus was the first prototype strain of coxsackievirus A (CV-A).

CV-B

A series of novel viruses were isolated from specimens from asymptomatic poliomyelitis patients' specimens via analysis of special poliomyelitis epidemic patterns. In 1949, Curnen Shaw and Melnick et al. reported the discovery of multiple viruses that were lethal in mice [2] and remarkably different from the virus identified in New York State in terms of antigenicity as well as manifestations in suckling mice. Infection with the New York virus resulted in universal inflammatory myopathies, especially in striated muscle, whereas the newly isolated virus-induced regional inflammatory myopathies were limited in striated muscle; however, injuries to the brain, pancreas, myocardium, and adipose layer also occurred. Later, it was understood that this isolated virus, which was different from the New York virus, was the prototype strain of coxsackievirus B (CV-B) [3].

ECHO Viruses

Enders, Weller, and Robbins innovated the tissue culture platform for poliovirus culture in human cells, which contributed greatly to the development of cellular and molecular biology [4]. Using the tissue culture platform, scientists isolated a

number of previously unknown viruses from specimens collected from several hundred nonparalytic poliomyelitis patients and healthy people worldwide; these viruses were found to be frequently present in human excrement and were capable of proliferating in tissue culture but failed to cause illness in experimental animals. These viruses were called “human orphan viruses” because no corresponding diseases could be identified. Later, they were renamed enteric cytopathic human orphan (ECHO) viruses [5].

It was understood that ECHO virus and coxsackievirus infections could cause multiple diseases, but scientists failed to identify corresponding links between particular viruses and resulting diseases. With further studies of polioviruses, coxsackieviruses, and ECHO viruses, scientists realized that the common features of these viruses were not limited to their residence in the human intestinal tract. Thus, they were named the genus Enterovirus.

Structure and Physical and Chemical Features of EVs

The genome of EVs is single-stranded, positive-sense RNA consisting of approximately 7500 nucleotides, of which approximately 750 are located in the 5′ untranslated region (UTR) and contain RNA secondary structural elements required for replication and translation; moreover, approximately 6700 are located in the open reading frame (ORF) region, and approximately 70–100 are located in the short 3′ UTR, which is needed for replication control. The ORF region encodes a polyprotein consisting of P1, P2, and P3, which is cleaved into 4 structural capsid proteins (VP1–VP4) and 7 nonstructural proteins (2A-2C, 3A-3D) by enterovirus proteases 2A and 3C during and after translation. These viral proteins and some intermediary products play different roles in viral replication. EV capsids consist of 60 subunits arranged in an icosahedral shape; each subunit consists of 4 polypeptides, VP1–VP4. VP1, VP2, and VP3 have a deep groove-like structure that serves as the binding site for receptors, while VP4 is cleaved from VP2 during capsid assembly and located on the internal side of the capsid. Polymerase 3Dpol, protease 3Cpro, and helicase 2C are the most conserved nonstructural proteins [6], whereas helicases 2A, 2B, and 3A are usually highly variable with no homology in the small RNA virus genus [7]. Proteins 2A and 3C are required for viral replication and play essential roles in the interactions between the virus and host. Protein 3D is a type of RNA-dependent RNA polymerase (RdRp) [8].

EVs lacking lipid capsids can remain stable under acidic conditions in the human stomach and survive for several days at room temperature. EV71 and other Evs have been detected in superficial water, groundwater, and thermal water. Evs are resistant to organic solvents (diethyl ether, chloroform), alcohol, and freezing but can be inactivated by temperatures higher than 56 °C, chloride, formaldehyde, and ultraviolet radiation.

Evolution of EVs

EVs are among the fastest evolving viruses [9]. As RNA viruses, EVs have high genetic variability depending upon two different evolutionary mechanisms: mutation and recombination. The lack of 3D polymerase proofreading activity underlies the high mutation rate. Due to the availability of good database references, the major antigen protein VP1 genome region (approximately 900 nucleotides for the whole gene, or gene fragments) is frequently used for evolutionary analyses. Bayesian molecular clock analyses of the VP1 gene sequence showed that the evolutionary rate of the VP1 gene is 4.1×10^{-3} to 3.07×10^{-2} nucleotide substitutions/site/year [10], and the average number of VP1 substitutions accumulated per site per year is 0.9×10^{-2}. Approximately 1 nucleotide substitution is introduced when a single genome is synthesized by RNA virus polymerase. As a result, the virus population usually exists as quasi-species [11], which comprise viruses that have a relatively similar genome with a difference of only one or several substitutions. Gene sequence variability among complicated virus populations is indispensable for EV proliferation. A mutation may be harmful to a single isolate but may have a positive impact on the whole virus population. Mutations generated during certain viral infections are essential for pathogenicity, such as neurovirulence in poliovirus [12]. The D31G mutation rate in the VP1 gene was found to be higher in those who died due to EV-A71 infection of the central nervous system [13].

Another force driving viral evolution is recombination, in which genome fragments belonging to different RNA strands recombine into a single genome. Recombination among naturally circulating EVs belonging to the same species occurs every couple of years [14, 15]. Interestingly, the evolution of the genome region that encodes viral capsid proteins is independent from that encoding nonstructural proteins [16]. Genes encoding capsids of different types of viruses seldom recombine with each other [17, 18], while genes encoding nonstructural proteins freely recombine with those of different types of viruses belonging to the same species [19]. The viral evolution resulting from mutation and recombination is essential for viral adaptation, transmission, and pathogenicity [12, 20–23]. In infected hosts, mutation and recombination are required to overcome tissue-specific innate immune responses, establish high infectivity, and enhance virulence [24, 25].

Classifications of EVs

EVs belong to the Picornaviridae family, which is one of the most ancient and diverse viral families in the world. The Picornaviridae family consists of genuses Enterovirus, Cardiovirus, and Aphthovirus, among others, and the genus Enterovirus comprises 3 rhinoviruses (RVs) and 10 EVs. EV species are further grouped into different serotypes by serological assays or genotypes by molecular biological assays, and each type has a prototype defined as the first identified virus of that type.

The vast majority of EV prototypes were isolated between 1947 and 1955. Initially, an isolated virus serotype was identified based upon the results of infectivity neutralization assays as well as by its pathogenicity in humans and animals; for example, ECHO viruses were named on the basis of the geographical locations of the isolates. Based on the observations of movement difficulty, skeletal muscle impacts, spastic paralysis, and extensive impact on tissues including the central nervous system caused by infection in a mouse model, coxsackieviruses were classified into groups A and B. The reason ECHO virus was originally classified into one group was due to its unclear association with human disease at the time of discovery. With the discovery of an increasing number of new EVs, it has become very difficult to classify EVs based only upon clinical manifestations because the high diversity of clinical manifestations can easily be attributed to the viral genotype being altered by small differences in the viral genome. Thus, since 1974, all newly discovered EVs with different serological characteristics have been numbered in the order in which they are identified starting from EV68. Thereafter, in addition to RV-A (74 serotypes) and RV-B (25 serotypes), RV-C was identified, and no significant differences in the genomic and virion structures between RVs and human EVs were found [26–28]; therefore, the genus Rhinovirus was reclassified as a species under the genus Enterovirus.

In recent decades, on the basis of phylogenetic analysis of the major EV antigen protein VP1, the International Committee on Taxonomy of Viruses (ICTV) has recommended classifying viruses belonging to the genus Enterovirus into 7 species of true EVs, EV-A to D, and 3 species of RVs, A–C. Since 2012, the modern EV classification scheme has been widely accepted. Later, a number of new classification proposals were included in the 9th volume of viral taxonomy published by the ICTV [29–33].

To date, the criteria for defining EV types rely on differences in the nucleotide sequences of the VP1 genomic region. A virus for which the VP1 gene sequence has less than 75% similarity with known EV types is defined as a new type. Homology of amino acid sequences over 85% is recommended as a supplemental classification criterion [34]. The identification of isolates in clinical specimens was conducted by sequencing approximately 300 nucleotides in the VP1 gene with universal PCR primers [35].

An increasing number of new types of EVs are gradually being identified and defined. Whether a newly discovered EV type recently evolved or jumped from an animal to humans remains unclear. For example, many new types of the 4 human EVs (HEV-A through -D) were originally transmitted from primates to humans [36, 37], whereas poliovirus evolved from CV (EV-C) [38]. Studying the emergence mechanisms of new EV subtypes is vitally important for understanding newly reported EV infections and forecasting alterations in EV virulence.

Currently, in addition to human RVs, the genus Enterovirus consists of 4 human EV species (HEV-A, -B, -C, and -D) and 6 animal EVs: bovine EV (EV-E and -F); porcine EV-B (EV-G); simian EV-A (EV-H); camel EV, discovered in 2015 (EV-I); and macaque EV-J.

The species of EV-A comprise 25 serotypes, including CV-A, EV-A, simian EV, and baboon EV. The major pathogens (CV-A16, EV-A71, CV-A6, CV-A10, etc.) causing HFMD are included in this species: coxsackievirus A (CV-A2, CV-A3, CV-A4, CV-A5, CV-A6, CV-A7, CV-A8, CV-A10, CV-A12, CV-A14, CV-A16), EV-A (EV-A71, EV-A76, EV-A89, EV-A90, EV-A91, EV-A92, EV-A114, EV-A119, EV-A120, EV-A121), simian EV (SV19, SV43, SV46), and baboon EV-A13 (BA13).

The species of EV-B has the largest number of serotypes at 63, including CV-B, CV-A9, ECHO (E), EV-B, and simian EV: coxsackievirus B1, CV-B2-B6, CV-A9, ECHO virus 1 (E-1), E-2–E-7, E-9, E-11–E-21, E-24–E-27, E-29–E-33, EV-B69, EV-B73–EV-B75, EV-B77–EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B110 (chimpanzee), EV-B111, EV-B112 (chimpanzee), EV-B113 (mandrill), and simian EV-SA5.

The species of EV-C consists of 23 serotypes in the poliovirus, CV-A and EV-C: poliovirus 1 (PV-1), PV-2, and PV-3, coxsackievirus A (CV-A1, CV-A11, CV-A13, CV-A17, CV-A19, CV-A20, CV-A21, CV-A22, CV-A24, EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116, EV-C117, and EV-C118).

The species of EV-D is a rare group consisting of 5 serotypes: EV-D68, EV-D70, EV-D94, EV-D111 (human and chimpanzee), and EV-D120 (baboon).

The species of EV-E consists of bovine EV-A, with 4 serotypes: EV-E1, EV-E2, EV-E3, and EV-E4.

The species of EV-F consists of bovine EV-B, with 6 serotypes: EV-F1, EV-F2, EV-F3, EV-F4, EV-F5, and EV-F6.

The species of EV-G consists of porcine EV-B, with 16 serotypes: EV-G1 to EV-G16.

The species of EV-H consists of 3 simian viruses (SV4, SV28, and SA4) isolated in 1950 and an A-2 plaque virus. They are grouped into a single serotype, EV-H1, due to their extensive similarities at the molecular level.

The species of EV-J consists of 6 simian EVs: SV6, EV-J103, EV-J108, EV-J112, EV-J115, and EV-J121.

The species of Rhinovirus A contains the vast majority of 80 serotypes: rhinovirus A1, A2, A7–A13, A15, A16, A18, A19–A25, A28–A36, A38–A41, A43, A45–A47, A49–A51, A53–A68, A71, A73–A78, A80–A82, A85, A88–A90, A94, A96, and A100–A109.

The species of Rhinovirus B consists of 32 serotypes: B3–B6, B14, B17, B26, B27, B35, B37, B42, B48, B52, B69, B70, B72, B79, B83, B84, B86, B91–B93, B97, and B99–B106.

There are some challenges and resulting disagreements regarding the classification of rhinovirus C. It is speculated that these viruses are closely correlated to rhinovirus A (thusly named rhinovirus A2) based on abundant sequencing results [39, 40], while other studies tend to name them rhinovirus C [41–44] or rhinovirus X [45]. To date, rhinovirus C consists of 55 serotypes (C1-C55).

In addition to the abovementioned 12 species, the genus Enterovirus contains other EVs that are not classified: simian EV (SV-47) (designated as a certain species

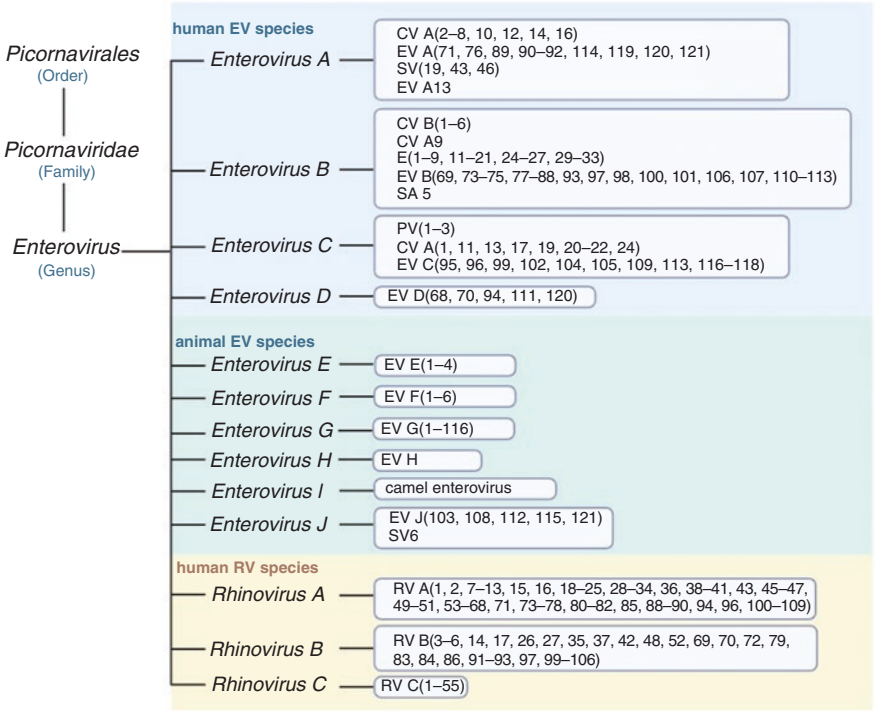

Fig. 2.1 Taxonomy of enteroviruses

due to a lack of genomic sequencing) and EV-122 and EV-123, which do not match any of the existing species (Fig. 2.1).

2 EV-Associated Diseases

The genetic diversity among EVs contributes to the variation in the manifestations of EV infection. EVs classified into the same species with similarity at the amino acid level might induce manifestations that vary widely upon infection, including vesicles on the skin mucosa, flu-like symptoms, and neurological disorders. EVs frequently replicate in the mucosa of the oropharynx and intestinal tract due to their resistance to body temperature and acidic conditions after fecal–oral or direct contact transmission. Then, EVs pass through the intestinal barrier and travel through the lymph nodes to the blood, causing primary viremia and subsequent symptoms in multiple tissues. In some instances, viral replication might lead to persistent viremia and the development of neurological disorders with unknown pathogeneses. The vast majority of EV infections are asymptomatic, but more than 20 clinical syndromes are widely recognized to be attributed to EV infections. Clinical

manifestations and disease severity are associated with age, sex, host immune status, etc. Such syndromes are characterized by short-term disease or central nervous system disease, paralysis, or even death.

Hand, Foot, and Mouth Disease

EV-associated vesicular stomatitis, i.e., hand, foot, and mouth disease (HFMD), is an acute infectious disease caused by coxsackievirus A and B and EV-A71. The incubation period of HFMD is 2–10 days, with a general disease course of 7–10 days. The common manifestations include fever and discomfort. Painful sores in the mouth and a blistering rash on the distal end of the hands and feet (fingers, palms, toes) and occasionally on the buttocks and groin develop 1–2 days later, which is followed by the rapid development of small vesicles. In most cases, the disease is mild and self-limiting and resolves within approximately 1 week without sequalae. Occasionally, aseptic meningitis, encephalitis, acute flaccid paralysis, neurogenic pulmonary edema, myocarditis, and other neurological disorders may develop and subsequently lead to permanent paralysis and even death.

The clinical features of HFMD were first described in New Zealand and Canada in 1957. In April 1957, Seddon submitted to the General Practitioner Institute of New Zealand Research Council a descriptive report on 8 clinical cases of a new childhood illness that occurred in the Mangakino region of Northern Island, New Zealand. No virological studies were conducted at that time. Robinson, Doane, and Rhodes reported the outbreak of a febrile illness characterized by a sore throat and skin rash over a period of approximately 4 weeks in June and July 1957 in an area encompassing 2300 acres located 10 miles from the northeastern part of Toronto. A total of 60 persons among 115 members of 27 families developed clinical symptoms, typically characterized by fever, oral, and faucial lesions and a bullous rash caused by macular rash. All the clinical cases were mild, and patients were not admitted to the hospital and recovered completely. The highest prevalence of this disease occurred in children 1–9 years old, and these children were considered to have transmitted the disease to their family members. A type of coxsackievirus A was isolated from 71% of the patients and was shown to be serologically associated with CV-A16 [46].

In 1960, Alsop, Flewett, and Foster reported an outbreak of a mild infectious disease characterized by maculopapular or vesicular exanthem with oral and pharyngeal and hand, foot, and buttock lesions that occurred in Birmingham from June to the beginning of August 1959. CV-A16 was isolated from vesicular fluid from the hand of a patient; the disease was then named “hand, foot, and mouth disease” for the first time. Later, a number of sporadic cases were reported by Flewett et al. in Birmingham, neighboring Walsall and Bristol, from 1960 to 1961. The clinical features were similar to those previously described. However, the viral isolates from these cases were identified as CV-A5 instead of CV-A16. Similarly, this disease was described as “hand, foot, and mouth disease” [47, 48].

In the following decades, multiple HFMD outbreaks occurred in many countries throughout the world; in the 1960s, several outbreaks occurred in New Zealand, the United Kingdom, and the United States of America (USA) [48, 49]. Forty-four and 47 deaths resulted from HFMD outbreaks in Bulgaria in 1975 and Hungry in 1978, respectively [50, 51]. In the 1970s, HFMD first emerged in Japan [52], and multiple outbreaks were subsequently reported in Japan, mainland China, Thailand, Vietnam, Singapore, Malaysia, Brunei Darussalam, and other Asia-Pacific countries [53–60]. In 2008 and 2009, large outbreaks occurred in mainland China and resulted in the inclusion of HFMD as a category C infectious disease in the national notifiable communicable disease surveillance system.

Improvements in viral detection assays and disease surveillance are greatly facilitating a better understanding of the etiology of HFMD. In the past 50 years, at least 23 EV serotypes belonging to 2 different species have been identified to cause HFMD. CV-A16, isolated in 1958, was the 1st pathogen confirmed to cause HFMD, followed by EV-A71 isolated in the USA in 1969. Since then, periodic HFMD epidemics have been reported worldwide. These outbreaks were alternatively caused by infections with CV-A16 and EV-A71, and they have been generally recognized as the most relevant pathogens of HFMD to date.

Poliomyelitis

The illness caused by poliovirus infection has been recognized since the time of ancient Egypt [61, 62]. In 1840, German orthopedist Dr. Jacob von Heine first classified poliomyelitis as a specific clinical disease entity. In 1890, the Swedish pediatrician O. Medin suggested the infectious nature of this disease based on its epidemic dissemination pattern. Poliomyelitis mainly affects children under 5 years old, and there is no antiviral drug for the treatment of poliomyelitis; only prevention is possible.

Poliomyelitis is associated with several clinical manifestations. Asymptomatic poliomyelitis is mild and resolves within approximately one week, with no symptoms of nervous system damage [63]. Nonparalytic poliomyelitis is a type of serous meningitis caused by poliovirus infection, but patients can fully recover. In 1% of poliomyelitis patients, poliomyelitis may rapidly progress to the most dangerous form, paralytic poliomyelitis, with damage to the central nervous system [64]. The recovery period may last up to 2 years, with permanent stable paralysis, contractures, and deformations. Irreversible paralysis usually develops in the legs in 1 out of 200 patients. Patient mortality can reach 10% due to the expansion of paralysis to the respiratory muscles. In particular, contracting the disease during the early stage of pregnancy may lead to miscarriage or preterm birth [65]. However, most women infected with poliomyelitis during pregnancy can give birth at full term. The fetus is typically affected by toxins and hypoxia rather than by direct transmission of the infection. Respiratory disorders pose a great threat and remain even after the acute phase of the disease.

Since the end of the nineteenth century, poliomyelitis epidemics have become very common. In the mid-twentieth century, poliomyelitis vaccines were successfully developed and widely used. Active prevention measures, including the extensive administration of vaccines, contributed to a 99% decrease in the incidence of poliomyelitis by 1988 [66]. In 1988, the World Health Organization established the target of worldwide poliomyelitis eradication by the year 2000 [67]. Currently, only Afghanistan and Pakistan are considered to exhibit high risks of poliomyelitis outbreaks. In 2013, a new strategic plan for poliomyelitis eradication by 2018 was promoted at the global vaccine summit in Abu Dhabi.

Other Diseases

According to a report published in 1874, there was an outbreak of pleurodynia in Iceland in 1856, which was the first reliable description of the disease caused by nonpolio EV infection that was subsequently named epidemic myalgia [68]. The disease is usually caused by CV-B infection and occasionally by infections with CV-A and certain types of ECHO viruses [69]. The common clinical symptoms include myositis of the upper abdominal muscles and pectoral muscles, fever, and headache, and the prognosis is generally good; patients usually recover in 7–8 days, although some of the patients may develop severe complications that lead to death.

The clinical manifestations of EV infections also include flu-like symptoms, such as nasopharyngitis, rhinopharyngitis, rhino-nasopharyngitis, epipharyngitis, or those associated with the common cold, all of which have a good prognosis and generally resolve within 1 week. These symptoms are frequently caused by rhinovirus infections. Some EV infections, such as those caused by EV-68, may lead to severe complications, such as pneumonia [70].

Aseptic meningitis is a viral infectious disease that affects people of all ages. The most common pathogens of this disease are nonpolio EVs [71], such as CV-A and CV-B, ECHO viruses, and EV69 and EV73 [72, 73]. The clinical manifestations include headache, fever, muscle aches, stomachache, stiffness in the neck, and other possible symptoms, such as minor sensitivity, rash, nausea, diarrhea, sore throat, and cough. Generally, this disease resolves within 7–10 days, with a good prognosis. However, in some newborn patients, the disease may progress to encephalitis with focal neurologic symptoms, and the prognosis may be very poor due to heart failure, liver damage, or even death [74].

Enteroviral encephalitis accounts for approximately 5% of cases of EV infections [75]. The major pathogens of this disease are CV-A and -B, ECHO viruses [76], and EV-A71 [75, 77]. The disease involves inflammation of the brain with common symptoms, including fever, vomiting, headache, weakness, consciousness disorders, cramping, behavioral disorders, and mild paralysis, and severe cases may result in coma. Acute cerebellar ataxia, drop attacks, and hemichorea may occur in

children. Enteroviral encephalitis is a severe disease that poses a great threat to life [78]. Encephalitis caused by EV-A71 infection is usually associated with high mortality [75, 79].

3 Common Pathogens of HFMD

EV-A71

Discovery of EV-A71

EV-A71 was first isolated in California, USA, in 1969 [80]. Large outbreaks of EV-A71-associated HFMD were reported in the USA, Europe, Australia, and Asia [81–84]. Severe neurological disorders attributed to acute EV-A71 infection were first reported in Bulgaria [50] in 1975 and Hungry [51] in 1978. More than 20 years later, such large outbreaks of HFMD with high mortality re-emerged in Malaysia, Taiwan, and mainland China. Since then, multiple EV-A71-associated HFMD outbreaks with high incidences of neurological disorders and high mortality have been reported in the Asia-Pacific region, and EV-A71-associated HFMD has become an essential public health issue in Asia-Pacific countries.

Structure and Genomic Features of EV-A71

EV-A71 belongs to the genus Enterovirus, family Picornaviridae. The EV-A71 virion has an icosahedral structure consisting of a nonenveloped capsid with a positive-sense RNA genome of approximately 7.5 kb. Picornavirus capsids comprise 60 subunits (prokaryon), and each subunit contains four structural viral proteins—VP1, VP2, VP3, and VP4. The surface of a picornavirus capsid is formed by subunits VP1, VP2, and VP3, whereas VP4 is a small protein attached to the inner surface of the capsid that is not impacted by the host antibody response.

According to the nucleotide sequences of the EV-A71 prototype BrCr, the whole genome of EV-A71 comprises an open reading frame (ORF) encoding a polyprotein made up of 2194 amino acids flanked by 5′ and 3′ untranslated regions (UTRs). A poly-A fragment with variable length is located on the terminus of the 3′UTR. The polyprotein is further cleaved into 3 precursor regions, namely P1, P2, and P3. P1 encodes 4 structural proteins, including 1A-1D (VP1-4); P2 encodes nonstructural proteins 2A-C; and P3 encodes nonstructural proteins 3A-D.

The VP1 protein is exposed and usually the target of host neutralization antibodies, which subject VP1 to persistent immune selection pressure, driving the adaptive evolution of multiple EVs. In addition, the VP1 gene is deemed to play a critical role in the pathogenicity and virulence of EV-A71 [85–87].

Genogroups and the Evolution of EV-A71

Based upon the comparisons and analysis of complete VP1 sequences for EV-A71 isolates worldwide in the past 30 years, an EV-A71 phylogenetic tree was generated, dividing EV-A71 into 3 distinct genotypes, namely A, B, and C [88]. Genotype A consists of a single EV-A71 prototype, BrCr-CA-70, with 16.5–19.7% divergence from other isolates. A total of 65 isolates from the USA, Australia, Colombia, and Malaysia (Sarawak state) detected from 1972 to 1997 represent genotype B. A total of 47 isolates from the USA, Australia, Taiwan, Canada, and mainland Malaysia represent genotype C. According to the amino acid sequences, the homology of the VP1 nucleotide sequence of genotype A (prototype BrCr-CA-70) with those of other EV-A71 isolates is 94.2–96.0% and at least 97.9% and 98.9% for genotypes B and C, respectively.

EV-A71 genotypes B and C are further divided into five subgenotypes each, namely B1-5 and C1-5 [88–93]. Based upon retrospective studies [88, 94–97], the B0 subgenotype (a possible precursor of B1 and B2) emerged in the 1960s and was identified as the predominant circulating strain together with the now-extinct genotype A during that time. In the European and American regions, subgenotype B1 was the predominant circulating strain in the 1970s, and subgenotype B2 was the predominant circulating strain in the 1980s. In the Asia-Pacific region, the circulating strains in Australia and Japan [89, 98] were similar to those in the European and American regions. The B3 and B4 subgenotypes were first identified during an outbreak in Sarawak state, Malaysia, which occurred from April to August 1997 and was the first large outbreak of HFMD in the Asia-Pacific region [99]. Then, the B3 subgenotype emerged in Singapore and Australia and in Hong Kong, China, and subsequently disappeared from the Asia-Pacific region. The B4 subgenotype then became the predominant circulating strain in the Asia-Pacific region starting in the late 1990s, leading to multiple large outbreaks in Malaysia and Taiwan, China, from 1999 to 2003 [100]. The B5 subgenotype was first identified during the EV-A71-associated HFMD outbreak in Malaysia in 1999 and became the predominant circulating strain in Malaysia, causing 2 large outbreaks in 2003 and 2005–2006 [101]. Since 2003, the B5 subgenotype has been one of the most important predominant circulating strains in the Asia-Pacific region.

The C1 subgenotype of EV-A71 genotype C started to circulate at the end of 1980 and was the predominant strain in the European and American regions before 1995. The C2 subgenotype emerged in 1995 and has been circulating alternately with the C1 subgenotype in the European and American regions since that time. In the Asia-Pacific region, the C1 and C2 subgenotypes have been reported in multiple outbreaks in Malaysia since the end of 1990. The C2 subgenotype was the predominant strain of EV-A71 in the large outbreak of HFMD in Taiwan, China, in 1998 [55, 102]. The C3 subgenotype was the first identified genotype C strain in the Asia-Pacific region, with only sporadic cases in Japan and mainland China. The C4 subgenotype was first isolated in Japan, and its first corresponding large outbreak occurred in Shandong Province, mainland China, in 2007 [103]. Then, it caused a

severe outbreak with high mortality and morbidity in Fuyang, Anhui Province, mainland China, in 2008 [104]. Since then, the C4 subgenotype-associated HFMD has been endemic in mainland China, and its incidence and mortality were ranked the highest of the category C infectious diseases in Law of the People's Republic of China on prevention and control of infectious diseases. The C5 subgenotype first emerged in Vietnam and became the predominant strain, causing sporadic cases mainly in Taiwan, China. Currently, the C4 and B5 subgenotypes are the major predominant circulating strains in the Asia-Pacific region.

Almost all the EV-A71-associated HFMD outbreaks in the Asia-Pacific region in the past 10 years were caused by new, not yet classified EV-A71 subgenotypes, which have aroused scientists' attention to their origins and genetic evolution. Phylogenetic and population genetic analyses [105] of VP1 sequences of isolates collected over more than 40 years from around the world demonstrate that the common ancestor of human EV-A71 diverged from its close ancestor CV-A16 in approximately 1941 and subsequently differentiated into genotypes B, C and now-extinct A. Bayesian MCMC analyses were performed using a relaxed molecular clock model to estimate the origin date of each subgenotype. The evolution speeds of the VP1 gene of genotypes B and C are estimated to be 4.5×10^{-3} to 4.6×10^{-3} and 4.2×10^{-3} substitutions/site/year, respectively. The molecular clock model showed that the common ancestors of the B1, B2, B3 and B4, and B5 subgenotypes could be traced back to approximately 1967, 1978, 1993–1994, and 2001, respectively, while those of the C1, C2 and C4, C3, and C5 subgenotypes could be traced to 1983, 1992, 1998, and 2002, respectively. Different subgenotypes of viruses usually circulate for 2–5 years before causing large outbreaks. As an RNA virus, new periodic outbreaks of EV-A71 infection are continuously reported in Asian regions due to the lack of a proofreading mechanism for genome replication and transcription and the resulting rapid mutation, whereas the cocirculation of different subgenotypes in the same region might contribute to potential recombination.

EV-A71-Associated Diseases

HFMD is a common disease caused by EV-A71 infection. There is a close genetic relationship between EV-A71 and CV-A16, and HFMD caused by EV-A71 infection is not significantly different from that caused by CV-A16 clinically, except for a few clinical observations in the Asia-Pacific region indicating that the skin rash in HFMD caused by EV-A71 and CV-A16 infection might be different [57, 106]: the skin rash related to CV-A16 infection is characterized by large vesicles, whereas the skin rash related to EV-A71 infection is characterized by papules, petechiae, and frequent diffuse erythema on the trunk and limbs. However, the tendency of neurological complications in the acute stage of EV-A71 infection is a unique feature that is not observed in CV-A16 infection.

Since its first discovery, EV-A71 has long been considered highly neurotropic and associated with a variety of neurological disorders, including aseptic

meningitis, brainstem encephalitis, and acute flaccid paralysis. However, the factors that determine whether the clinical outcome of EV-A71 infection is asymptomatic, HFMD, or severe neurological disorders remain unclear. There were significant differences in the incidence of mild HFMD and severe neurological disorders caused by EV-A71 infection in different outbreaks. For example, only 7% of HFMD cases had concomitant neurological symptoms in HFMD outbreaks in Perth, Australia [57], while 80% had concomitant neurological symptoms in Taiwan, China [107]. The most common clinical features of sporadic EV-A71 infection cases are mild HFMD signs, with few reports of neurological symptoms [108]; this finding most likely suggests that EV-A71 strains prevalent during a particular EV-A71 outbreak differ widely in terms of dermatotropism and neurotropism and that there is no strict link between these two pathogenic features.

According to previous epidemiological data on EV-A71, the incidences of neurological disorders and other complications tend to vary among different HFMD outbreaks in Asian countries, indicating a difference in virulence among different EV-A71 subgenotypes. However, there is no single "neurotoxic" subgenotype that can be directly linked to severe illness or even death. Thus, exploring the determinants of the virulence of the strains would play a key role in elucidating an understanding of the pathogenesis of severe neurological disorders. A molecular genetics study on virulence in poliovirus, which belongs to the Enterovirus genus as EV-A71, showed that a minor sequence difference in the genome-restriction region (in particular, the 5′ nontranslated region and the VP1 gene) is sufficient to cause large variation in neurovirulence among different viral strains, suggesting that the neurovirulence phenotype of EVs might be determined by slight genetic differences [109]. For example, the closely related EV-A71 viruses isolated in Japan and Bulgaria in the mid-1970s belonged to the same subgenotype [110], but mild HFMD cases were reported in the outbreak in Japan [52, 111], while poliomyelitis-like neurological disorders rather than typical HFMD symptoms were reported in the outbreak in Bulgaria [50]. In addition, genome homology between the C1 and C2 subgenotypes was high, with VP1 nucleotide sequence homology at 92%; however, the C1 strains were only isolated from mild HFMD cases with potentially lower neurovirulence [89], while the C2 strains led to substantial acute flaccid paralysis and brainstem encephalitis in outbreaks in Taiwan, China, and Western Australia. Based on strict alignment of the incidence of neurological disorder during infection with EV-A71 strains isolated in specific outbreaks or specific patient populations, some virulence determinants of EV-A71 strains have been identified. For example, the strains causing severe neurological disorders in the outbreak in Perth, Australia belonged to the C2 subgenotype, of which the amino acid 170 of the VP1 gene mutated from alanine in other lineages to valine (A → V) [88, 112]. This locus was located on the joints of VP1, VP2, and VP3 and was considered to be the binding site of the virion to the cellular receptor [113]. The A → V mutation exhibits increased hydrophobicity at this site, which may lead to a subsequent conformational change and enhanced neurovirulence.

CV-A16

Discovery of CV-A16

CV-A16 is another important pathogen of HFMD [114]. CV-A16 was first isolated in South Africa in 1951 [115]. The first HFMD outbreak described in Toronto, Canada, in 1957 was caused by CV-A16 infection. The relationship between HFMD and CV-A16 infection was confirmed in 1959, and HFMD was first named according to the clinical manifestations [47]. Later, multiple HFMD outbreaks caused by CV-A16 infection were subsequently reported, including outbreaks in Sydney, Australia, in 1991 [116]; England and Wales in 1994 [117]; Taiwan, China, in 2002–2003 [118]; Singapore in 2002, 2005, and 2007 [119]; Vietnam in 2005 [93]; Odisha, India, in 2009 [120]; Beijing, mainland China, in 2007 [121]; and Guangzhou, mainland China, in 2009 [122].

Structural and Genomic Characteristics of CV-A16

CV-A16 is classified as human enterovirus type A (HEV-A) belonging to the genus Enterovirus of Parvoviridae. CV-A16 is an unenveloped icosahedral particle approximately 30 nm in diameter that contains a 7.4 kb single-stranded, sense, polyadenylated viral RNA genome. The genome contains an open reading frame (ORF) encoding a large polyprotein precursor that is subsequently processed into structural proteins P1 and nonstructural proteins P2 and P3. P1 is cleaved into the viral capsid subunit proteins VP0, VP1, and VP3 by virus-encoded protease, and VP0 is further cleaved into VP2 and VP4. VP1, VP2, and VP3 are located on the outside of the capsid, while VP4 is located on the inside of the capsid. The neutralizing epitopes of the virus are mainly located on VP1. The coding regions of the genome are flanked by the 5' and 3' nontranslated regions. The 5' nontranslated region consists of approximately 740 nucleotides and contains sequences that control genome replication and translation, such as the internal ribosome entry site (IRES). The 3' nontranslated region contains the poly-A tail, which is critical to the infectivity of the virus [123].

Genotypes of CV-A16

Currently, based upon the phylogenetic tree and genetic diversity of the VP1 gene, CV-A16 is classified into 3 genotypes, A, B and D, and genotype B is further divided into subgenotypes B1, B2, and B3. Before 2000, B2 was the predominant subgenotype, but it has been replaced with B1 in recent years as the predominant circulating subgenotype worldwide [124, 125]. The B1 subgenotype consists of the B1a, B1b, and B1c branches. B1a was first reported in Japan in 1995 and subsequently became the predominant subgenotype in mainland China, Malaysia, and Thailand

[126–128]. B1b is the recombinant of CV-A16, CV-A4, and EV-A71. It was cocirculating with B1a in China from 1999 to 2008 [129, 130] and has become the predominant strain in China since 2010 [131–133]. The B1c branch was first identified in Malaysia between 2005 and 2007 and has since been reported in Russia, France, India, Japan, and other countries and caused a HFMD outbreak in India in 2013 [126, 134, 135]. In 2017, 2 new CV-A16 strains were isolated in Shenzhen, mainland China, and were classified as the B3 subgenotype [131]. The D genotype was first identified in Peru in 2009, and this newly emerged genotype has been circulating in multiple countries in recent years [135–137]. The D genotype has undergone multiple recombination events during transmission, which may alter the biological characteristics of the virus, thus altering its transmissibility and pathogenicity [138]. In 2016, the first outbreak and transmission of the CV-A16 D genotype was reported in Shanghai, mainland China [137].

From the epidemiological data, CV-A16 fails to show a high-frequency transmission between different regions as do other viral pathogens with global distribution, such as influenza and norovirus. The geographical limitation of transmission might be due to the fact that the majority of patients infected with CV-A16 are younger than 5 years of age, with a large proportion aged 1–3 years [139–141]. However, imported transmission may also lead to regional prevalence of CV-A16-associated HFMD, suggesting the importance of the close surveillance of viral prevalence in geographically adjacent areas.

CV-A16-Associated Diseases

CV-A16 was the first identified HFMD pathogen. The symptoms of CV-A16 infection are usually mild and self-limited, such as blisters and ulcers on the hands, feet, and mouth and pharyngitis. However, a few cases of severe HFMD caused by CV-A16 infection have also been reported.

The first CV-A16-associated death was reported in the summer of 1959 in California, USA. The patient was a boy aged 7 weeks in whom the primary sign was a tongue ulcer. The patient died 4 days after the development of allergies, dyspnea, tachycardia, cyanosis, convulsions, and other clinical symptoms. The autopsy results indicated the development of encephalitis and myocarditis, and CV-A16 was isolated from the myocardium, blood, and intestinal contents [142]. The second case of death associated with CV-A16 infection occurred in the summer of 1959 in California, USA; this death was attributed to respiratory symptoms accompanied by severe heart failure. The autopsy results revealed severe inflammatory injury in the myocardium, and CV-A16 was isolated from the intestinal tissue of the patient [143]. In 2004, in Taiwan, China, a boy aged 15 months was admitted to the hospital due to convulsions, with symptoms of fulminant myocarditis accompanied by refractory shock. The patient died 6 days after the onset of the disease, possibly from myocardial failure. CV-A16 was isolated from the pharynx and larynx as well as rectal culture [144]. In 2007, France reported a case of fatal adult pneumonia caused by CV-A16 infection. A man aged 76 years was admitted to the hospital due

to acute fever, osphyalgia, and dyspnea without myocarditis or left ventricular dysfunction. The patient died of refractory hypoxemia 28 days later. The autopsy results showed diffuse alveolar damage and fibrosis. CV-A16 was isolated from lung tissue [145]. In 2009, in Japan, a baby aged 23 months developed HFMD accompanied by rhombencephalitis; the patient was diagnosed by magnetic resonance imaging (MRI) based on clinical signs of allergies, dystaxia, myoclonia, and nystagmus occurring 3 days after the onset of typical HFMD, which was caused by CV-A16 virus infection as indicated by the etiology [146]. In addition, of the 92 HFMD cases with neurological disorders reported in Shenyang city, China, 19 were identified to be caused by CV-A16 infection (20.7%), including 2 cases of brainstem encephalitis and 1 case of acute flaccid paralysis [147]. A retrospective study of approximately 20,000 suspected pediatric HFMD patients aged 1 month to 14 years old in a hospital in Shanghai city, China, from 2014 to 2016 showed that a total of 234 patients were diagnosed with EV-A71 or CV-A16 infection with neurological complications (aseptic meningitis, encephalitis, encephalomyelitis, acute walking difficulty, and autonomic nervous system disorder), of which 90 cases and 144 cases were caused by EV-A71 and CV-A16 infection, respectively [148].

EV-A71 and CV-A16 Cocirculation

In recent years, frequent alternating or concomitant circulation of EV-A71 and CV-A16 virus in the Asia-Pacific region has become an essential public health issue. In a retrospective epidemiological survey of serum samples collected from children under 5 years old in different provinces of China in 2005, positivity for anti-EV-A71 and anti-CV-A16 antibodies was found in approximately 30% and 40% of samples, respectively, implicating extensive previous transmission of EV-A71 and CV-A16 in multiple provinces in China had occurred before the large outbreak of HFMD in China in 2008 [149]. Another study involving serum samples collected from healthy children under 9 years old in Guangdong Province, China, in 2007 indicated an anti-EV-A71 antibody positivity rate of 44.6%, an anti-CV-A16 virus antibody positivity rate of 70.3%, and an anti-EV-A71 and CV-A16 antibody double positivity rate of 32.4% [150]. All these results likely suggest widespread asymptomatic or unidentified EV-A71 or CV-A16 infection occurred before the large outbreak of HFMD in China in 2008.

The cocirculation of EV-A71 and CV-A16 might lead to coinfection [151, 152]. The incidence of EV-A71 and CV-A16 coinfection was reported to be 0.62% in Hunan Province from 2008 to 2010, 14.3% in Hangzhou city in 2009, and 7.4% in Beijing city in 2010 [153–155]. In 2011, a study of HFMD patients with central nervous system complications in multiple regions of China showed that coinfection with EV-A71 and CV-A16 caused more severe central nervous system complications than single infection with either EV-A71 or CV-A16 [156]. Additionally, coinfection of EV-A71 and CV-A16 might lead to an increased possibility of genetic recombination [104, 157]. Mutation and recombination are essential aspects of EV

evolution. The lack of proofreading mechanisms in 3D polymerases of EV makes them mutate at an average rate of one mutation per genome synthesized. Recombination of a poliovirus vaccine strain and a wild-type strain by template conversion in negative-strand synthesis has been demonstrated [158, 159]. Recombination between cocirculating EV-B viruses [160], recombination between circulating CV-A9 strains in their nonstructural gene-encoding regions [161], and heterologous recombination between EV-A71 genotypes B and C [162] have been observed and confirmed. In addition to homologous recombination between EVs of the same genotypes, recombination between EVs of different genotypes has been reported. For example, 2 EV-A71 strains (SHZH98 and SHZH03) isolated from mainland China were products of recombination between EV-A71 subgenotype C2 and CV-A16 prototype G-10; the CV-A16 strains (Tainan/5079/98) isolated from Taiwan, China, were products of recombination between EV-A71 subgenotype A and CV-A16 prototype G-10. Phylogenetic analysis, similarity mapping, and promoter analysis based on the whole genome sequencing of 2 EV-A71 strains (SZ/HK08-5 and SZ/HK08-6) and 2 CV-A16 strains (SZ/HK08-3 and SZ/HK08-7) isolated from samples collected during the large HFMD outbreak in Shenzhen city, mainland China, in 2008 confirmed that in EV-A71 strains, recombination occurred between EV-A71 subgenotypes B and C at the 2A and 2B joint regions and between EV-A71 subgenotype B and the CV-A16 G-10 strain in the 3C region; in CV-A16 strains, recombination occurred between the CV-A16 G-10 strain and EV-A71 subgenotype A at the 2A and 2B joint regions [157]. These results most likely suggest the homologous as well as heterologous recombination of EV-A71 and CV-A16 during circulation.

Other Enteroviruses

The pathogenicity of HFMD has changed greatly in the past decade, with new pathogens being identified continuously. Other HEV-A family members, such as CV-A2, CV-A4, CV-A5, CV-A6, CV-A8, and CV-A10, as well as HEV-B family members, such as ECHO1, ECHO4, ECHO6, ECHO7, ECHO19, and ECHO25, CV-A9, CV-B3, CV-B4 and CV-B5, have been identified to be associated with HFMD breakouts or sporadic cases [58, 119, 164–169]. Of these, CV-A6 and CV-A10 have received the most attention. CV-A6 infection is rarely noticed, and its clinical features are mainly associated with herpangina [170, 171]. CV-A10 was detected in some small HFMD outbreaks, while other CV-A were only detected in sporadic HFMD cases [172, 173]. It is worth noting that CV-A6 and CV-A10 are becoming the primary pathogens of HFMD, as their infection incidence has significantly increased in recent years.

In autumn 2008, the first CV-A6-associated HFMD outbreak was reported in Finland [174]. Thereafter, CV-A6-associated HFMD outbreaks occurred in France, the United Kingdom, Spain, Finland, and other European countries [174–180]. CV-A6-associated HFMD cases have also been reported in the Asia-Pacific regions,

where the predominant pathogens of HFMD were EV-A71 and CV-A16 in the past, such as mainland China, India, Singapore, Taiwan China, Japan, Thailand, and Israel [181–194]. Other regions throughout the world, such as the USA, New Zealand, and Brazil, have also been affected [195–199].

CV-A10 has also been identified as a novel pathogen of HFMD [200], which commonly co-occurs with other enteroviruses, such as CV-A6 and CV-B3, worldwide [175, 182, 201–203]. In addition to the HFMD outbreak attributed to CV-A6 and CV-A10 cocirculation in Finland in 2008, CV-A6 and CV-A10 infections accounted for 35.3% of the total tested cases in the largest HFMD outbreak in Singapore in 2008 [192]. CA6 and CA10 were also the major serotypes documented in a French study, causing the outbreak in 2010 [175]. A study conducted in India in 2012 indicated that the primary pathogens of HFMD were CV-A16 and CV-A6, while CV-A10 and EV-A71 were the rare pathogens [188]. Thus, it is necessary to conduct broader regional surveillance to forecast potential HFMD outbreaks caused by CV-A10.

A study of clinical specimens collected from HFMD patients during the outbreak in Finland in 2008 showed that 71% of cases were positive for CV-A6 infection and 28% of cases were positive for CV-A10 infection [204]. The clinical manifestations included typical HFMD-like symptoms and central nervous system complications in some patients. It is difficult to differentiate the clinical manifestations of these two viruses in the acute infection phase. Specifically, it is worth noting some non-typical HFMD symptoms associated with CV-A6 infection, including a sporadic rash around the mouth and on the buttocks, trunk, knees, hands, back and side of the hands and feet, and perianal region; bullous skin reaction similar to an adverse drug reaction; vesicular, eruption-like rash; severe vasculitis-like rash, eczema and blebs or varicella-like rash; and primary immune-related skin-disease-like bullous rash. Furthermore, a typical symptom in this outbreak was fingernail detachment in some patients several weeks post-HFMD [204], and CV-A6 was detected in the detached fingernails. More severe neurological disorders, such as aseptic meningitis and encephalitis, as well as epididymitis, were reported to be associated with HFMD attributed to CV-A6 infection. CV-A10-related HFMD is frequently associated with herpangina; however, CV-A10 was also found to trigger fingernail detachment after HFMD development [205]. In addition, CV-A10 infection can lead to severe HFMD [114].

4 Rapid Diagnosis and Genotyping of EV Infection

The pathogens of HFMD have varied substantially in the past 10 years, and coinfection and genetic recombination of multiple EVs might contribute to the worldwide transmission of HFMD and the resulting diversity of symptoms. All these changes bring challenges to the early diagnosis, treatment, and prevention of HFMD. Rapid identification of HFMD pathogens helps to predict possible symptoms and complications and subsequently facilitates timely and effective clinical treatment as well as

the initiation of proper public health prevention strategies during large HFMD outbreaks.

EV infection is diagnosed through virus isolation and culture, serological assays, PCR identification or supplementary analysis, such as microarray. Isolation of EVs from nasopharyngeal swabs or fecal specimens from patients is deemed the gold standard for diagnosis [79]. Currently, the cell lines used for EV isolation and culture include human striated muscle sarcoma cells, human lung fibroblasts, and Vero cells [206, 207]. EV infection can be confirmed by observed cytopathic effects (CPE) in human striated muscle sarcoma cells 7-10 days post-inoculation, followed by serological confirmation via neutralization assay using specific anti-sera or indirect immunofluorescence assay using specific monoclonal antibody [206]. Additionally, amplification of different VP1 gene regions of the cultured virus with specific or universal PCR primers and subsequent sequencing of the amplification products can be performed to identify and genotype EVs at the molecular level [208].

However, in clinical practice, it is difficult to perform virus isolation and neutralization assays in community clinics in developing countries due to the requirements for high-level biosafety facilities and professional operation skills. An IgM detection assay based on enzyme-linked immunosorbent assay (ELISA) can overcome these difficulties. A study on EV-A71 and CV-A16 IgM showed that specific anti-EV-A71 and CV-A16 IgM could be detected in some patients on the 1st day of illness; 100% IgM detection could be achieved on the 5th day of EV-A71 infection and 8th day of CV-A16 infection, and both IgM titers could be sustained for weeks [209]. ELISA for measuring IgM levels has widely been promoted and used in community clinics for the detection and diagnosis of multiple serotypes of EVs due to the advantages of simple operation, rapid speed, and low cost [210]. Real-time PCR of clinical specimens using EV-specific primers has also been widely applied for clinical diagnosis [211–213] due to the advantages of simple operation, rapid speed, and high throughput. It is the most frequently used molecular approach to etiological diagnosis in certain public health emergencies [214–217]. Multiplex RT-PCR technology can be used to detect and analyze multiple EVs in samples with a single amplification using compatible EV-A71 and CV-A16 and universal EV-specific primers [218]. The efficacy and accuracy of etiological diagnosis based on RT-PCR amplification are mostly dependent upon appropriate sampling time and method as well as optimal sample transportation conditions, all of which require close cooperation among laboratory experts and clinical doctors. Additionally, the microarray assay can be used to detect multiple pathogens in parallel by using pathogen-specific probe hybridization. Some studies have demonstrated significant increases in the levels of proinflammatory cytokines and inflammatory cytokines, such as TNF-α and IL-6, during EV infection [219–223]. In particular, increases in the levels of IL-1, IL-6, IL-10, IL-13, IFN-γ, and TNF were positively correlated with the development of severe HFMD [223–225]. Hence, the detection of cytokines can be used to supplement RTPCR results and provide a reference for disease progress forecasting.

In summary, each detection method has inevitable shortcomings and cannot achieve a 100% detection rate due to the rapid progression of HFMD. For example,

the RT-PCR detection rate may be reduced in severe HFMD patients due to elimination of the virus in the nasopharyngeal region by a strong immune response. Other studies found that only 20% or even fewer EV-A71-infected patients with positive RT-PCR results can be confirmed with virus isolation and cultures from samples [226]. ELISA-based IgM detection requires the prior development of IgM detection kits targeting specific pathogens, which are not sufficiently effective when dealing with emergency outbreaks of unknown infectious diseases. In this regard, the first-line etiological assay in practical clinical treatment and public health prevention is RT-PCR. In some specific settings, immunological assays for the detection of IgM and cytokines can be used to supplement RT-PCR findings and provide a reference for clinical diagnosis. However, virus isolation, culture and serological identification are still nonnegligible approaches in large-scale epidemic situations.

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Chapter 3
Mechanisms Underlying HFMD Clinical Pathology in Children

Xingli Xu

Abstract Hand, foot, and mouth disease (HFMD) is generally recognized as a contagious viral disease that commonly occurs in children worldwide, with a high incidence in Asia–Pacific regions in the past 20 years (Koh et al., Pediatr Infect Dis J 35(10):e285–e300, 2016; Puenpa et al., J Biomed Sci 26(1):75, 2019). Since 2000, major epidemics have emerged in East Asia (Huang et al., Emerg Infect Dis 24(3):432, 2018), and numerous large outbreaks have been reported in Taiwan (Chang et al., Pediatrics 109(6):e88, 2002), China; Anhui Province, mainland China; Australia (McMinn et al., Clin Infect Dis 32(2):236–242, 2001); and Southeast Asia (Khanh et al., Emerg Infect Dis 18(12):2002–2005, 2012). Several million HFMD cases have been reported in children. In approximately 1.5–2% of fatal cases, evidence of neurological disorders is identified (Koh et al., BMJ Glob Health 3(1):e000442, 2018; Gonzalez et al., Int J Mol Sci 20(20):5201, 2019), and in some cases, death may be potentially attributed to neurogenic pulmonary edema (PE) or cardiovascular complications occurring within a short time of damage to the nervous system (Wang et al., Pathology 48(3):267–274, 2016). Although cases of fatal HFMD account for only 0.1% of all cases, because HFMD primarily affects children under 2 years of age (Chan et al., Clin Infect Dis 31(3):678–683, 2000), HFMD, especially fatal HFMD, is a public health issue worthy of attention.

Keywords Clinical manifestation · Pathogenesis · Immune · Inflammatory · Injury

1 Clinical Manifestations of HFMD

Because of the occurrence of long-term epidemics of HFMD, numerous causative pathogens have been successively identified, such as coxsackievirus and echovirus, which both belong to the genus Enterovirus [1–5]. A specific type of enteroviral

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infection can lead to more than one clinical manifestation, and the same clinical manifestations can be observed in patients with different types of enteroviral infections. Thus, the clinical manifestations of HFMD tend to vary and are categorized primarily as typical mild HFMD, atypical HFMD, and fatal HFMD in children.

Typical Mild HFMD in Children

Typical mild HFMD is frequently seen in children under 5 years of age, accounting for approximately 90% of all HFMD cases, with the number of boys infected being greater than the number of girls. Typical HFMD is commonly attributed to EV-A71 or CV-A16 infection, with a small number of HFMD cases attributed to infections with CV-A6, CV-A10, CV-A4, or other coxsackieviruses and echoviruses [6–10]. Typical HFMD patients usually present with sudden fever with body temperatures higher than 39 °C, although some HFMD patients present with no or low fever. Moreover, some patients present with forms of skin rash, such as maculopapules, papules, and blisters, numbering from several to dozens, on the mouth, hands, feet, buttocks, etc., co-occurring with fever or presenting 1–2 days post-fever. The area may be flushed with inflammatory cells surrounding the skin rash, and there may be little liquid inside the blisters, no pain or itching, and no scab or scar formation after resolution of the skin rash. Additionally, some patients cough or vomit or develop diarrhea, rhinorrhea, or anorexia; other patients develop only a skin rash or herpangina; and very few patients fail to develop skin rash (Fig. 3.1, Chinese guidelines for the diagnosis and treatment of hand, foot, and mouth disease (2018 edition)) [11, 12].

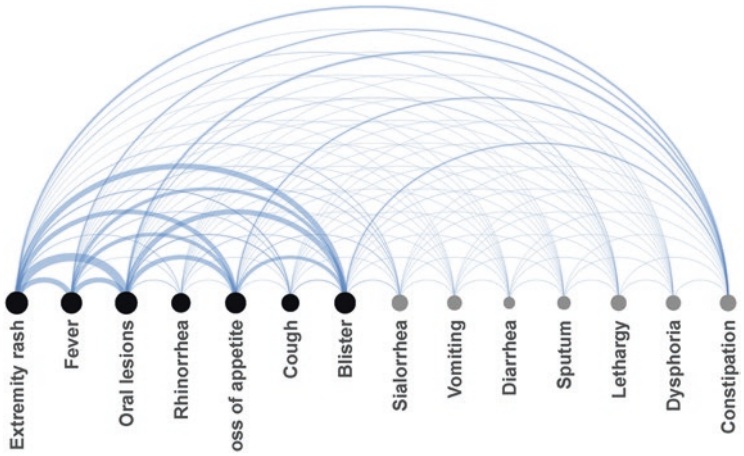

Fig. 3.1 The arc diagram of symptoms and signs of patients with mild HFMD [11]. Reused with permission from [11]

Atypical HFMD in Children

With the continuous changes that have occurred in the causative pathogens of HFMD in recent years, an increasing number of atypical HFMD cases have been reported, with an increase of approximately 7% in total HFMD cases [13, 14]. Atypical HFMD is commonly caused by CV-A6, CV-A10, or CV-A16 infection [8, 9]. Atypical HFMD in children progresses slowly, usually with mild clinical symptoms, such as a body temperature lower than 39 °C and a skin rash similar to blisters, eczema, or varicella. An atypical skin rash, characterized by few or very few, small, thick, and hard petechiae and the presence of ecchymosis, may be observed in some patients. The severe skin rash associated with infection with certain enteroviruses, such as CV-A6, shows bullous alterations accompanied by pain and itching not only in the mouth and on the hands and feet but also occasionally on the trunk and in other regions of the body [13, 15–17] (Fig. 3.2).

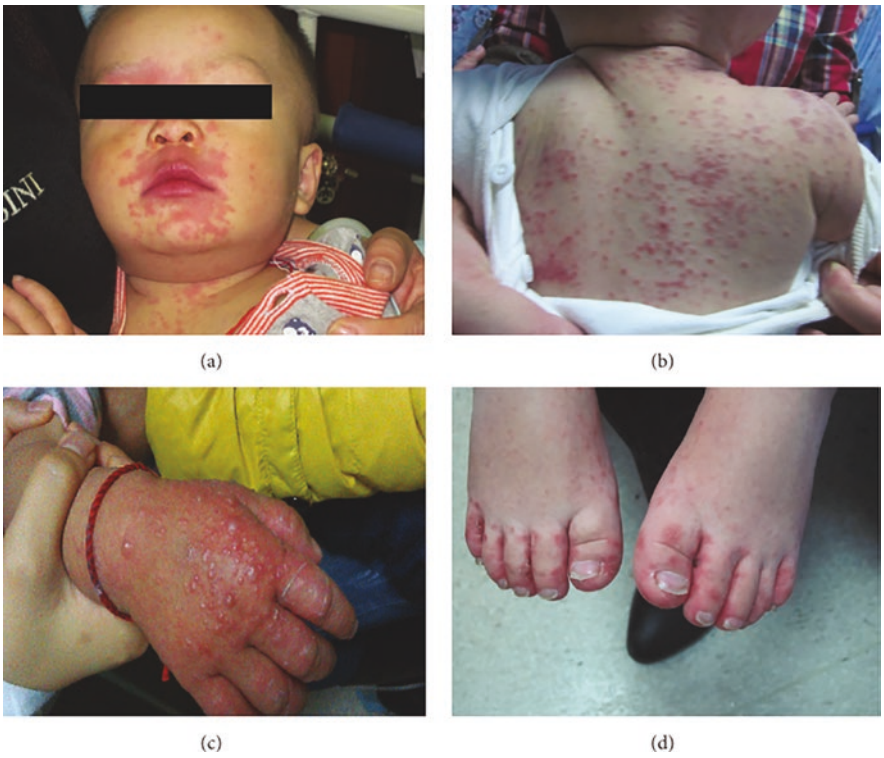

Fig. 3.2 Characteristics of rashes in atypical HFMD. (**a**) The papulae were mainly distributed on the perioral area and face; (**b**) the papulae/vesicles were found at the back; (**c**) the papulae were found on the hand; (**d**) the erosions were noted in both feet. Reused with permission from [13]

Fatal HFMD in Children

Fatal HFMD usually occurs in children 5 years of age or younger who develop a high-grade fever in addition to the typical HFMD symptoms involving the nervous system, respiratory system, cardiovascular system, and gastrointestinal system. Nervous system disorders include brainstem encephalitis, poliomyelitis, and typical aseptic encephalitis, with the major clinical symptoms of lethargy, general weakness, coma, epileptic seizure, hyperphagia, neck stiffness, and dysregulated pupil dilation [18–20]. Respiratory system symptoms usually include tachypnea, dyspnea, hemoptysis, cyanosis, moist rales in the lungs that are obviously exacerbated within a short time, frothy sputum, pneumorrhagia, PE, and even alveolar consolidation [21]. Cardiovascular system symptoms include pale complexion, tachycardia, cyanosis, cold extremities, prolonged capillary refill time, hypotension, hypertension, and even rapid progressive heart and lung function failure. Some patients develop gastrointestinal symptoms, such as vomiting and diarrhea, and others develop hepatic and renal dysfunctions [22].

In China, the initial diagnosis and treatment of classic severe HFMD is based on the central nervous system (CNS) symptoms, which include lethargy, weakness during sucking, jumpiness, headache, vomiting, irritability, limb tremors, myopathy, and nuchal rigidity, although most of these manifestations will resolve. However, when symptoms of early cardiopulmonary failure, including increased heart and respiratory rates, cold sweat, cold extremities, mottled skin, increased blood pressure, etc., are evident, patients are considered to be in the critical stage of HFMD. Early diagnosis and proper treatment are critical for reducing mortality. Classic severe HFMD may rapidly progress to the early cardiopulmonary failure stage. More seriously, some patients may develop cardiopulmonary failure characterized by tachycardia (bradycardia is also occasionally seen), tachypnea, cyanosis, cough with pink foamy or bloody sputum, hypotension and, ultimately, cardiovascular collapse. Some patients develop severe encephalopathy, convulsions, or coma, and this stage constitutes critical HFMD and is frequently accompanied by high mortality (Chinese guidelines for the diagnosis and treatment of hand, foot, and mouth disease (2018 edition)).

Approximately 70% of severe HFMD cases are attributed to EV-A71 infection [23]. With the administration of inactivated EV-A71 vaccines, more severe HFMD cases are frequently reported to be caused by CV-A16 infection. Of all severe HFMD cases, approximately 0.1% are fatal [24]. In summary, HFMD poses a great threat to the health and life of children.

2 HFMD Pathogenesis

HFMD is a contagious disease attributed to multiple and diverse causative pathogens, and the molecular mechanism underlying HFMD pathogenesis is largely unclear. According to existing case data, viral infection tends to lead to a variety of

clinical manifestations and disease states in different patients. These differences have been linked to the presumption that during infection all of the following variables likely affect the pathological course and ultimate disease outcomes: differences in virus genotypes or serotypes; the biological features of the virus itself; stability of the host internal environment; host gene polymorphism(s); host cell interactions with the virus, especially the interactions between host immune system and the virus; and the related host microenvironment. Therefore, the pathogenesis of HFMD is described on the basis of this presumption.

Biological Features of Enteroviruses and HFMD Development

Etiology of Enterovirus Infection

The effective replication of viruses in host cells is key to pathogenicity. Through the interactions between viral self-coding proteins or noncoding regions and multiple proteins in host cells, viruses may inhibit transcription and translation in host cells to promote self-proliferation (Fig. 3.4). In the EV-A71 viral genome, the 5′ coding region consists of a VPg protein covalent ligation site, the 5′ noncoding region consists of an internal ribosomal entry site (IRES), and the 3′ untranslated region (UTR) is followed by a polyadenylation tail. The IRES can ensure that RNA is synthesized in the complete clover-like structure and determine the direction of mRNA transfer [26]. The 3′ noncoding region is the major binding site for the transcriptional complex. Additionally, other noncoding regions, precursor proteins, and mature proteins also play roles in viral infections [27].

An increasing number of studies have demonstrated that the VP1 protein plays an essential role in EV-A71, CV-A16, and CV-A6 infection and virulence and that VP1 protein mutation might lead directly to failures in viral infection or changes in viral virulence. The VP1 region contains some essential neutralization antibody-targeted sites, and these sites are essential targets for HFMD vaccine development. Structural analysis of the EV-A71 virion indicated that VP1-98, VP1-145, and VP1-242 are located on the BC loop, DE loop, and HI surface loops [28, 29]. The residues of these three amino acids are closely related to variation in multiple EV-A71 phenotypes, including PSGL-1 binding (VP1-145 and VP1-242) [30], putative heparan sulfate binding (VP1-242) [31], neutralization antibody binding (VP1-98, VP1-145, and VP1-242) [32], and virulence (VP1-145). Several in vivo studies with mice showed that the amino acid at the 145th site in the VP1 protein directly determined viral virulence, and the virulence of the VP1-145E strain was obviously higher than that of the VP1-145G or VP1-145Q (VP1-145G/Q) strain [33–35]. Chikako et al. noticed that in a cynomolgus macaque model of infection with VP1-145G (PSGL-1-binding receptor dependent) virus, VP1-145G was easily mutated to VP1-145E. VP1-145G was detected only in the peripheral blood mononuclear cells (PBMCs) of some of these macaques, whereas macaques expressing VP1-145E exhibited viral viremia and nervous system injury [36]. Nevertheless, a molecular biology study designed to identify the causative pathogen(s) of clinical

HFMD revealed an obviously higher level of the VP1-145G/Q strain than the VP1-145E strain in fatal HFMD cases with nervous system injury [37–39]. Taken together, the results of analyses of mice, nonhuman primate cynomolgus macaques, and clinical cases indicated that amino acid 145 in the VP1 protein determines whether VP1 binds to the PSGL-1 receptor and thus determines viral virulence and infection outcome. However, a great variety of PSGL-1 receptors are expressed in model animals and humans; therefore, the differences in VP1-145 mutant virulence in animal models and humans are obvious [40].

Unique EV-A71 Structure and Related Pathogenicity

The EV-A71 virion has a nonenveloped structure with icosahedral symmetry. Rao et al. determined the precise EV-A71 viral structure through X-ray crystallography analysis and discovered features common to other associated enteroviruses, such as poliovirus. Enteroviruses have a specific structure, called the "canyon," which is located on the viral surface. And the "canyon" structure is often the binding site of many cell receptors [41]. Besides, there is other specific structure of EVA71 virus named "the pocket factor." It is a natural lipid, located on the viral capsid. During EVA71 infection process, EVA71 virus binds with cell receptors followed by pocket factor squeezing, which leads to destabilization and disintegration of the viral particle, releasing its genetic substances into the cells, where they replicate [42]. Plevka et al. [28] also conducted a study on the EV-A71 crystal structure. The results showed that, in contrast to those observed in other enteroviruses, the EV-A71 pocket molecule is a small-molecule chemical structure exposed to the surface of the canyon of the capsid protein (VP1) pentamer. The pocket molecule mainly stabilizes the virion and protects it from invasion from outside substances (Fig. 3.4). Thus, the pocket factor not only facilitates replication of the virus and accelerates disease progression but also plays an important role in the fine-tuned self-protective mechanism in EV-A71, subsequently abrogating the effectiveness of antiviral drugs used to treat HFMD. In summary, the discovery of the EV-A71 pocket provided important support for further exploration into the mechanisms underlying viral infection of cells, as well as for the study of viral pathogenesis and the development of antiviral drugs.

Host Gene Susceptibility and HFMD

As infection with a given virus may lead to a variety of clinical manifestations, from asymptomatic or mild HFMD to severe neurological complications and even death, it is presumed that differences in host gene expression and immune state might be important factors in determining disease severity. Recently, studies on host susceptibility genes have revealed that 2′-5′oligo adenylate synthetase (OAS) and human leukocyte antigen (HLA) genes may be related to HFMD pathogenesis.

OAS Genes

OAS genes, consisting of OAS1, OAS2, and OAS3, mainly encode antiviral proteins, the production of which is induced by interferons, and these genes are located on chromosome 12q24. OAS genes are involved in nonspecific and specific immune responses and are critical to antiviral effects and signal transduction. Studies have shown that individuals carrying the OAS1 rs10774671 single nucleotide polymorphism (SNP) GG genotype exhibited an increased risk of developing CV-A16-associated HFMD with a high level of IFN-γ expression, while HFMD patients with an AA+AG genotype showed an increased risk of developing encephalitis [43]. Using the sequence-specific amplification PCR (PCR-SSP) method, Chinese scientists found that Han individuals carrying the OAS1 rs1131476 variant (in which G is replaced with A) with an AA or A genotype show a high risk of developing severe HFMD after EV-A71 infection. The OAS1 gene polymorphism is presumed to impact OAS1 activity to repress its inhibitory effect of EV-A71 viral protein synthesis and subsequently lead to disease progression after EV-A71 infection.

HLA Gene

The HLA gene is located on chromosome 6p21.31 and consists of a number of closely linked loci, and it encodes the human major histocompatibility complex (MHC), which is closely related to human immune system function. The HLA-A33 genotype is common in Asian populations and rare in populations of European descent. According to the results of early studies, individuals carrying the HLA-A33 allele show susceptibility to EV-A71 infection, while HFMD patients carrying the HLA-A2 allele show a high risk of developing cardiopulmonary failure [44]. HLA-G is a critical immune tolerance allele that can inhibit the functions of multiple immune cells, including T lymphocytes, natural killer (NK) cells, and antigen delivery cells. HLA-G expression can stimulate the proliferation of inhibitory immune cells, such as regulatory T cells (Tregs), immune tolerance-related dendritic cells (DCs), and NK cells, and maintain persistent inhibition of immune functions [45]. In contrast to other HLA I antigens, HLA-G is expressed as 7 isoforms, of which HLA-G1-G4 is a membrane-bound protein and HLA-G5-G7 is a soluble molecule [46]. Due to the inhibitory immune function induced by HLA-G expression, the expression of HLA-G inhibits the functions of multiple immune cells involved in the defense against EV-A71 infection. For example, in EV-A71-infected patients, the HLA-G expression level on the monocyte surface and the serum HLA-G (sHLA-G) level in plasma have been found to be significantly increased, and the increase in the HLA-G expression level was closely related to the severity of EV-A71 infection. Moreover, the HLA-G gene can undergo a 14-bp insertion/deletion mutation at the 3741 bp site. In severe EV-A71-infected HFMD patients, the frequencies of the HLA-G1 14 bp gene insertion/deficiency polymorphisms 14 bp−/−, 14 bp+/−, and 14 bp+/+ have been shown to be sequentially decreased. The HLA-G 14 bp−/− genotype has been shown to be closely related to susceptibility to

EV-A71 infection, suggesting the involvement of HLA-G in EV-A71 infection. Furthermore, the sHLA-G level in the plasma of severe HFMD patients has been shown to be significantly higher than that in the plasma of healthy children, and the sHLA-G level in the plasma of critical HFMD patients has been shown to be significantly higher than that in severe HFMD patients [47, 48]. The results indicated a potential relationship between the sHLA-G level in plasma and disease severity in EV-A71 infection and suggested that the sHLA-G level in plasma may be measured to diagnose EV-A71 infection.

Receptors Involved in Viral Interactions with Host Cells

Host receptors susceptible to viral binding are involved in the initial stage of infection. Cell receptors play essential roles in determining the specificity of cell and tissue infection, the effect of addiction-related viral species and viral pathogenicity. Viral infection of cells and viral transmission between cells depend on different cell surface molecules. In infected tissues, viral transmission between cells plays a critical role in pathogenesis. Receptors mediate a number of processes, such as the binding and internalization of viruses, viral conformational alteration and uncoating, and other mechanisms associated with viral entry into host cells [49].

Host Immune Response and HFMD

Disease progression and severity are generally correlated with the host immune state after viral infection. Notably, viral infection may activate innate and adaptive immune responses and stimulate the secretion of multiple cytokines. Cytokines are not only involved in host immune protection but can also induce immunopathological injury. During the evolution of viruses and hosts, viruses have been found to form a self-defense mechanism against host immune responses, and they subsequently developed the ability to promote the self-infection process [50, 51]. With the progression of viral infection and viral proliferation in tissues and organs, disease progression is accelerated. An excessive response by the immune system might lead to an imbalance in the immune system and subsequent disorder of the inflammatory response, including the overstimulation of cytokine-inducing molecules, which might exacerbate the immunopathological process during viral infection.

Viral Infection and Innate Immune Escape

Elevated cytokine levels are frequently recognized in EV-A71-, CV-A16-, and CV-A6-infected patients. Cytokines play crucial roles in viral clearance through terminal effector molecules activated during innate antiviral immune responses.

Nevertheless, the cytokine response is initially generated after the recognition of viral pathogen-associated molecular patterns (PAMPs) by host cellular pattern recognition receptors (PPRs), which initiate a corresponding signal cascade response to activate the adaptive immune response, facilitating the prevention of the invasion of and infection with foreign pathogens. During the development of HFMD, the innate immune response is initiated by the host in the early stage of viral infection to defend against viral invasion and promote viral clearance; however, when completing their life cycle in host cells, viruses proliferate and spread to escape the innate immune response through viral proteins, host proteins, and other molecules [52].

HFMD and the Inflammatory Response

The inflammatory response, which is primarily characterized by the infiltration of inflammatory cells and the secretion of proinflammatory cytokines and chemokines and by cellular edema and vascular permeability, plays a crucial role in the development of HFMD. Studies have demonstrated that multiple cellular signaling pathways are involved in the pathogenesis of inflammation. The related cell signaling pathways are described in this section [53].

Oxidative Stress (OS) Signaling

Oxidative stress (OS) typically develops when reactive oxygen species (ROS) generation overwhelms the cellular antioxidation defense [54]. The ROS production induced by EV-A71 infection is essential for viral replication in host cells. One study indicated the presence of mitochondria as major ROS sources in EV-A71-infected cells in vivo [55]. The antioxidation capacity of glucose-6-phosphate dehydrogenase (G6PD)-deficient cells is weaker than that of wild-type cells [56]. Therefore, G6PD-deficient cells are sensitive to EV-A71 replication and exhibit clearer cytopathological effects, exhibiting loss of viability and remarkable upregulation of NF-kB signaling. Previous in vitro studies have demonstrated that ROS generation triggered by EV-A71 infection may activate multiple inflammatory signaling pathways. For example, curcumin can inhibit the ROS production induced by EV-A71 infection and control the activation of ROS-mediated extracellular signal-regulated responses [57]. Additionally, the ROS production and Jun N-terminal kinase (JNK) activation induced by EV-A71 infection was inhibited in cells treated with apigenin [58]. These results most likely suggest that the induction of ROS production might be a potential biological mechanism triggered by EV-A71 infection that subsequently results in an inflammatory response.

NF-kB Signaling

The NF-kB signaling pathway plays important roles in EV-A71 replication and the EV-A71-induced inflammatory response. For example, in different cells, EV-A71 infection leads to the initial activation of NF-kB signaling and then regulates the secretion of downstream differentially expressed inflammatory cytokines [59–61]. When the NF-kB pathway is overactivated, substantial levels of regulatory response products such as TNF-α are produced to coordinate the inflammatory response and thus aggravate tissue injury. In vascular smooth muscle cells, EV-A71 infection can stimulate the degradation of the NF-kB inhibitor IkB, leading to the transfer of NF-kB to the nucleus to activate and promote the expression of vascular cell adhesion molecule-1 (VCAM-1) mRNA [62]. Additionally, resveratrol, which inhibits EV-A71 replication and cytokine secretion in rhabdomyosarcoma (RD) cells, induces anti-inflammatory effects by blocking IKK/NF-kB activation. Additionally, CV-A16 may induce an inflammatory response by activating the PERK/STAT3/NF-kB signaling pathway [63].

Epidermal Growth Factor Receptor (EGFR) Signaling

The epidermal growth factor receptor (EGFR) is an ERBB receptor family member and a tyrosine kinase receptor. EGFR-dependent signaling pathways are involved in the inflammatory response and pathogenicity after viral infection by regulating inflammatory gene expression. In EV-A71-infected cells, EGFR may be transactivated by cellular Src kinase to stimulate cyclooxygenase-2 expression and prostaglandin E2 release [64]. Another study revealed that EV-A71 infection induces the generation of ROS and inactivation of NADPH oxidase via integrin 1/EGRF to regulate EV-A71 replication in SK-N-SH cells [65]. Thus, host cells can regulate the inflammatory response and EV-A71 replication via EGFR signal transduction after infection.

Mitogen-Activated Protein Kinase (MAPK) Signaling

Mitogen-activated protein kinase (MAPK), a serine/threonine proteinase with a phosphorylation function, is critical for extracellular-to-intracellular signaling [66]. Among MAPKs, extracellular signal-regulated kinase (ERK), P38, and c-Jun N-terminal kinase (JNK) constitute the major MAPK components [67]. These kinases are involved in multiple physiological processes, such as inflammation and cell stress, growth, development, proliferation, and death, through multiple substrates, such as phosphorylated transcription factors and enzymes. Several studies have indicated that the MAPK signaling pathway may be activated after EV-A71 infection. Xie et al. reported that the p38, ERK1/2, and JNK1/2 signaling pathways may be activated in human tonsillar epithelial cells to induce the overexpression of IL-8, IL-1β, IL-6, and IL-12p40 upon EV-A71 infection [59]. Similar observations

have been reported with respect to human intestinal epithelial cells, vascular smooth muscle cells, and premature DCs [62, 68, 69]. EV-A71 infection can activate JNK1/2 and p38 and successively induce phosphorylation of the downstream transcription factors c-Fos and c-Jun in premature DCs and subsequently induce upregulation of IL-2, IL-6, IL-10, and TNF-α expression levels. In vivo, JNK1/2 and p38 inhibitor treatment can reduce levels of EV-A71 replication and inflammatory cytokine secretion [68]. Some studies have demonstrated that cell apoptosis and p38-mediated proinflammatory cytokine secretion were induced in human astrocytes infected with EV-A71 [70]. In summary, EV-A71 infection can activate major components of the JNK1/2 and p38 MAPK signaling pathways and lead to the secretion of corresponding inflammatory cytokines.

Phosphatidylinositol 3-Kinase (PI3K) Signaling

Phosphoinositide 3-kinase (PI3K) is a phosphatidylinositol kinase with the dual functions of a proteinase and phosphokinase that can be classified as type I PI3K (PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ), type II PI3K subtype (PI3KC2α, PI3KC2β, and PI3KC2γ), and type III PI3K. PI3K signaling is mainly involved in the regulation of cell proliferation and growth and can regulate the inflammatory response by activating the downstream proteinase Akt [71, 72]. EV-A71 infection has been shown to activate the PI3K/Akt signaling pathway and further regulate the transcription of IL-8, IL-1β, IL-6, and IL-12p40 proinflammatory cytokines [59]. Shi et al. detected inflammatory cytokine secretion in human RD cells after EV-A71 infection [73]. In addition, EV-A71 infection was shown to induce COX-2 expression and PGE2 production in mouse astrocytes via the c-Src/PDGFR/PI3K/Akt/p42/p44 MAPK signaling pathway and subsequently contribute to the expression of the transcription factor AP-1 [61]. Overall, EV-A71 infection has been shown to regulate the inflammatory response by activating the PI3K/Akt signaling pathway.

Calcium (Ca2+)-Dependent Signaling

As an important messenger in eukaryotic cells, calcium is mainly involved in the transmission and regulation of cell proliferation, differentiation, growth, and aging. Upon viral infection, the Ca2+ concentration has been shown to increase in mitochondria; Ca2+ influx has been shown to activate calcium proteinase and be involved in caspase-independent apoptosis mediated by apoptosis-inducing factor (AIF). Specifically, inhibitors of Ca2+ influx have been shown to inhibit calcium proteinase activation and AIF function [74]. Additionally, the EV-A71-encoded VP1 protein has been shown to activate calmodulin-dependent protein kinase II (CaMKII), which is involved in Ca2+ homeostasis to induce phosphorylation of the serine 82 residue in the N-terminal structural domain, which promotes EV-A71 replication [75]. In summary, EV-A71 infection activates Ca2+-dependent signaling to promote host cell apoptosis and subsequent EV-A71 replication.

Cytokines Associated with the Development of HFMD

In addition to innate immune response escape and inflammatory responses, some cytokines activated during the immune response in host cells triggered by viral infection are presumed to be involved either in the antiviral immune response or promotion of disease development. Interestingly, the cytokines that were found to be different between healthy volunteers and patients with mild or severe HFMD with complications likely played important roles in the EV-A71 infection process. Furthermore, they are likely potential target candidates for the diagnosis of viral infection and the development of antiviral drug treatments [76–79] (Table 3.1).

Table 3.1 Cytokines associated with EV-A71 infection

Cytokines	Expression during EV-A71 infection	Potential functions in EV-A71 infection
TNF-α	Critical HFMD cases > severe HFMD cases > mild HFMD cases; expression level peak in severe phase and decline slightly during recovery	Associated with the disease severity
IFN-γ	Expression level increased in some severe cases; higher expression levels in patients with neurogenic pulmonary edema (PE) than in patients with central nervous system (CNS) complications	Associated with the disease severity
IL-1β	Expression level increased in acute phase but decreased in the recovery phase in patients with severe HFMD with complications	Essential role in triggering severe HFMD with complications
IL-18	Expression level increased in HFMD cases with PE and gastrointestinal syndrome complications, the 1st and highest peak occurred 3 and 10 days after severe disease development	Expression level might be used as a prognosis indicator and be related to immune injury caused by EV-A71 infection
IL-33	Critical HFMD cases > severe HFMD cases > mild HFMD cases	Associated with inflammatory injury
IL-37	Expression level significantly increased in typical HFMD patients	Unclear
IL-4	Expression level increased in different courses of HFMD in infants and young children with complications; HFMD cases with encephalitis > HFMD cases without complications	Associated with the HFMD encephalopathy development
IL-13	Peaks in the early stage in hospitalized HFMD patients, expression level significantly increased in HFMD patients with PE	Associated with anti-inflammatory responses and pathological injury caused by PE
IL-6	Expression level significantly increased in severe HFMD patients, especially HFMD patients with encephalitis and severe HFMD with cardiopulmonary failure	Markers for microbiological infection, associated with disease severity

(continued)

Table 3.1 (continued)

Cytokines	Expression during EV-A71 infection	Potential functions in EV-A71 infection
IL-12	Severe HFMD patients > mild HFMD patients > healthy people; HFMD with encephalitis and PE > HFMD with encephalitis	Associated with the disease severity and complication development
IL-23	HFMD with encephalitis cases >HFMD without complications	Associated with the HFMD encephalitis development
IL-27	Higher expression level in the early stage of cardiopulmonary injury but not the late stages in nervous system injury and cardiopulmonary injury	Associated with the early stage of the cardiopulmonary injury
IL-35	IL-35 secreted regulatory T-cell (Treg) reduced in severe HFMD, positive correlation to the variety of Treg-to-Th17-cell ratios	Associated with the HFMD pathogenesis
IL-10	Significantly higher expression level in PE patients than in patients with nervous system injury, critical HFMD patients > severe HFMD patients > mild HFMD patients	Protective function in the PE development
IL-22	Severe HFMD cases > Mild HFMD cases	Positive correlation to increased cTh22 level in severe HFMD patients and mild HFMD patients
IL-17	Either increased or decreased in HFMD cases with encephalitis	IL-17F gene polymorphism is correlated to severe HFMD susceptibility
IL-8	Critical HFMD cases > severe HFMD cases > mild HFMD cases	Associated with the disease severity
IP-10	HFMD patients < healthy people	Beneficial factor in EV-A71 infection
MCP-1 and RANTES	Expression levels increased in mild and severe HFMD cases and HFMD cases with complications	Associated with disease progression, expected prognosis indicator
G-CSF	Expression level significantly increased in severe HFMD patients with pulmonary failure; expression level in the cerebrospinal fluid significantly higher rather than in the plasma in HFMD patients with nervous system symptoms	Predominant mediators in the cerebrospinal fluid induced during neurological injury
HMGB1	Disease development phase > recovery phase in severe and critical HFMD patients	Positive correlation to the variety of IL-6 and TNF-α expression levels; an indicator of the HMFD severity

Overall, EV-A71 infection first stimulates susceptible cells and nonspecific immune cells to secrete TNF-α, IFN-γ, IL-6, etc., which are cytokines that play vital roles in viral replication and early infection control. In parallel, the cells activated by cytokines can promote the secretion of proinflammatory cytokines and cytokines that interfere with viral replication and kill infected host cells. However, the activities of many cytokines and chemokines, such as TNF-α, IFN-γ, IL-1β, IL-18, IL-33,

IL-37, IL-4, IL-13, IL-6, IL-12, IL-23, IL-27, IL-35, IL-10, IL-22, IL-17F, IL-8, IP-10, MCP-1, G-CSF, and HMGB1, are closely correlated with the development of severe HFMD attributed to EV-A71 infection.

Viral Infection and Host Pathological Injury

In general, viral-encoded proteins can directly interact with the host immune system to promote escape of the host immune defense. In addition, enterovirus can directly or indirectly interfere with normal host physiological activities to disturb the host immune response to facilitate viral infection in host cells. For example, enterovirus can directly or indirectly interfere with cell autophagy and apoptosis involved in maintaining cellular homeostasis to facilitate viral infection in host cells [80, 81].

Autophagy

Autophagy is a life-sustaining activity in eukaryotes that is involved in the lysosome-dependent degradation pathway. Autophagy is a form of programmed cell death involving the phagocytosis and degradation of excess, damaged and aged intracellular components and exogenous microorganisms. Autophagy is important in the metabolism of cellular components and maintenance of the cellular homeostasis and immune defense of the host. In recent years, many studies have demonstrated that phagocytosis is involved in essential regulatory functions in the immune responses of the host involving lymphocyte development and innate and adaptive immune response regulation [80].

Autophagy helps TLRs on lysosomes recognize a corresponding ligand and promote the antiviral innate immune response after viral infection. During primary T-cell differentiation into T helper 1 (Th1) and Th2 cells, many abnormal autophagosomes form in the cell, which may impact pro-B-cell development toward pre-B cells. In summary, autophagy determines cell fate during a specific phase of cellular development. Autophagy can be used to degrade pathogenic microorganisms invading the cell and deliver antigens from microorganisms to MHC class II molecules for subsequent identification by T cells or NKT (NKT) cells in the immune system, which may provide the foundation for the induction of a pathogen-specific adaptive immune response [82–84].

However, viruses, especially RNA viruses, can rapidly evolve to adapt to host autophagy via a mechanism that enables their escape from autophagy pathways. Some studies have reported that EV-A71 induces autophagy during the viral infection process. EV-A71 promoted the formation of autolysosomes via a virus-encoded nonstructural 2BC protein interaction with host proteins STX17 and SNAP29 that subsequently led to the infection of cells [85]. Yang et al. discovered that the EV-A71-encoded 3A protein inhibited interferon 1 production by upregulating the expression of the autophagy-associated protein LRRC25 [86]. You et al. found that

an EV-A71-encoded 3D nonstructural protein interacted with ACOX1 to downregulate its expression, reduce peroxisome numbers, increase ROS generation, attenuate DJ-1/NRF2/HO-1 pathway activation, and subsequently induce autophagy in neuronal cells [87]. EV-A71 infection upregulated the phosphorylation levels of p38 MAPK and ERK and subsequently induced autophagy and IL-6 production [88]. EV-A71 infection promoted autophagy in the brain, lung, and muscle tissues of mice via mTOR inhibition and ERK pathway activation [89]. Autophagy induced by EV-A71 infection has been shown to promote viral replication and proliferation in host cells and to be positively correlated with pathological outcomes [81, 90].

Apoptosis

Apoptosis is the typical process of programmed cell death that can be used to clear excessive, damaged, and aged cells and thus maintains the stability of the internal environment. The apoptotic mechanism is very complicated and involves many energy-dependent signaling cascades. To date, two major apoptosis pathways have been identified: the external or death receptor route and the internal or mitochondrial route. An additional pathway involves T-cell-mediated cytotoxicity and perforin/granzyme-dependent apoptosis [91]. The perforin/granzyme route can induce apoptosis via granzyme A or granzyme B. Ultimately, external, internal, and perforin/granzyme routes induce apoptosis via the digestive activities of the caspase-3 pathway. The apoptosis process includes DNA fragmentation; degradation of the cytoskeleton and ribonucleoproteins and cross-linking proteins; apoptotic body formation; phagocyte-binding ligand expression; and phagocytic uptake [91]. During the viral infection process, apoptosis, especially which induced through the T-cell-mediated cytotoxicity killing pathway, plays an essential role in the anti-infection immune response of the host. Furthermore, as a super TNF/NGF receptor family member, Fas/CD95 plays a vital role in apoptosis and immune regulation in vivo. Fas ligand is a cytokine expressed on the surface of activated T lymphocytes and NK cells. The binding of Fas protein and FasL can initiate downstream apoptotic pathways. Cytotoxic T lymphocytes (CTLs) and NK cells, including other effector cells, can simultaneously kill target cells via Fas- and FasL-mediated apoptosis [92].

Nevertheless, viral infection can interfere with apoptotic signaling to promote its own infection. Apoptosis is a significant outcome of pathogenic CVB3 infection, and CVB3 virulence declines when apoptosis is inhibited [93]. In addition, EV-A71 infection leads to the targeted regulation of SOS1 and GADD45 expression through the regulation of miR-146a or miR-370 expression, modulating apoptosis [94]. EV-A71 infection is also involved in a caspase-independent apoptotic route triggered by Ca2+ flux activation of calcium protease activity [74]. Furthermore, EV-A71 cleaves eukaryotic initiation factor 4G via the viral-encoded 2A protease, abrogating host translation and inducing host apoptosis [95, 96]. Moreover, EV-A71 can induce apoptosis via 3C protease activity [97].

Overall, autophagy and apoptosis are closely correlated with pathological processes during EV-A71 infection. EV-A71 infection promotes autophagy and

apoptosis via various mechanisms. However, due to the limited amount of study data, some conclusions are based on presumptions, and the signaling pathways induced by EV-A71 infection await further exploration.

3 Pathogenesis of HFMD-Associated Disease

Pathogenesis of HFMD-Associated Skin Rash and Blisters

A skin rash and blisters on the mouth, hands, and feet are typical signs of mild HFMD. To date, no conclusion on the pathogenesis of these symptoms, except an assumption of transmission through blister fluid, has been identified. By referring to the literature on HFMD-associated skin rash and blister pathology, viral-specific immunohistochemistry, and molecular biology, Liu et al. conducted pathological examination on blisters in the mouth of EV-A71-infected neonatal rhesus macaques, and inflammatory cell infiltration, edema, blood, and muscle fiber injury were noticed in the blister region. In parallel, the analysis of viral titers of samples taken from the blister region revealed live EV-A71 at 7 days post-infection, and the peak value was identified 10 days post-infection, after which it started to gradually decline [98]. Hooi et al. detected viral antigen and viral RNA in the oral squamous epithelia and epidermis of hamsters at 3–4 days post CV-A16 infection [99]. Immunohistochemical staining of blister fluid taken from an atypical CV-A6-infected HFMD patient failed to indicate live virus; however, CV-A6 was identified in the RT–PCR amplification reaction [100]. Chung et al. conducted pathological examination of specimens taken from CV-A6-associated HFMD patients, and the nucleic acids of CV-A6 were detected in the blistering skin lesion samples of 28.6% (6/21) of these patients. Furthermore, viral particles were noted in the blister region of a few patients through electron microscopy. Additionally, CD8+ CTLs, CD20-positive B lymphocytes, and CD56-positive NK cells or NKT cells were identified by immunohistochemical staining in blister region samples [101]. The expression of the granulysin protein, which is expressed by CTLs and NK cells, was found in the injury region. Zhao et al. identified viral replication and proliferation in the respiratory epithelial cells and peripheral lymph nodes of rhesus macaques infected with recombinant EV-A71 expressing EGFP (fluorescent) protein via the respiratory tract; EV-A71 was shown to invade the preconventional DC populations in the infected region [102]. Nishimura et al. reported that EV-A71 can infect Jurkat T cells [103].

In summary, it can be preliminarily concluded that EV-A71, CV-A16, CV-A6, and other HFMD-associated viruses can directly infect epithelial tissues, DCs, and lymphocytes. Referring to pathological examinations of skin rash and blistered regions in HFMD patients, the potential pathogenesis is presumed to be due to direct epithelial cell infection resulting from the injury and congestion of local tissues, with or without overexpression of inflammatory cytokines and the granulysin protein secreted by overactivated DC cells, NK cells, CTLs, and B lymphocytes.

The overexpression of inflammatory cytokines and the granulysin protein may ultimately contribute to pathologies characterized by localized redness and swelling, increased lymphatic capillary permeability and inflammatory cell aggregation, leading to inflammation-related injuries.

Pathogenesis of HFMD-Complicated CNS Disorders

In addition to skin rash and blisters in the mouth and on the hands and feet, severe injury of the CNS, usually accompanied by neurological symptoms, such as headache, vomiting, lethargy, limb fatigue, ataxia, and convulsion, might be presented by some HFMD patients. Previous epidemiological and molecular pathogenic studies have demonstrated that EV-A71 is the common causative pathogen of CNS injury in HFMD patients, while CNS disorders of HFMD caused by CV-A16, CV-A10 and CV-A6 infections have also been reported in recent years [104]. A total of 70.7% and 20.6% of HFMD cases accompanied by CNS injuries were attributed to EV-A71 and CV-A16 infection, respectively [104]. MRI examination of HFMD patients (Fig. 3.3) indicated that the major inflammatory injuries in the CNS were found in the diencephalon, midbrain, pons, medulla oblongata, and medulla, while parenchymal opacification was identified in the medulla oblongata, red nucleus, substantia nigra, oculomotor nucleus, trochlear nerve, frontoparietal, parietal lobe,

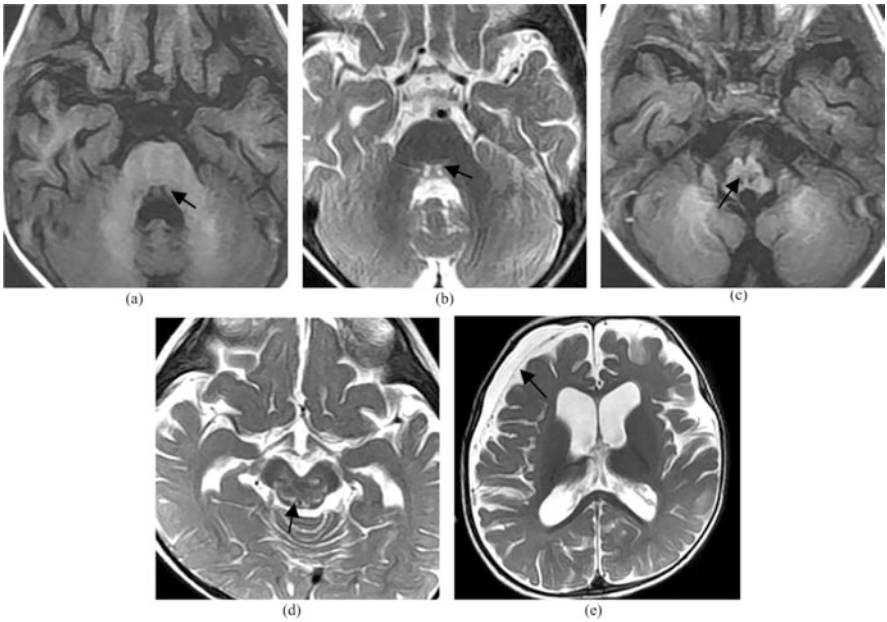

Fig. 3.3 MRI examination results of EVA71 infected HFMD of a male, seven months patient. The major inflammatory injuries were detected on Pontine tegmentum (**a**, **b**), posterior oblongata (**c**), red nucleus, substantia nigra (**d**), and frontoparietal regions (**d**). Reused with permission from [105]

and spinal cord regions. Therefore, major clinical manifestations of acute aseptic meningitis, brainstem encephalitis, cerebellitis, and poliomyelitis were observed [105, 106].

Furthermore, a histopathological examination of the CNS in patients who died or had severe disease caused by EV-A71 infection showed substantial and aggravated inflammatory cell infiltration, neuron degeneration and necrosis, tissue liquefaction necrosis, nerve cell phagocytosis, astrocyte proliferation, and glial nodule formation, resulting in severe pathogen-related injuries (Fig. 3.4). Referring to clinical manifestations, by using MRI and pathological tissue examinations in patients with

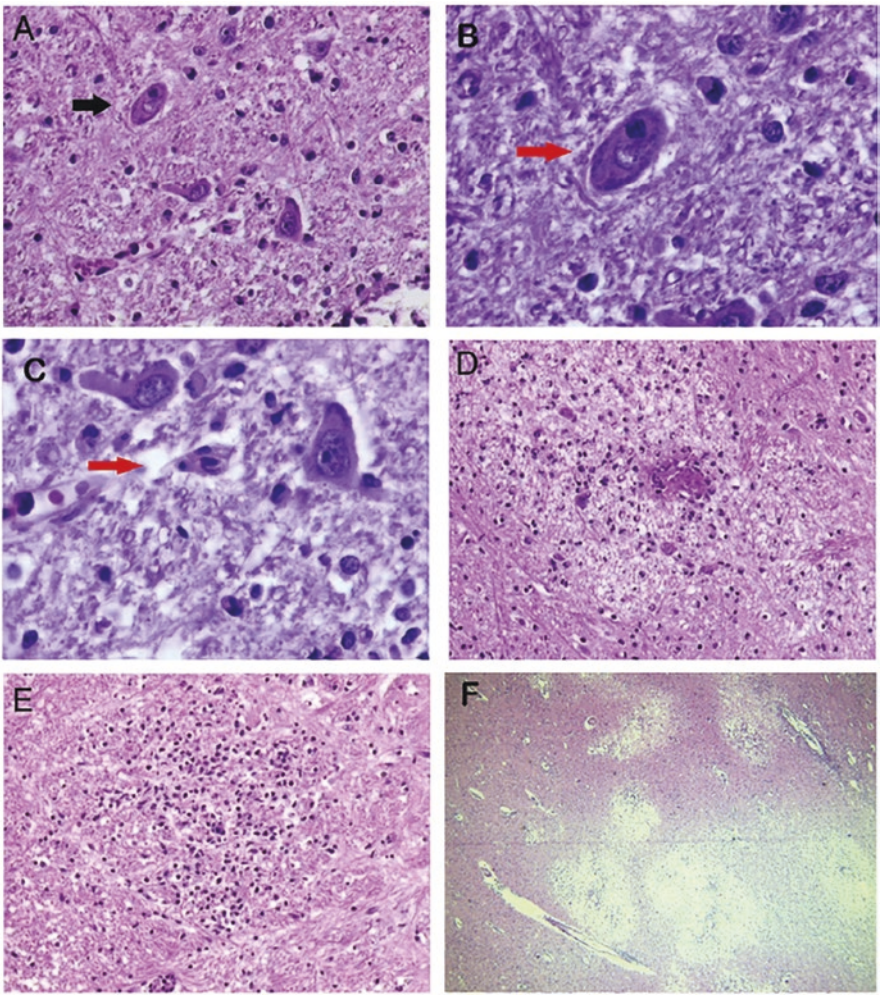

Fig. 3.4 Histopathological features of the CNS in fatal HFMD patients. Neuron degeneration and necrosis ((**a**) ×100, (**b**) ×400, (**c**) ×400), nerve cell phagocytosis ((**d**) ×40), astrocyte proliferation and glial nodule formation ((**e**) ×40), and brain tissues Necrosis ((**f**) ×40) were observed. Reused with permission from [25]

severe HFMD with encephalopathy due to EV-A71 infection, EV-A71 neurotoxicity was very clearly observed. EV-A71 is the most important neurotropic virus after polioviruses. In addition, CV-A16 virus pathology, including viral protein expression or viral RNA-positive responses, has been identified in the CNS tissues of mice and hamsters [99, 107].

Although the pathogenesis of HFMD-associated viral infection in the CNS remain largely unknown, recent pathological, immunohistochemical, and other viral detection analyses have indicated that there the following three mechanisms are involved in HFMD-associated CNS disorders [25].

Direct Pathogenicity Induced by Viral Infection

Similar to poliovirus, EV-A71, CV-A16, CV-A6, and other enteroviruses are transmitted via the fecal-oral route starting with proliferation in the nasopharynx, gastrointestinal mucosa-associated lymphatic tissue, followed by release in blood, two peaks in viremia, and final entry into target tissues; a portion of the entering virus contributes to CNS infection by breaking through the blood–brain barrier or through retrograde axonal transport in peripheral nerve pathways [108]. Viral entry into neuronal cells leads to substantial viral replication and direct tissue-specific cell damage [109–111]. Xing et al. conducted an autopsy on a patient who died after EV-A71 infection, and their analyses indicated neutrophil infiltration into the meningeal surface, neutrophil and neuronal cell necrosis in large areas of the parenchyma, and severe injuries in the brainstem and spinal cord. More directly, immunohistochemical staining of the EV-A71-infected CNS with a specific antibody against the structural protein VP1 showed the presence of EV-A71 viral antigen in neuronal soma, different brainstem regions, anterior and posterior horn cells of spinal ganglion and gastrointestinal autonomic ganglion [111]. All these data suggest that infectious EV-A71 breaks through the blood–brain barrier and enters the CNS tissues, where the virus survives and replicates, resulting in pathological injuries to nervous tissues. Furthermore, substantial inflammatory cell infiltration has been noticed in the CNS tissues of patients with severe EV-A71 infection, implying that a potential immunological mechanism mediates CNS tissue injury [112].

Immune Injury

Immune injury usually occurs in the late stage of EV-A71 infection as an excessive immune response further deteriorates the injured CNS. The excessive immune response is characterized by neutralization antibody titer increases along with severe HFMD development; autoimmune injury resulting from the cross reactions between viral antigen and host cell epitopes; immune injury resulting from the enhancement of certain antibody-dependent processes; and injury resulting from a

significant increase in the expression levels of some cytokines, including proinflammatory cytokines.

Xie et al. reported that in the acute phase the B-cell numbers and ratios and serum total IgG levels in EV-A71-infected patients with severe HFMD were higher than those observed in mild HFMD patients. Throughout the progression of infection to the recovery phase, the neutralization antibody level was found to be increased, and B-cell numbers and ratios returned to normal levels, suggesting a potential association between the humoral immune response and nervous system complications [113]. Jia et al. found a cross reaction between EV-A71-specific IgG and human brain tissues. The localization of the corresponding antigen peptides indicated that 4 peptides (VP2, VP1, 2A, and 2C) of EV-A71 displayed a strong cross reaction with human brain tissues [114]. Thus, a common epitope between the viral antigen and host cell may lead to autoimmune pathological injury via cross reaction. Furthermore, the study of an EV-A71-infected suckling mouse model indicated that viral infection promoted blood–brain barrier permeability and led to the entry of EV-A71-specific IgG into brain tissues, which triggered a cross reaction. How these viral antibodies respond to viral proteins needs further investigation. Fan et al. compared the database of EV-A71 and human proteins via bioinformatic techniques and identified a common epitope, and the corresponding monoclonal antibody was effectively bound to mediator complex subunit 25 (MED25), which is highly expressed in brainstem tissues. In vivo, this antibody was distributed to the brainstem region of the mice at 7 days post-EV-A71 infection followed by intravenous injection of the monoclonal antibody. A similar antibody was identified in the sera of EV-A71-infected patients. All these results suggest the potential involvement of autoimmune factors in the pathogenic mechanism underlying the nervous system injury attributed to EV-A71 infection [115].

In addition, certain specific antibodies exhibit antibody-dependent enhancement (ADE) in multiple viral infections, such as dengue virus, respiratory syncytial virus, and Ebola virus infections [116, 117]. ADE has also been reported in some small RNA virus family members, such as foot and mouth disease virus, poliovirus, and coxsackievirus B. Han et al. demonstrated that neutralizing antibodies may enhance EV-A71 infection of THP-1 cells and increase the death rate of suckling mice. They established an EV-A71-reinfected suckling mouse model, and they reported that the antibody generated from the 1st infection was shown to increase the death rate of the reinfected mice [118]. Based on this conclusion, Cao et al. further analyzed the association between different subtypes of neutralizing antibodies and ADE effects and found that IgG3 exhibited no neutralization activity but exhibited remarkable ADE effects. The mechanism underlying ADE is frequently mediated by the IgG Fc receptor (FcγR) on the surface of immune cells or other host cells. FcγR is also expressed in CNS tissues [119]. The determination of whether ADE is triggered after a virus enters the CNS awaits further exploration.

In addition, it has been demonstrated that TLR7 plays a vital role in the first step of the innate immune response after EV-A71 infection. However, substantial data

have shown the role played by the TLR7 death receptor in multiple noninfectious CNS injury-related diseases, such as ischemic stroke, Alzheimer's disease, and morphine-mediated neurodegeneration [120–122]. Therefore, TLR7 likely exhibits a diverse function in infectious diseases regarding its involvement in the host defense against viral infection and the inflammatory responses and neuronal activity dysfunction observed in the CNS. Luo et al. observed damage to neurofilament integrity in the cerebral cortex and remarkable inflammatory cell infiltration and neuronal degeneration in the cerebral cortex of wild mice infected with EV-A71. These results were not observed in TLR7-knockout mice. In parallel, levels of IL-6 production, caspase-3 cleavage, and apoptosis in EV-A71-infected TLR7-knockout mice were significantly lower than those in EV-A71-infected wild-type mice. Moreover, upregulation in TLR7 expression, IL-6 induction, and astrocyte apoptosis were identified in EV-A71-infected human astrocyte U251 cells. All these results suggest the involvement of pathological injury in EV-A71 infection via the upregulation of TLR7 and IL-6 expression [123]. Furthermore, many studies have demonstrated remarkable increases in the levels of IL-6, IFN-γ, IP-10, and MIG cytokines or proinflammatory cytokines in the sera and cerebrospinal fluid of patients with neurogenic PE, brainstem encephalitis, and somatoform autonomic dysfunction. These results suggested the potential involvement of a cytokine-mediated immune mechanism in the pathological injury of the nervous system.

Inflammatory Injury

Inflammatory injury is usually attributed to either the passage of proinflammatory cytokines through the blood–brain barrier or local brain tissue inflammation-associated cell activation. Inflammation-associated cells in brain tissues include astrocytes, vascular endothelial cells, ependymocytes, macrophages, neutrophils, and lymphocytes. In a rat model, EV-A71 was shown to promote prostaglandin E generation and be involved in the inflammatory response by activating the transcription factor NF-kB and inducing cyclooxygenase-2 (COX-2) gene expression in astrocytes [61].

The clinical features of EV-A71 infection-associated inflammatory brain injury or inflammatory cerebropathy include early injury development and rapid clinical progression; the symptoms occur along with the clinical progression of the systemic inflammatory response and the development of sepsis; the degree of brain injury is positively correlated with the inflammatory response or severity and duration of sepsis; and the inflammatory response leads to either aggravation of the primary brain injury or secondary cranial nerve injury or damage, with the exception of the potential brain cell protection induced by certain inflammatory responses, and subsequently leads to aggravation of brain injury or death by secondary or accompanying bacterial infection.

Pathogenesis of HFMD-Complicated Neurogenic PE

Neurogenic PE is a major cause of death in HFMD patients. More than 80% of severe HFMD patients present with PE or lung bleeding [124]. The mortality of patients with PE is very high, and death may occur within 24 hours after the development of PE [125]. The development of neurogenic PE is a complicated pathological and physiological process that is associated with neural factors, humoral factors, and biological activity factors followed by CNS injury. The histopathological examination of animals and severe and fatal HFMD patients has indicated the aggregation and infiltration of a substantial number of inflammatory cells, such as a variety of lymphocytes and phagocytes in the lung; thickening of the alveolar septum, congestive edema of the lumen accompanied by focal hemorrhage, and incomplete bronchiolar walls resulting from partial bronchiolar epithelium injury in EV-A71-infected lung tissues have also been observed. However, no EV-A71 pathogens have been isolated from these damaged lung tissues, and the EV-A71-positive antigen has not been detected by EV-A71-specific immunohistochemical staining of these tissues. Further pathological examination of the heart tissues often indicates no remarkable inflammatory changes. The PE attributed to EV-A71 infection is thus presumed to be neurogenic, with neurogenic urinary retention, enteroplegia, hyperhidrosis, insomnia, tachycardia, etc., which are symptoms of somatoform autonomic dysfunction in the development of PE.

Angiopoietin-2 (Ang-2) expression is closely correlated with vascular permeability and is quickly released to mediate endothelial cell activation and enhance endothelial cell sensitivity to inflammatory cytokines upon external stimulation. The increase in Ang-2 levels observed in septic shock is correlated with vascular leakage and the development of acute respiratory distress syndrome [126, 127]. Qi et al. found remarkably higher Ang-2 expression levels in EV-A71-infected HFMD patients with PE than in non-PE and control groups; sera taken from PE patients can damage cell junctions between human pulmonary microvascular-endothelial cells and subsequently increase vascular permeability to promote PE development [128]. Jin et al. identified mast cell accumulation, activation and allergy-related inflammation in the brain, lung, and skeletal muscles of EV-A71-infected mice, and the damage to lung tissues was the most severe. In addition, the expression levels of histamine, platelet-activating factor, IL-4, IL-5, IL-13, TNF-α, nitric oxide, endocrine gland-derived vascular endothelial growth factor, and noradrenaline in EV-A71-infected lung tissues were markedly increased. Additionally, in EV-A71-infected lung tissues, the numbers of T cells, DCs, and monocytes were decreased, whereas the numbers of eosinophils, Tregs, and mast cells were somewhat increased. Interestingly, the mast cell numbers and trypsin levels in the target organs or tissues tended to be higher in the severely infected mice than in the control mice. Thus, mast cells are presumed to be directly involved in the development of PE induced by EV-A71 infection [129]. Endothelin-1 (ET-1) is a vasoconstrictor consisting of 21 amino acids. ET-1-related mRNA-binding sites and receptor binding sites are

expressed in multiple brain regions; thus, ET-1 could act as a neurotransmitter to regulate neural functions [130, 131]. Multiple animal studies have indicated that the intrathecal injection of ET-1 not only temporarily increases arterial blood pressure, heart rate, and sympathetic nervous system (SNS) activity [132] but also enhances lung vascular permeability and results in PE development. Hence, PE is attributed to α-adrenaline receptor-mediated severe lung pulmonary vasoconstriction developed after ET-1 receptor activation and catecholamine release after EV-A71 infection of the CNS. Kao et al. reported that EV-A71 infection can induce pathological injuries mainly in the cerebral cortex, pons, medullar substance, cerebellum, spinal cord and ventral, medial and caudal medullary substance of the brainstem. These regions are involved in the central depressor and sympathetic inhibitory mechanisms in the vasomotor center [133]. Injuries in these regions may lead to SNS excitement [134].

In summary, the potential pathogenesis of the neurogenic PE attributed to EV-A71 infection might include the following: upon EV-A71 entry into the CNS, complicated brainstem encephalitis develops and results in the dysfunction of the hypothalamus and medulla oblongata nucleus of the solitary tract; the stress reaction of the human body leads to SNS excitement and markedly increased catecholamine (adrenaline and norepinephrine, etc.) expression levels in the blood. Then, systemic vasoconstriction and hemodynamics change rapidly, arterial blood pressure increases rapidly, and a large volume of blood flow from the general circulatory system drains into the circulatory system of the lung. These factors may lead to a rapid increase in the filtration pressure of effective pulmonary capillary blood flow; a large volume of fluid then remains in the gaps in lung tissues and leads to PE. In addition, angiogenin and other proteins in sera lead to vascular endothelial cell injury and an increase in vascular permeability. These findings may finally be attributed to high levels of plasma protein extravasation and aggravated PE.

Pathogenesis of HFMD-Complicated Injury of Other Organs

Recent clinical data demonstrate that the clinical manifestations of HFMD include fever, skin rash, and blisters in the mouth and on the hands and feet, accompanied by symptoms in other organs, such as lymphadenopathy, myocarditis, diarrhea, liver injury, and kidney injury. Liu et al. conducted a pathological examination of the systemic organs of EV-A71-infected rhesus macaques and found injuries and pathological changes in the CNS, trachea, lung, heart, muscle, and brown adipose tissue. Similarly, a pathological examination of a vital HFMD revealed moderate inflammatory cell infiltration, focal hepatic necrosis of liver cells and inflammatory cell infiltration, kidney injury, tonsil lymph node and germinal center necrosis, jejunal epithelial cell necrosis, and inflammatory cell infiltration in the regions near myocardial cells, in addition to CNS and lung injuries [111].

Pathogenesis of HFMD-Complicated Myocardium Injury

Previous pathological and pathogenic data have demonstrated that HFMD-associated viruses can directly infect myocardial cells, leading to the aggregated infiltration of inflammatory cells but no severe pathological injuries in myocardial tissues. Thus, HFMD-complicated myocardial injury is presumed to be correlated with viruses, including direct injury induced through its toxicity, myocardial cell immune injury via viral infection, or neurogenic injury due to CNS disorder.

Some CV-A16-infection case reports in China have indicated abnormal levels of myocardial zymogram indicators in HFMD patients, including CK-MB levels of >25 U/L, AST levels of >40 U/L, CK levels of >190 U/L, LDH levels of >245 U/L, and troponin I (cTnI) levels of >0.1 ng/mL, accompanied by elevated levels of malondialdehyde (MDA), decreased levels of superoxide dismutase (SOD), and marked increase in the levels of programmed cellular apoptotic factors serum Fas (sFas) and sFasL [135]. In addition, some studies have revealed that the potential myocardial injury induced by coxsackievirus and other enterovirus infections may be attributed to abnormal levels of oxygen-free radicals (ROS) and cell apoptosis [136, 137]. MDA is the final product of lipid peroxidation induced by the targeting of cell membrane polyunsaturated fatty acids by ROS, and the MDA level is indicative of lipid peroxidation or indirect ROS generation and cell injury in the tissue. SOD is an oxygen-free radical scavenger that forms the enzymatic front line of defense against ROS-mediated injury [138, 139]. Overall, HFMD-associated myocardial injuries are primarily characterized by abnormal levels of myocardial enzymes, oxidation and antioxidation disequilibrium, and increased levels of myocardial cell apoptosis. Abnormalities in the levels of myocardial enzymes are key indicators of myocardial injuries.

Under long-term stress, the levels of myocardial cell energy metabolism and enzyme synthesis increase, and oxygen consumption in cardiomyocytes increases, followed by ROS accumulation and cell membrane damage and an increase in permeability. Additionally, recurring and long-term fever and inflammatory responses have been shown to lead to increased levels of cell membrane damage and permeability, which can manifest as increased creatine kinase isoenzyme and cTn levels. In addition, a blood lactic acid level increase is another sensitive biochemical indicator of myocardial anoxia. MDA level elevation and SOD level decline likely suggest a decline in myocardial ROS scavenging capacity and ROS accumulation. In turn, ROS accumulation leads to the loss of the structural integrity of the cell membrane and to increased cell membrane fluidity and calcium ion permeability, transmembrane calcium ion transport into the membrane, and calcium ion overload. These changes ultimately cause cell damage and death-associated myocardial ischemia and injury, creatine kinase isoenzyme abnormalities, and even cardiac arrhythmia and heart failure.

In summary, the mechanism underlying HFMD-associated myocardial injury is presumed to include 2 aspects. First, direct attack of the myocardium by viruses with inflammatory cytokines contributes to creatine kinase isoenzyme abnormalities and ROS accumulation, which lead to cell membrane damage and an increase

in permeability followed by cell damage and death. Another aspect is that the brainstem encephalitis-triggered catecholamine storm contributes to coronary spasm, myocardial ischemic injury, and myocardial necrosis.

Pathogenesis of HFMD-Complicated Gastrointestinal Injury

Enteroviruses are transmitted via the fecal–oral route and proliferate in the intestine, shedding into feces. However, their mechanisms of pathogenesis differ from those of viral gastroenteritis. As previously mentioned, diarrhea is frequently identified as an accompanying major gastrointestinal inflammatory symptom in HFMD patients. Pathological examination of fatal EV-A71 infection has revealed inflammatory cell aggregation in the intestine and enterocyte necrosis, and immunohistochemistry staining has shown that viral antigen could be detected in enterocytes and enteric autonomic ganglia [111]. Live virus has also been isolated from the mesenteric lymph nodes of EV-A71-infected rhesus macaques [98]. Hence, EV-A71 infection has been shown to directly invade enterocytes, mesenteric lymph nodes, and enteric autonomic ganglia. The intestinal injuries attributed to EV-A71 infection include the following aspects: (1) Virus-infected intestinal epithelial cells are observed and lead to intestinal necrosis; (2) Virus-infected mesenteric lymph nodes can lead to regional inflammatory cytokine release, inflammatory cell aggregation, bleeding, and intestinal mucosa necrosis and ulceration; (3) Viruses infect gut autonomic ganglia directly and lead to diarrhea or gastrointestinal injury. The gut nervous system is located within the wall of the gastrointestinal tract. It comprises neurons, neurotransmitters, and proteins and supporting cells to form a network structure system that coordinates the movement and secretion of the gastrointestinal tract. Functional and/or structural changes in the gut nervous system can affect gastrointestinal function, leading to diarrhea or gastrointestinal damage; (4) EV-A71 infection-related CNS injury, especially brainstem encephalitis, may cause an increase in sympathetic nerve excitability and high stress in all organs of the body. The stress response in the digestive tract manifests as gastrointestinal stress ulcers with bleeding and is frequently accompanied by sustainable sudden contractions, spasms, ischemia, and stress-related ulceration of gastrointestinal submucosal vessels.

Pathogenesis of HFMD-Complicated Acute Kidney Injury

Acute kidney injury (AKI) refers to abnormal kidney function or structure, which manifests as reversible elevations in creatinine levels and imbalances in water–electrolyte regulation for a period of less than 3 months. Mild kidney function injury may contribute to the increased incidence and mortality of acute kidney function failure. HFMD accompanied by kidney injury is occasionally reported. Xu et al. [140] reported one EV-A71-infected HFMD patient with accompanying kidney injury. The patient's blood pressure was 145/102 mmHg with a heart rate of 93/min, and the patient showed moderate eyelid edema and altered levels of kidney function

indicators, namely serum creatinine (Scr) levels of 243.6 μmol/l, blood urea nitrogen levels of 10.3 mmol/l, and skin rash and blisters in the mouth and on the hands and feet but no other complications. As early as 1986, one CV-A16-infected HFMD adult patient with AKI had been reported to exhibit major clinical manifestations of severe amyosthenia and low blood pressure, creatine phosphokinase levels of 133,000 U/L (the normal range is 60–270 U/L), and subsequent development of rhabdomyolysis [141]. Zhou et al. [142] reported a CV-A16-infected HFMD patient with accompanying kidney injury presenting with the following symptoms: high blood pressure and moderate eyelid edema as indicated by physical examination and hematuria, severe proteinuria, hypoalbuminemia (11.3 g/l), mild hyperglobulinemia (37.0 g/l), and hyperlipemia as determined by laboratory tests. Additionally, a CV-B family member has also been found to be a causative pathogen for glomerulonephritis. Pathological examination has revealed cytopathic effects of human renal proximal tubule cells and podocytes attributed to coxsackievirus B1-6 infection [143, 144].

In conclusion, we can presume that HFMD-associated kidney injury is rarely accompanied by CNS and heart and lung dysfunction, and the common complications are skin rash, fever, and kidney dysfunction, in addition to other HFMD symptoms. Inflammatory cell infiltration in the kidney is occasionally found in animal models. Hence, we can assume that, on the one hand, HFMD-associated kidney injury is attributable to the activation of the human immune system and substantial inflammatory cytokine release after viral infection, while, on the other hand, in kidney injury and even the development of nephritis involving renovascular contraction, decreases in kidney blood volume and subsequent decreases in quick glomerular filtration rate result from multiple regulatory disorders of kidney function caused by regional autonomic nervous system injury.

Pathogenesis of HFMD-Complicated Acute Liver Injury

It has been demonstrated that severe HFMD is frequently accompanied by liver injury manifesting in markedly higher levels of aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and high-sensitivity C-reactive protein (hs-CRP) than those observed in mild patients [145]. Furthermore, a histopathological examination conducted using the EV-A71-infected rhesus macaque model indicated regional blood vessel congestion and mild inflammatory cell infiltration in the liver. Thus, the pathogenesis of enterovirus hepatitis is primarily understood as follows: EV-A71 enters the body through the oral cavity and moves into the digestive tract, proliferating in the pharynx and intestinal lymph nodes. It then invades regional lymph nodes and causes viremia and then invades the liver and spleen and other organs, where it undergoes substantial proliferation, causing direct disintegration of liver cells after entering the liver via the blood circulatory system, ultimately resulting in liver cell injury and liver dysfunction.

4 Conclusion

In summary, the diversity of enteroviruses is presumed to contribute to the diversity of the clinical manifestations of HFMD, making the pathological development and pathogenesis of HFMD very complicated. During viral infection, the biological features of the viral pathogens themselves and a variety of host genes and processes of viral infection in different tissues and organs and the state of the immune system of host cells and interaction of viruses with host immune system molecules are all speculated to contribute to the range in HFMD disease outcomes. Multiple virus-encoded nonstructural proteins, such as 2A, 2B, 2C, and 3D, and the structural protein VP1 play roles in HFMD development. Regardless of the basic questions about the development of HFMD and its related pathogenesis, the current understanding of these processes might provide many clues for the prevention and treatment of HFMD. In particular, the target proteins of virus-encoded proteins interacting with the host cell may be potential targets for the research and the development of drugs and vaccines for the treatment and prevention of HFMD. Furthermore, multiple cytokines have been identified to be closely related to HFMD severity and progression. Therefore, these cytokines can be considered potential targets for the diagnosis of HFMD and the development of antiviral treatments.

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Chapter 4
The Characteristics of EV-A71-CV-A16 Infection and Interaction with a Host

Shengtao Fan

Abstract Recently, the number of HFMD outbreaks in the Asia–Pacific region has been increasing every year. Relationship between EVs and activation of the host immune response is still not completely clear. In addition to the innate immune response, the host may launch an adaptive immune response, which the virus can escape to proliferate and replicate within the host. EVs have evolved multiple strategies to interfere with the recognition by these receptors that developed during evolution with the host. EVs also encode host cytokines and receptor mimetic factors to create a favorable microenvironment for pathogen survival and to escape the protective immune response of the host. During the coevolution of humans with other organisms, viruses participated in many host functions through viral encoded proteins or microRNAs (miRNAs), which can adapt to a new environment or host. The antiviral immune responses induced by different EVs are different. To answer these questions, more research is needed. In this chapter, EV-A71 and CV-A16 virus infections were analyzed to understand the characteristics of EV infections.

Keywords EV-A71-CV-A16 infection · Host · Immune response

1 Preface

In recent years, the incidence of hand–foot–mouth disease (HFMD), a common acute viral infectious disease in children, has been increasing in the Asia–Pacific region and poses a serious threat to the life and health of infants and young children [1, 2]. The interaction between this pathogen and the host is a key component of infection that urgently needs to be understood for the future elucidation of HFMD

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pathogenesis and the subsequent research and development of drugs and vaccines [3]. The characteristics of enterovirus (EV) pathogen infection are closely related to the interaction between the virus and host, which involves many processes, mainly viral invasion, replication, nucleocapsid assembly, shedding, and maturation [4]. The growth and reproduction of viruses cannot progress without the support of the host cell, but the host cell does not passively accept viral infection. Through long-term evolution with viruses, host cells have developed immune mechanisms to suppress viral infection, but evolution is reciprocal and selective, and viruses have also evolved immune escape mechanisms to counter the host antiviral response [5]. Understanding the biological mechanisms of viral replication is important for understanding the interactions between EVs and their hosts, providing a basis for HFMD treatment and prevention, including vaccine development.

2 Host Characteristics of EV-A71-CV-A16 Infection

Viral infection and pathogenesis are complex processes involving many factors, namely those of the virus and host, and host receptors are the first key factors in determining the degree of viral infection. A specific cell receptor is the main factor that determines the course of virus-infected tissues. Because of the complexity of various EV types, the corresponding receptors show specific characteristics. However, the EV infection pathway in a host is not unique. The target organs of EV include intestine, myocardium, respiratory tract, and CNS. Four different types of receptors, namely including EV-A71, CV-A7, CV-A14, and CV-A16, bind EVs. Scavenger receptor 2 class B, member 2 and (SCARB2) [6, 7] and coxsackie viruses A2–6, A8, A10, and A12 bind kringle (KR)-containing transmembrane protein 1 (KRM1) [8]; coxsackie viruses B1–3 and B5 bind coxsackievirus and adenovirus receptor (CAR) and the EV-E3, E6, E7, E11, and E12 strains bind LRB-decay-accelerating factor (DAF)/(neonatal Fc receptor) [4, 9, 10]. Of course, this classification has limitations. A virus does not solely bind host membrane receptors during infection; for example, EV-A71 infection involves multiple host proteins, including human P-selectin glycoprotein-1 (PSGL-1), annexin II (Annexin II), sialylated glycans, heparan sulfate, vimentin, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) [11]. CARB2 and PSGL-1 support viral replication, but the other aforementioned proteins have been shown to aid early viral binding. After host receptor-mediated entry through the lipid bilayer membrane of the cell, the viral particles adsorb the receptor, and the cell membrane begins to sag around the viral–receptor complex to facilitate endocytosis. After hydrolyzation by cell proteases, the viral particles carrying the viral protein VP4 are separated from the host receptor and the noninfectious viral subunit [12]. The RNA genome then enters the cytoplasm and initiates infection. During this process, host protein synthesis is diminished, and the virus exhibits higher protein synthesis activity. The positive RNA viral strand is the template by which the virus synthesizes complementary negative RNA viral strands in the cytoplasm, and then, the

negative RNA strand is the template for the synthesis of a large number of positive RNA strand in the endoplasmic reticulum. During this process, multiple-stranded intermediate complexes (often carrying a negative-strand template and many copies of the positive strand) are produced as large amounts of positive RNA viral strands accumulate in the cytoplasm. Then, the viral proteins are translated and progeny viral particles are assembled and are ultimately released through host cell lysis. The morphological changes in the cell leading to lysis are called cytopathic effects (CPEs) and include chromatin condensation, nuclear and plasma membrane vesicle formation, membrane permeability changes, leakage of intracellular components, and cell shrinkage. In addition, viral infection of host cells can interfere not only with host DNA replication but also host gene transcription and protein synthesis. In general, the inhibition of host DNA replication and gene transcription by viruses often leads to a secondary cause of host protein synthesis termination, with the low rate of protein generation further inhibiting host transcription [13]. The study of these mechanisms will help to clarify the mechanisms underlying viral infection and provide a scientific basis and theoretical basis for the prevention and treatment of viral disease. In this chapter, EV-A71 and CV-A16 virus infections were analyzed to understand the characteristics of EV infections.

Characteristics of EV-A71 Infection

Studies of EV-A71 infection etiology showed that the virus belongs to a group of nonenveloped protein capsids with positive-strand RNA genes and 70 polyhedrons. After entry into the host cytoplasm, the viral capsid protein and functional protein are generated by translation of the gene RNA, and complete genome replication and viral progeny particle assembly are accomplished through the action of relevant functional proteins with specific biological characteristics [14]. In 2009, Satoshi Koike et al. demonstrated for the first time that SCARB 2 is a functional receptor for EV-A71. They enabled cells to support infection with EV-A71 by expressing human SCARB 2 in an otherwise viral-insensitive mouse fibroblast (L929 cells), and the infection was blocked by the SCARB 2 protein and polyclonal antibodies [6]. Later, they demonstrated that other pathogens that cause HFMD, namely strains CV-A7, CV-A14, and CV-A16, infected the susceptible cells via SCARB 2 mediation [7]. In addition, SCARB 2 transgenic mice have been successfully infected with EV-A71 virus and were found to develop neurological symptoms such as ataxia, dysregulation, paralysis, and even death in mice with severe infection. The most severely EV-A71-infected cells were nerve cells in the spinal cord, brain stem, cerebellum, hypothalamus, thalamus, and brain. This finding comported with the pattern of severe EV-A71 infection in children [15, 16]. Additionally, in 2009, Hiroyuki Shimizu et al. enabled human PSGL-1 cell infection with EV-A71 by overexpressing human PSGL-1 on otherwise unsusceptible L929 cells [17]. The results suggested that PSGL-1 was a functional receptor of this virus and mediated cell infection. Early studies have shown that nonhuman primates, including macaques

and rhesus monkeys, are generally susceptible to EV-A71 infection. Since the outbreak of HFMD in the 1990s, nonhuman primates have been used to study the characteristics of EV-A71 neurovirulence throughout infection. However, in general, mice are not sensitive to EV-A71 infection and do not exhibit definitive clinical symptoms [18]. Interestingly, mice infected with EV-A71 virus or inoculated with an inactivated EV-A71 virus antigen showed an immune response characterized by elevated serum neutralizing antibody levels. Moreover, the offspring of mice immunized with EV-A71 virus or a viral antigen resist EV-A71 infection after exposure through intracerebral injection [19]. This result provides a preliminary basis for studying the immunological effects of EV-A71 vaccines. Due to the limitations of a mouse-suckling mouse model, nonhuman primates are important models for EV-A71 vaccine evaluation. For example, a researcher in Japan developed an infection model using an EV-A71 strain adapted to infect cynomolgus monkeys. Our previous results showing the effects of EV-A71 infection on rhesus macaques revealed that infant rhesus macaques can be used to simulate the clinical course of EV-A71 infection in humans [20]. Our preclinical studies with an inactivated EV-A71 vaccine were based on data showing the immunogenicity of the vaccine in rhesus macaque models and the immune response to infection. In addition, studies have shown that EV-A71 infection can lead to the activation of multiple molecules in the central nervous system (CNS) of animals, reducing the neural conduction threshold and leading to abnormalities in the conduction function of the CNS system. Although vaccine-immunized animals did not effectively respond to viral challenge, no abnormal changes in CNS functional molecules have been observed. The mechanisms related to these outcomes are still unknown.

Characteristics of CV-A16 Infection

The mechanisms by which CV-A16 invades the body have not been fully elucidated, but it has been inferred from studies on the molecular biological processes and structural properties of poliovirus (PV) entry into cells; PV and CV-A16 are in the same EV genus [21]. CV-A16 may bind to a specific cell membrane receptor through its canyon-like recessed structure that is formed by a quintuple axis. However, CV-A16 binds the receptor in a dynamic state, and the interaction is influenced by pH, temperature, and ionic strength. After the virus binds to its specific receptor, a series of structural changes follow. To date, the known CV-A16-binding receptors include O-linked sialylated glycoproteins or glycolipids, PSGL-1, and DC-SIGN. CV-A16 virus can infect rhesus macaques through administration in a nasal spray. Typical clinical symptoms, including increased body temperature and fatigue, appear 3–5 days after infection. Further testing of infected animals revealed viremia development between days 4 and 6 and detoxification of the nasal cavity, oral cavity, and anus. The virus was isolated during infection (1–10 days) from various tissues and organs, mainly respiratory organs, and viral proliferation was

characterized by a dynamic process distribution [22, 23]. However, our observations suggest that CV-A16 may induce mild elevation of serum neutralizing antibody levels in rhesus monkeys that have recovered from the clinical symptomatic stage; however, this immune response did not protect animals from clinical symptoms or prevent viral proliferation in various tissues and organs of the body, especially the respiratory system, after second infection with the same virus [23]. Because rhesus macaques can be repeatedly infected with CV-A16, a unique EV, researches face difficulty stimulating effective immune protection even though the virus proliferates in the tissues of the body. Moreover, the exact mechanism of CV-A16 remains unclear.

Previous studies of CV-A16 infection in rhesus macaques revealed certain similarities between CV-A16 and EV-A71 infection; neither virus infects adult mice but can cause neurological symptoms and death in suckling mice after injection into the brain. These studies show the difficulty in studying the mechanism of CV-A16 infection in mice and the mechanism of the interaction between the virus and immune system, even though it is similar to that of EV-A71. The immune response to adult mice induced by a CV-A16 virus antigen caused nervous system symptoms and death in the offspring of mice that had been previously challenged with CV-A16 through injection in the brain as suckling [24]. Therefore, these mice can be used for the immunological evaluation of CV-A16 virus and a CV-A16 antigen. Considering this evidence with mice, we analyzed the clinical characteristics and pathological process of CV-A16 infection in rhesus macaques. We established an infection model of the virus with young rhesus macaques after analyzing the infection in rhesus macaques in different age groups. We found that CV-A16 caused herpes of the oral mucosa and increased body temperature, similar to clinical symptoms observed in humans. Moreover, the animals showed detoxification in the nasal cavity, oropharynx, and anus with typical viremia; in addition, the virus proliferated in all tissues and organs [23].

3 Characteristics of the Immune Response to EV-A71-CV-A16 Infection

Characteristics of the EV-A71-CV-A16 Innate Immune Response

Innate immunity, as the first line of defense against infection, involves recognition of foreign pathogens and eliminating infection in a few minutes or hours, and it is effective against all foreign pathogens. Innate immunity is important for the early control of EV-A71 infection, as evidence showed that type I interferon (IFN)- or IFNAR-knockout mice exhibited a substantial increase in EV-A71 morbidity and mortality [25, 26]. Furthermore, treatment with type I IFN increased viral load and

mortality due to EV-A71 infection, whereas type I IFN treatment increased mouse survival [26]. These results suggest that the innate immune response is closely related to the infection and pathogenesis of EV-A71.

Innate immunity is based on at least three strategies for recognizing foreign pathogens, the first of which relies on pattern recognition receptors (PRRs) to recognize conserved nonself molecular structures, such as peptidoglycans, lipopolysaccharides, virus single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), and viral proteins of pathogens. A second, the innate immunity approach involves monitoring dangerous immune molecules, known as; these molecules contribute to the development of infections and sterile inflammatory responses by inducing the upregulation or release of immune response molecules during cell lysis or tissue damage. A third approach of the innate immune response involves receptors detecting "missing molecules" in normal, healthy organisms that are not present in infected cells or microbes. When these molecules are recognized, the immune response is not triggered, but when an abnormal signal is detected by the host, the innate immune response is triggered by the host to suppress the infection [27].

EV-A71 Infection and Host Cell Pattern Recognition Receptor (PRR)-Related Signaling Pathways

The molecular patterns associated with viral pathogens are recognized by PRRs, including retinoic acid-inducible gene 1-like receptors (RLRs), Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain-like receptors (NLRs), also known as NOD-like PRRs. The host can recognize different components of the viruses and activate natural immune responses through different downstream signaling pathways. RLRs are RNA helicases that recognize viral RNA in the cytoplasm. After recognizing invading viruses, RLRs and other viral nucleic acid recognition receptors activate downstream signaling cascades to amplify their effects; eventually, these pathways are induced to produce large amounts of IFN, tumor necrosis factor α (TNF-α), and interleukin 1β(IL-1β), which exert innate immune effects [28, 29]. In EV-A71 infection, both RIG-1, an RLR, and melanoma differentiation-associated 5 (MDA5/IFIH1) are involved in the anti-infection immune response. Although RIG-1 is structurally and functionally similar to MDA5, the viral spectrum identified by these proteins slightly differs. RIG-1 mainly recognizes the 5′-triphosphate-containing RNA and dsRNA groups during RNA viral infection. MDA5 recognizes long, >2 kb, dsRNAs. RNA binding by RIG-I and MDA5 depends on the length of the RNA, and RIG-I and MDA5 recognize different viruses probably because they recognize different RNA ligands [30, 31]. Specifically, the length of the dsRNA strand produced after virus infection is the basis of RLR recognition and activation differences. Once bound to viral RNA, RIG-I and MDA5 undergo a conformational change that leads to the release of the CARD domain, which interacts the CARD domains located in the N-terminus of mitochondrial antiviral signaling molecule (MAVS) located on the outer mitochondrial membrane

[32–34]. MAVS can bind to TNF-receptor-associated factor 3 (TRAF3) directly and specifically. Upon recruitment by MAVS, TRAF3 recruits and activates two IKK-associated kinases, namely TANK-binding kinase 1 (TBK1) and inducible IKB kinase (IKK-i; also known as Ikke); these kinases subsequently phosphorylate IRF-3 and IRF-7 [35]. Phosphorylated IRF-3 and IRF-7 induce the formation of homodimers and/or heterodimers that are translocated to the nucleus for binding to IFN-stimulated response elements (ISREs). The coactivation of hundreds of IFN-stimulated genes (ISGs) by these dimers initiates the body's anti-infection immune response [36]. NF-ƙB can also be activated by MAVS, mainly through FADD- and CASPASE-8/CASPASE-10-dependent pathways. NF-ƙB plays an important role in the activation of innate immune responses by activating several components of the innate immune system, such as proinflammatory cytokines, adhesion molecules, chemokines, acute-phase proteins (such as serum amyloid A), and inducible enzymes (such as iNOS and COX-2) [37, 38].

In addition, the TLRs comprise the first known family of PRRs and play important roles in host anti-infective immunity. TLRs recognize different pathogen-associated molecular patterns (PAMPs) of pathogens, including viruses, bacteria, mycobacteria, fungi, and parasites. PAMPs include lipoproteins (recognized by TLR1, TLR2, and TLR6), dsRNA (recognized by TLR3), lipopolysaccharide (LPS) (recognized by TLR4), Flagellin (recognized by TLR5), ssRNA (recognized by TLR7 and TLR8), and DNA (recognized by TLR9). Both the TLR3 and TLR7 signaling pathways are associated with picornavirus recognition. After binding to viral RNA, TLR3 or TLR7 recruits a TIR domain-containing ligand molecule, such as MYD88 (TLR7) or TRIF (TLR3), and induces the secretion of type 1 IFNs, inflammatory factors, and chemokines. In addition, TRIF interacts with receptor-interacting protein 1 (RIP1), which in turn activates NF-ƙB and subsequently induces inflammatory cytokine release [39]. Type I IFN binding to an interferon receptor (IFNAR) activates the receptor-related proteins Janus kinase 1 (JAK1) and TYK2 tyrosine kinase (TYK2), leading to phosphorylation of potential cytosolic transcription factor signaling molecules and STAT1 and STAT2 transcriptional activators. Phosphorylated STAT1 and STAT2 can form dimers and be translocated into the nucleus, where they interact with IRF9 to form a three-molecule complex called IFN-stimulated gene factor 3 (ISGF3). Then, ISGF3 binds to a homologous DNA sequence and becomes an SRE, which directly activates the transcription of hundreds of ISGs and drives the body into anti-infection immunity activation. In contrast to the actions of TLRs, inflammasomes formed during NOD-like receptor signaling play important role in the inflammatory response; they can induce the activation of caspase-1 and secretion of inflammatory factors, including IL-1β and IL-18, which are involved in the regulation of innate immune responses following viral infection [40]. Previous studies have shown that HFMD-causing virus infection can induce the production of IL-1β and activate the formation of NLRP3 inflammasome, which plays an important role in the innate immunity inflammatory response.

EV-A71 Infection- and Injury-Associated Molecules

In 1994, French immunologist, Polly, and colleagues put forward the danger theory; that is, in addition to heterologous infectious substances, endogenous factors released because of stress produced by injury or cell death can induce natural immune signals, and at the same time, the adaptive immune response can be initiated directly or indirectly. The aforementioned endogenous molecules are called danger-associated molecular patterns (DAMPs) and are generally found in the nucleus, cytoplasm, or extracellular matrix; for example, heat shock protein (HSP), high-mobility group box 1 (HMGB 1), ATP, SP100, cytosolic RNA, and cytosolic DNA (including mitochondrial DNA released by damaged or stressed cells) are all perceived by a range of PRRs as risk-associated molecular patterns, triggering natural immune responses and proinflammatory responses. However, the roles played by DAMPs in pathogen infection, in which they function as a double-edged sword, has attracted considerable attention. DAMPs can lead to the elimination of pathogens and infected and damaged cells, which contributes to host repair. In contrast, DAMPs can aggravate inflammation, leading to tissue damage, enhancing the susceptibility of pathogens, and increasing the severity of infection. Therefore, whether from the perspective of prevention or treatment, DAMPs have shown good research value and application prospects. In fact, the expression level of DAMPs is the basis of evaluating the severity of disease and predicting a prognosis, providing new and important clues for research on the pathogenesis, prevention and treatment of infectious diseases such as HFMD, and the development of related vaccines.

Characteristics of the EV-A71-CV-A16 Adaptive Immune Response

The adaptive immune response, including cellular immunity and humoral immunity, is the host's second line of defense against pathogens. The components that play roles in cellular immunity are mainly T lymphocytes, which can be categorized into $CD4^+$ T cells (including subsets of T-helper cells, such as Th1, Th2, and Th17, and regulatory T cells [Tregs]) and $CD8^+$ T cells (including TC1, TC2, and TC17 cells). The main components of humoral immunity are B lymphocytes and the antibodies they secrete. Among the $CD4^+$ T cell population, cells that secrete cytokines such as IFN-γ, IL-2, and TNF-α are generally considered to be Th cells, with Th1 cells mediating T-cell cytotoxicity and delayed hypersensitivity, and cells that secrete IL-4, IL-5, cytokines such as IL-6 and Il-10, are defined as Th2 cells, which mainly promote the activation and maturation of B cells. $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTLs), can directly and specifically bind to and kill infected target cells, which is one of the most effective ways to eliminate pathogens in a host. B cells are the main cells involved in humoral immunity. After stimulation by an antigen, B cells are transformed into plasma cells, which synthesize immunoglobulins. Antibodies are immunoglobulins that bind to targeted antigens. Little is

currently known about the role played by adaptive immune cells through antibody-dependent cell cytotoxicity (ADCC) in the pathogenesis of HFMD.

In a study that utilized EV-A71 to infect mice in different immune states, mice lacking $CD4^+$ or $CD8^+$ T cells exhibited more severe disease and pathological outcomes than wild-type mice. Moreover, data have indicated that $CD4^+$ and $CD8^+$ T cells are associated with the development and outcome of EV-A71 infection that causes HFMD [41]. A study that on infected people in a phase III trial of the inactivated EV-A71 vaccine showed that significant increases in the Th2-associated cytokines IL-4 and Il-10 were detected in the serum of EV-A71-infected children [42]. The levels of Th1 cell cytokines (IL-2 and IFN-γ) and IL-10 in the serum of CV-A16-infected children were higher than those of other EV-infected children. However, the levels of Th1 and Th2 cell cytokines (IL-2, IL-16, IFN-γ, IL-4, and Il-10) were increased in the serum of children infected with other types (non-EV) viruses. Chen et al. found that the proportion of Th17 cells was significantly elevated in the peripheral blood of EV-A71-infected children with HFMD, suggesting that Th17 cells may be associated with the pathogenic mechanism of EV-A71 infection [43]. In contrast, another study of a clinical cohort of patients with HFMD found significantly higher percentages of Th1 and TC1 in the peripheral blood of children with mild and severe HFMD compared with the controls; moreover, the ratio of Th1/Th2 and the expression level of IFN-γ in the peripheral blood of the children with HFMD was also increased. Furthermore, the proportion of Th17 cells and the IL-17A expression levels were highest in critically ill patients and lowest in negative controls, with the same trend represented by the Th17 cell /Treg ratio. Similarly, the levels of Th1, Th17, TC1, and TC17 cells in peripheral blood, as well as IFN-γ and IL-17A levels in serum, were significantly higher in CV-A16-infected children than in negative controls [44]. In terms of humoral immunity, no significant difference in EV-A71-specific neutralizing antibodies was found in different infected patients. In summary, although the data available are not consistent with respect to the state of the host immune response after EV-A71 and CV-A16 infection, these results clarify that cellular immune responses play important roles in the course of infection.

In the development of an inactivated vaccine against EV-A71, immunization with an inactivated EV-A71 virus antigen induced an increase in neutralizing antibody levels and IFN-γ-specific T lymphocytes in rhesus monkeys. Rhesus monkeys immunized with an inactivated EV-A71 vaccine and then infected with wild-type virus showed a significant increase in IFN-γ and TNF-α levels in serum. The antigen levels after 7 days of infection showed a particular pattern. In addition, the level of IL-4 returned to normal 14 days after infection, while IL-6 showed only a slight change. The Th1 immune response, mainly involving IFN-γ and TNF-α secretion, may have played a dominant role in anti-EV-A71 activity in the immunized monkeys. In addition, an increase in the number of IFN-γ-secreting cells was also detected in the bronchi, hypothalamus, and spinal cord in the infected monkeys; however, 27% of the IFN-γ-secreting cells were detected in nervous system tissues of the positive virus-infected control group, which was significantly higher than that of the immune group, and the treatment group exhibited necrosis, retrogression, many aggregating glial cells, and vascular cuff formation. Therefore, we were

convinced that Th1-mediated T-cell cytotoxicity played an important role in the virus defense of these monkeys during EV-A71 infection; however, the excessive proliferation of these cells may be related to the pathological damage caused by the viral infection, and the number of details regarding these findings needs to be increased. In combination with the results observed in the aforementioned clinical cases, we can speculate that host resistance to EV-A71 virus infection is mainly realized through Th1-cell-mediated cytotoxicity; however, the proportion of Th1, Th2, Th17, and Treg cells must be maintained within a reasonable range to promote host defensive action. After the proportion of these cells is imbalanced or a subset of these cells excessively proliferates, the pathological results of the virus infection may increase [44]. The molecular mechanisms needed to maintain cell stability during infection need to be further explored. Although EV-A71 and CV-A16 are both in the same EV genus and can cause similar clinical symptoms, the anti-infective immune responses induced by EV-A71 and CV-A16 are quite different. A CV-A16-inactivated antigen did not produce a protective effect against wild-type virus infection in the rhesus monkey model. However, the expression of the LEF1, NTNG1, ARHGEF 28, and PCDHA6 genes, which are related to the activation of the innate immune response, was significantly upregulated after infection of 16HBE cells with each of these viruses in vitro. These results were verified with rhesus monkey model. The upregulated expression of genes related to T follicular helper (TFH) and Treg functions was observed in rhesus monkeys infected with CV-A16. These results suggest that CV-A16 infection induces, at least, specific immune responses in rhesus monkeys. However, the CV-A16 antigen did not appear to induce an immune response at an intensity to protect the monkeys against future viral attack. In contrast, CV-A16 antigen immunization of animals by intradermal immunization induced an appropriate immune response and led to a protective effect in more than 95% of the animals [45]. The reason for the success of the latter vaccination test is that skin tissue contains abundant innate immune cells, including Langerhans cells (LCs) and dendritic cells (DCs), which play important roles in antigen presentation and immune response activation. In support of this explanation, recent studies have shown that antigens delivered to dermal or epidermal cells induced activation of innate immune responses. LCs and DCs in skin can capture antigens, transport them to lymph nodes, and present them to corresponding immune cells on the basis of the characteristics of the antigen. However, because of its weak immunogenicity, CV-A16 virus cannot induce the proper intensity of an immune response; therefore, improvements to its ability to induce an immune response may be realized by using the cell population in skin tissue.

4 EV-A71-CV-A16 Interacts with the Host

During the coevolution of humans with other organisms, viruses participated in many host functions through viral encoded proteins or microRNAs (miRNAs), which can adapt to a new environment or host. These proteins and RNA species

facilitate virus escape from a host's immune attack, enabling the virus to leverage a host's cell reproduction and replication machinery to create a favorable microenvironment, and ultimately promote host disease. Based on the mechanism of interaction between EV-A71-CV-A16 and the innate immune system, most instance of viral immune escape is caused by degrading or hijacking host proteins in signaling pathway or leveraging the protein signal needed for cell differentiation induced by proteases, structural proteins, and nonstructural proteins of a virus. Alternatively, a virus may directly affect the host's immune response by acting on certain virus-encoded mRNAs (affecting the transcription of genes involved in antiviral signaling pathways) in host cells. In addition, viral infection can stimulate changes in the expression profile of certain miRNAs in host cells, which in turn can affect mRNAs (affecting the transcription of genes associated with antiviral signaling pathways) in host cells.

The Mechanism by Which EV-A71-CV-A16 Escapes the Innate Immune Response

During the activation of the innate immune response, the secretion and activation of IFNs, especially type 1 IFNs and various cytokines, are key steps. As the main antiviral molecule in the host, IFN not only exerts an antiviral effect, that is, produce IFN α/β immediately after viral infection, but also protects neighboring cells from viral infection via paracrine signaling, reducing viral spread. Moreover, IFN play an immunomodulatory role, promoting macrophage phagocytosis of antigens by, natural killer (NK) cells killing of infected target cells and of T- and B-cell activation, to enhance the immune response of the host. Therefore, blocked production of IFN can directly increase viral survival in a host. In addition, virus escape from the innate immune response is mainly realized by the action of virus-encoded proteins or miRNAs, which can exert various respects, including inhibiting host protein synthesis, antigen recognition, and interferon signaling pathway activation.

Cessation of Host Protein Synthesis

Viruses are microorganisms that live strictly in the host cell. They need the host cell to provide the space, environment, and certain enzymes for reproduction. Viruses encode host cytokines and receptor mimetic factors to create a favorable microenvironment for pathogen survival and to escape the protective immune response of the host, which it achieves by interfering with the metabolic system of host cells. The mechanism of action of viral mimetic molecules mainly involves blocking or interfering with the host cell's immune defense system and regulating normal cell growth and metabolism pathways. In the case of PV, the host stops synthesizing host RNAs and proteins. In this process, eukaryotic translation initiation factor 4G (eIF4G) and

cap-binding protein complex P22 subunit are cleaved by the virus-encoded 2A protein kinase, which inhibits the translation of the corresponding host RNA. However, eIF4G cleavage only partially blocks host protein translational in EVs, suggesting that other mechanisms are also involved in EV-induced translational repression [46]. PV infection can inhibit RNA polymerase II-mediated transcription in host cells, which is achieved by the cleavage of TATA-binding protein (TBP) and cyclic AMP-responsive element-containing protein (CREB) by a virus-encoded 3C protease [47, 48]. Similarly, EV-encoded 3C protein-mediated protein cleavage of poly A-binding protein (PABP) plays an important role in the translational inhibition of apoptosis [49]. These studies suggest that EVs block the transcription and translation of host mRNA through, at minimum, 2A and 3c proteases and that this overarching cessation of host protein synthesis may, to some extent, inhibit the synthesis and secretion of IFN and cytokine signaling pathway components or other proteins associated with their secretion.

Interference with PRR Recognition

Because EVs are recognized by PRRs on the surface and inside of host cells, EVs have evolved multiple strategies to interfere with the recognition by these receptors that developed during evolution with the host. Previous studies have shown that both RLRs and NLRS, which are involved in the recognition of viruses, can be used as targets for virus escape from the host's innate immune response. Although TLRs play critical roles in targeted anti-EV response, to date, no evidence has shown that EVs can act directly on TLRs to escape the innate immune response. For example, after PV or human type retrovirus 1 (HRV1) infection, MDA5 degradation can be induced in a proteasome- and caspase-dependent manner, and degradation of this receptor is independent of 2A and 3c protease action [50]. In contrast, CV-B3, EV-A71, and PV 2A proteases have been reported to directly cleave MDA5 [51]. In addition to MDA5, RIG-I is a key target of action for CV-B3 and EV-A71, and RIG-I cleavage degradation can be induced by the 3C protease after viral infection [51, 52]. However, some studies have indicated that the 3C protease inhibits RIG-I-mediated production of type I IFNs by preventing the formation of functional complexes; this process is mediated by cytosolic RIG-I and MAVS adaptor molecules not by direct cleavage of RIG-I [53]. In conclusion, the mechanism of EV-encoded 2A and 3C proteases interact with the RLRS receptor members MDA5 and RIG-1 is currently unclear, virus-encoded proteases clearly can directly or indirectly mediate the cleavage-induced degradation of MDA5 and RIG-1 to inhibit interferon secretion by the host. Furthermore, the NLRP3 inflammasome is activated after EV-A71 infection and plays a protective role against infection with EV-A71 [54]. However, after EV-A71 infection, NLRP3 can be cleaved by 2A and 3C proteases, inhibiting NLRP3 inflammasome activation [54].

Interference with Molecules Involved in Innate Immune Signaling Pathways

In addition to interfering with PRRs, many molecules in innate immune signaling pathways are targets of viruses that evade antiviral responses, and EVs can interfere with viral recognition of downstream MAVS adaptors through other protective mechanisms [51, 55, 56]. For example, the EV-A71 2A protease directly targets and cleaves MAV at three different sites, none of which can activate type I IFN production [55]. Similar to EV-A71 infection, CV-B3 infection can cleave MAV in a similar pattern [51, 55]. The MAV cleavage site of PV 2A proteases is completely different from that of CV-B3 and EV-A71 2A proteases. Interestingly, CV-B3 3C proteases can cleave overexpressed MAVS in vitro, whereas EV-A71 3C cannot cleave MAVS [53, 55, 57]. These results suggest that MAVS cleavage is a common part of the process by which EVs inhibit the production of type I IFN. In a mouse model of PV infection, TRIF-mediated TRL3 signaling pathways played important roles in antiviral immune responses [58]. Similarly, activation of the TLR3 signaling pathway in macrophages was important in protecting aged mice against EV-A71 and CV-A16 infection [59, 60]. The EV-A71-encoded 3C protease interacts with TRIF and induces its cleavage, which in turn inhibits TLR3-mediated antiviral responses [61]. Furthermore, the 3C protease of CV-B3 cleaves TRIF at multiple sites, including the N- and C-terminal regions, and localizes to the signaling complex via TRIF, preventing the production of type I IFNs within the cytoplasm [57]. Furthermore, the 3C protease of EV-D68 can cleave TRIF, albeit at a different site from that targeted by EV-A71 or CV-B3 3C [62]. These results suggest that TRIF is a common target by which EVs inhibit activation of the TLR3 signaling pathway.

When an adaptor molecule is activated, a signal is transmitted to effector targets, including the IKB complex and TBK1/IKK complex, which then activate NF-ƙB and IRF3/7, respectively. After a signaling pathway is activated, NF-ƙB and IRF3/7 are translocated to the nucleus where they induce the production of type I IFNs [28, 63]. However, both EV-A71 and EV-D68 infection can reduce IRF7 expression [64, 65]. The EV-A71 3C protease can directly induce IRF7 cleavage by cleaving Q189 at a guide site, a process closely related to 3C proteolytic activity. Cleaved IRF7 fails to activate type I IFN production [64]. Similarly, Lee et al. found that the EV-A71 3C protease inhibited the production of type I IFN in mice [36]. In addition, viruses suppress innate immune responses by targeting the NF-ƙB signaling pathway, which plays an important role in activating interferon or inflammatory cytokine production. EV-A71 3C protease inhibited NF-substance activation by cleaving the transforming growth factor-activated kinase 1 (TAK1) complex [40]. In addition to the enzymatic activity of virus-encoded proteases 2A and 3C, the viral nonstructural protein 2C plays an important role in EV-A71 escape from innate immunity [66, 67]. Specifically, 2C proteins can interact with IKK to inhibit TNF-mediated activation of NF-ƙB [67]. It has also been reported that EV-A71, EV-D68, and PV-encoded 2C proteins inhibit the formation of heterodimers P65 and P50 by interacting with p65, thereby inhibiting the activation of NF-ƙB [66]. Taken together, these data show that EV-encoded proteases 2A and 3C can influence their cell

biological activity by cleaving adaptor and downstream effector molecules, thereby interfering with innate immune signaling pathways, subsequently inhibiting the secretion of IFN and escaping the innate immune response. In addition, EV-encoded nonstructural protein 2C can inhibit activation of NF-ƙB and inhibit secretion of IFNs by interacting with important molecules in the NF-ƙB signaling pathway.

Interference with Interferon-Mediated Signaling

The aforementioned research shows that EV-A71 can inhibit the production of IFN at different stages, such as by interfering with the translation of host mRNA, the recognition of antigen and receptor, and interfering with the adaptor and downstream effector molecules in the innate immune signaling pathway. Does EV-A71 affect the activation of the IFN-1 receptor IFNAR1? The activity of the IFN-1 receptor is realized through the Janus-activated kinase (JAK)-signaling sensor that induces ISG and transcription activator (STAT) signaling pathways. Indeed, Lu et al. [68] found that EV-A71-encoded 2A protease degrades IFNAR1, which in turn inhibited IFN-mediated phosphorylation of STAT1, STAT2, JAK1, and tyrosine kinase 2 (TYK2). Another protease, 3C, encoded by EV-A71, also inhibited JAK1-STAT signaling by cleaving the IRF9 protein. IRF9, an important protein in the JAK1-STAT signaling pathway, together with STAT1 and STAT2 proteins constitutes a heterologous complex that induces ISGS expression induced by ISGF3. The degradation of IRF9 can directly block the JAK1-STAT signaling pathway and subsequent inhibit IFN production. However, Liu et al. [69] found that EV-A71 can inhibit JAK1-stat signaling by downregulating JAK1 expression but not IFNAR1 expression. In summary, EV-A71 can inhibit the JAK1-STAT signaling pathway as well as subsequent interferon production either by enzymolysis of IFNAR1 or IRF9 molecules by virus-encoded proteases or by downregulating JAK1 expression.

MicroRNA (miRNA) Interference Regulation

Increasing evidence suggests that the development of a range of viral infectious diseases is associated with an imbalance in miRNA regulation [70]. When a virus invades the host, host cell-encoded miRNAs protect against viral infection, and structural or nonstructural viral proteins regulate host miRNAs to trigger a variety of pathophysiological processes, such as immune escape [71]. For example, hepatitis C virus HCV significantly increased intracellular miR-122 expression, and upregulated miR-122 in turn activated HCV genome replication, ultimately promoting disease progression [72]. Japanese encephalitis virus (JEV) induced the production of miR-301a by host cells to block the innate immune responses of neurons mediated by interferon regulatory factor (IRF)-1, thereby promoting their own survival within the host [73]. miR-21 promoted the replication of dengue virus serotype 2 (DENV2) in human laryngeal epithelial cells (Hepg2) [74]. In addition, the role played by miRNA in EV-A71 and CV-A16 infection is gradually being revealed;

for example, EV-A71 infection upregulated or repressed miRNA expression in certain hosts, either directly or indirectly by targeting genes associated with the immune response, facilitating EV-A71 escape from the host's innate immune response and other viral infection processes in the host [75]. Li et al. found that the miRNA-548 family, including miR-548b-5p, miR -548c-5p, miR -548i, miR -548J, and miR-548n, downregulated the expression of IFN1 by targeting the 3' UTR of IFN1, which in turn promoted EV-A71 infection [76]. Ho et al. reported that EVs induced the upregulation of miR-146A expression, which inhibited IFN production by targeting IRAK and tumor necrosis factor receptor-associated factor 6 (TRAF6) [77]. Hence, the pathological damage caused by EVs is clear. Xu et al. demonstrated that EV-A71 infection drove the downregulation of miR-526a expression, which in turn drove the upregulation of cylindromatosis tumor suppressor gene (CYLD) expression and ultimately inhibited RIG-I pathway activation, leading to the downregulation of IFN-I expression [78]; this mechanism allowed the virus to escape the innate immune response. After EV-A71 infection in HEP2 and rhabdomyosarcoma (RD) cells, the miRNA expression profile of the host cells exhibited significant changes, implying that altered miRNA expression may play an important role in EV-A71-host interactions [79]. Taken together, these studies have shown that miRNAs are involved in the innate immune response process in the early response to exogenous or endogenous stress signals in cells after viral infection. Viral infection can cause the upregulated or downregulated expression of certain miRNAs. These miRNAs then promote viral infection in host cells by targeting interferon secretion-related genes. Although the host cell encodes miRNAs to fight against viral infection, the virus genome also encodes miRNAs or alters the expression profile of miRNAs in host cells, directly or indirectly interfering with the expression of many host immune-related genes to escape from the host immune response.

The interactions between EVs and the host are complex. As mentioned above, during EV infection, EV-encoded structural proteins, nonstructural proteins, and miRNAs regulate the host's intracellular signaling pathways through a variety of strategies and thus evade the host's innate immune response, ensuring continued infection within the host. Of course, the mechanism through which EVs escape the innate immune response and include only those that have been reported thus far. In summary, the EV immune escape mechanism is still not completely clear; for example, which viral protein molecules are involved in immune escape, and what molecules do these viruses target? To answer these questions, more research is needed.

EV-A71-CV-A16 Escapes Adaptive Immunity

In addition to the innate immune response, the host may launch an adaptive immune response, which the virus can escape to proliferate and replicate within the host. The escape of viruses from adaptive immune processes is affected by two main factors: first, the differentiating and inhibiting influence of viruses on T-cell development, and second, the effect of viruses on B-cell development and differentiation that

enables their escape from the neutralizing antibodies produced by plasma cells. Although the mechanisms underlying EV induction of adaptive immunity and possible adaptive immune escape from the host are unclear currently, however, considering experimental clues, we have found the degree of Th1 cells and Th17 cell activation, the level of IL-17A secretion, and the ratio of Th17 cells/Tregs differed depending on whether patients presented with mild or severe EV-A71 infection. These results suggest that host seems to employ specific strategies that enable EV-A71 to escape the host's adaptive immune protective response, and this process of immune escape results in differences in the immune responses manifested by patients with mild and severe infection. Moreover, the immune status of cells in children and rhesus monkeys with CV-A16 infection is markedly different from that in models infected with EV-A71. However, similar to those of EV-A71, the mechanisms by which CV-A16 escapes the adaptive immune response of the host remain unclear.

Relationship Between EVs and Activation of the Host Immune Response

Research on the antiviral immune response has shown that the immune response caused by viral infection is, in fact, the comprehensive effect of the entire immune system based on the response to pathogens. This process involves structural recognition of different PAMPs by PRRs inside and outside cells of infected tissues, especially mucosal epithelial tissues. Therefore, the related signaling pathways induce the transcription of NF-ĸB, which leads to the production of different signaling molecules, such as immune factors or inflammatory cytokines; these molecules then stimulate and regulate various components of the innate immune system (including innate lymphoid cells (ILCs)) and various subsets of T and B cells in the acquired response system to release specific antibodies and induce CTL responses. Finally, the data have revealed a complete course of immune protection in the clinical rehabilitation stage. Considering our previous work, we have concluded that the antiviral immune responses induced by different EVs are different. The difference in PAMP recognition by PRRs in epithelial cells may be caused by differences in structural molecules on the surface of the cells infected by a virus, such as differences in the epithelial cells of the respiratory and digestive tracts. These differences in the activation and regulation of innate immune responses may lead to different interactions between the innate and acquired immune systems and ultimately to differences in antiviral immunity. In vitro, EV-A71 and CV-A16 infection of respiratory/digestive tract epithelial cells showed differences in VPS, 2A, 2B, 3A, 3B, mRNA virus gene expression and interactions of TLRs and NLRs in cells, which may explain differences in the expression profiles of signaling molecules in the innate immune response. With this possibility in mind, the activation of innate immune response signaling molecules on different innate immune cells was

observed, especially ILCs, and their cellular functions after activation in animal models; these functions included specific cell-killing effects by ILC1-like subsets and the regulatory effects of other secondary signaling molecules expressed by ILCs on DCs and T cells in tissues. Through experiments of EV-A71 and CV-A16 infection in the respiratory or digestive tract of nonhuman primate models, the properties of EVs that trigger DC chemotaxis in epithelial tissues were observed, and subsequently, $CD11c^{+}/CD141^{+}$ DC cells were identified as markers of infection [22]. The pathological manifestations of this infection included inflammatory damage in certain main tissues and organs accompanied by viremia; herpetic pathological lesions on the mucous membranes of the lips and limbs of the infected animals; and increased expression levels of certain inflammatory factors in the blood [20, 22]. Interestingly, despite the similarities in the pathological manifestations in EV-A71- and CV-A16-infected animals, distinct features of the antiviral immune responses to these viruses were identified. EV-A71 infection elicits potent immune responses, including elevated levels of neutralizing antibodies in serum and specific CTL responses. More importantly, the immune response confers effective clinical protection against challenge with wild-type viruses [24]. CV-A16 infection induces neutralizing antibody and specific CTL responses but does not lead to effective overall protection from clinical infection with the virus [22]. Further gene response map analysis of immune cells in EV-A71- and CV-A16-infected primates showed that CV-A16 infection caused marked upregulation of the transcriptional expression of genes associated with functional activity in TFH cells and Tregs in the animals [80]. In addition, a comparative analysis of gene response patterns of human bronchial epithelial cells after infection with EV-A71 and CV-A16 indicated that the expression of certain molecules related to innate immunity, including LEF1, NTNG1, ARHGEF28, and PCDHA6, was significantly upregulated by CV-A16 infection [81]. Although these data have not yet been systematically reviewed, they provide characteristic data on differences in the development of antiviral immune responses in EV-A71 and CV-A16 infection at different stages. These findings provide clues for further understanding of the immune system-related roles played by these two EVs.

5 Conclusion

Recently, the number of HFMD outbreaks in the Asia–Pacific region has been increasing every year, posing a serious threat to the life and health of infants and young children. EV-A71 and CV-A16 are EVs, which are small RNA viruses. The diseases caused by EV-A71 and CV-A16 can seriously affect the health of children. Therefore, the epidemiological characteristics, pathogenesis, and immune response associated with these viruses have been extensively studied, and the development for these viruses continues. PV and hepatitis A vaccines are typical examples of EV vaccines. However, differences in the infection modes, pathogenesis, and clinical pathological characteristics of the many known EVs are still not completely clear,

and their pathogenesis may be particularly complex. Therefore, because the specific pathological mechanism is not clear, explaining the cause of the common herpetic lesions on the whole body or hand, foot, and mouth caused by EV-A71 or CV-A16 infection is difficult. In particular, we ask, what is the relationship between these pathologies and the host? Only by systematically studying the infectious characteristics of EVs and the relationships between EVs and their hosts can resolve the challenges. When answer these questions, a theoretical basis for effective treatment and vaccine research of HFMD is acquired.

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Chapter 5
Pathogen–Host Interaction and Its Associated Molecular Mechanism in HFMD Pathology and Immunology

Qihan Li, Ying Zhang, and Yun Liao

Abstract Hand, foot, and mouth disease (HFMD) is caused by the enterovirus family, which includes EV-A71 and more than 10 other members. The disease involves a complex pathological mechanism that includes intricate and finely-tuned interactions between these pathogens and the host. The research on vaccines that can effectively be used to prevent HFMD caused by major pathogens suggested that the viruses presenting with the same structure but different antigenic traits in response to the immune system interactions enable to interact dynamically to cell surface receptors and to lead to similar pathological outcome through diverse mechanisms. This suggests that further understanding of the whole process of signal stimulation by viral antigen molecules and innate immune receptor molecules could improve our recognition about the events of pathological injury to the body and the characterization of antiviral immune responses with phenotypic differences during the pathogenesis. The accumulated data about process of interaction between virus structure and host in molecular level might provide the theoretical and technical support for next generation of vaccine against HFMD for public health initiatives.

Keywords Etiology · Molecular biology · Immunology

1 Introduction

In recent years, hand-foot-and-mouth disease (HFMD) has become prevalent in Asia–Pacific countries. It causes hand, foot, and mouth lesions, which are the primary clinical manifestations in children, and it can cause neurogenic

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cardiopulmonary failure or function-impairing epidemic viral diseases in certain cases [1–5]. Therefore, HFMD has become a public health issue that needs attention [6–9].

According to substantial etiological studies, a variety of HFMD-related pathogens are similar; they include EV-A71 coxsackievirus groups A and B in the enterovirus family, such as CV-A16, CV-A6, CV-A10, and CB3 [9–13]. However, these pathogens show subtle differences in the biological infection processes that cause pathologies with a similar mechanism underlying clinical manifestations [14, 15]. Therefore, to a great extent, HFMD reflects molecular mechanism characterized by complex and fine-tuned interactions between viral pathogens in the environment and their human hosts.

With an in-depth comprehension of interaction mechanisms, researchers are gaining specific insights into the human host response to viral infection and the related defense patterns, or their understanding of this process has been confirmed, especially in terms of clinical diagnosis, treatment, and prevention through the innate and adaptive immune responses. HFMD in children is caused by multiple pathogens, and the clinical characteristics range from moderate to severe because their lifespan in the organism and immune responses vary [9–11, 16, 17]. For example, an infection exhibits specific biological patterns that vary by viral strains and elicits different inflammatory and innate immune responses by triggering the expression of genes that perform different functions [18–20]. Hence, HFMD is a systemic model valuable for investigating the different effects of viral species or strains and the subsequent pathological and immunological outcomes. Furthermore, HFMD is a viral epidemiological model that can be used to distinguish clinical prognoses.

2 Etiological Significances of the Original Structural Characteristics of HFMD-Causing Viruses

The pathogens that cause HFMD in children are primarily members of the enterovirus family, as verified by etiological studies, and more than ten kinds of enterovirus have been thus identified, including EV-A71, CV-A16, CV-A10, CV-A6, and CB3 [9–13]. Poliovirus, which causes poliomyelitis in children, has attracted attention as a representative of the enterovirus family and has become the basis for the comprehensive study of these viruses in terms of their molecular biology, structure, and infection mechanism. These studies have provided a solid basis for understanding the behavior of various HFMD pathogens in children. In particular, the analysis of the novel seventy-member polyhedron nucleocapsid-encapsulated single-stranded RNA gene structure of this small virus, for which the thermodynamics have been analyzed based on crystallography techniques, has been particularly useful. These studies allow us to start our analysis by evaluating the structural dynamics of pathogens in combination with different receptors and subsequent intracellular events to

understand pathogenic infection, which is characterized by inflammatory responses, and relevant pathological outcomes.

Significance of the Structural Dynamics of Picornaviruses

Studies on viral molecular biology have confirmed that a specific cell surface receptor is vital to mediating virus entry into a cell and the subsequent tissue-specific tropism, pathological processes, and clinical outcomes of the infection [21–23]. The binding of viruses to specific corresponding receptors, which are structural proteins on the cell surface with signal-mediating or other physiological functions, is the basis of the structural correspondence between biological macromolecules involved in the interaction between viruses and human hosts [24, 25].

Therefore, investigating and analyzing the processes and results of infection caused by the binding of viruses and to specific receptors can provide a molecular biology basis for an in-depth understanding of the pathology caused by a class of infectious viruses. For example, viruses that cause HFMD in children belong to the picornavirus family, the structural characteristics of which are clearly understood. These enveloped viruses are composed of a single-stranded positive-strand RNA structure with a diameter of approximately 30 nm, and the most important structural feature for binding cell receptors is mainly an icosahedral nucleocapsid structure, which consists of the viral structural proteins VP1, VP2, VP3, and VP4. These VP proteins are composed of capsomers, and each capsomere unit forms a pentamer with 5 axes of symmetry, and 12 pentamers form a nucleocapsid. The complete virion formed by the viral nucleocapsid wrapped around the viral genome exhibits a characteristic surface structure, with a canyon-like trench structure that includes the binding site for a corresponding receptor (Fig. 5.1).

Although different picornaviruses generally comprise four structural proteins and an identical icosahedral particle structure with a characteristic canyon-like trench that harbors a receptor-binding site, differences in amino acid sequences of each structural protein determine different surface structures in the center of the canyon-like structure in the capsid; these structural differences are largely derived from different amino acids, particularly basic amino acids, which endow the virus with specific charge characteristics [27, 28]. Thus, a class of cell receptors is defined on the basis of the specific picornaviruses that bind to them; however, although the members in each class of cell surface proteins have similar structures, the receptors bound by specific viruses vary.

By comparing the structures of a certain number of primary picornaviruses, including the viruses that cause HFMD in children, the validity of an inferred virus identity can be verified (Fig. 5.2). Hence, an in-depth analysis of the structural thermodynamics of picornavirus virions enables us to develop a more complete concept of the molecular biological process by which these viruses interact with their receptors.

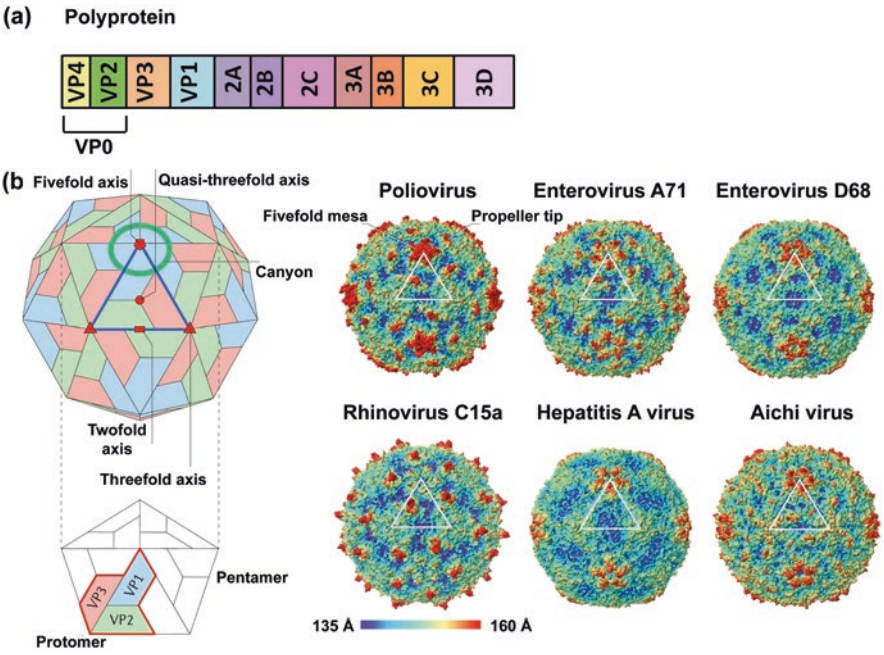

Fig. 5.1 The structural characteristics of picornavirus [26]. (**a**) The characteristics of vial polyprotein; (**b**) the three-dimensional structure of viral nucleocapsid. Reused with permission from [26]

According to previously reported data, the structural dynamics of the picornavirus, for which the poliovirus is a model, greatly depend on humidity and pH changes in the structural microenvironment when the virus binds to a receptor, especially at physiological temperature (37 °C). The conformational change of the virus allows the internal structural components (VP4 and part of the VP1 fragment) to flip outward to be exposed on the viral surface, which facilitates viral particle binding to a cell surface receptor, and when a virus or viral particles bind the receptor, small N-terminal fragments of VP4 and VP1 are cleaved. In summary, viruses leverage changes in their own structural dynamics to systematize the biological program of infection; that is, a virus binds to a receptor, and then, its genome enters the cell [34–39].

Furthermore, the dynamic structural changes in certain viral and cellular structures depend on pH changes (acidification) at the cell membrane or in vesicles [40, 41]. Therefore, viral entry into cells is based on the dynamic characteristics of structural viral proteins in a specific physical and chemical environment, and in any case, microscopic behaviors are determined by the molecular biology through which different picornaviruses bind to corresponding receptors, prompting viral entry into a cell and leading to specific pathological outcomes. That is, the infection process of each picornavirus differs and is an important basis for the molecular biological mechanism of the clinicopathological characteristics of the diseases it causes.

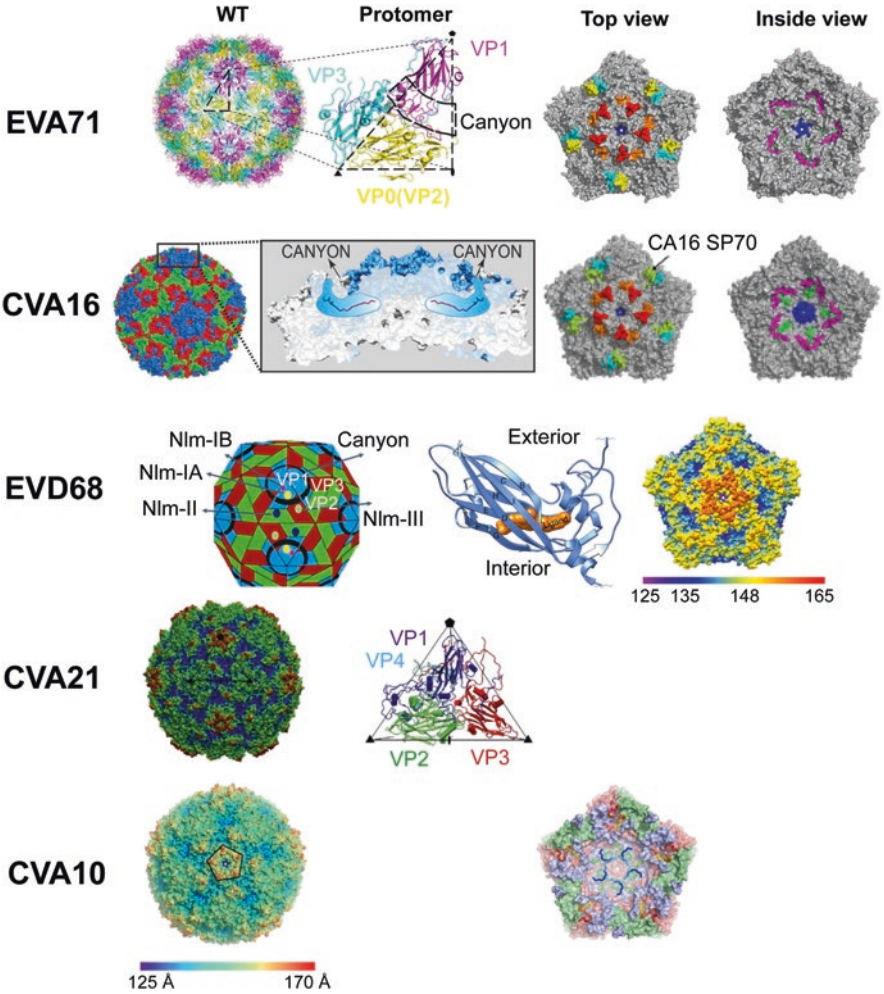

Fig. 5.2 The structural thermodynamics of picornavirus virions [29–33]. Reused with permission from [29–33]

Structural Characteristics of the Major Pathogens Causing HFMD and Their Host Receptors

As previously mentioned, recent research suggested that various enteroviruses exhibit a clear pathogenic association with clinical diseases such as HFMD in children. In other words, many viruses can be considered causative agents of HFMD; however, the main viral pathogens are EV-A71, CV-A16, CV-A10, and CV-A6, which are similar to other enteroviruses, in that they carry the characteristic picornavirus structure and a canyon-like trench structure that is unique to each species

(Fig. 5.2). Differences in the primary viral structures are based on their respective VP1, VP2, VP3, and VP4 amino acid sequences, which establish different characteristic spatial conformations in the trench. The response of receptors differs on the basis of the viruses that preferentially bind to them during infection.

Previous studies have shown that the entry of EV-A71 into the brain depends mainly on the cell surface receptor SCARB2 [23, 42, 43], the receptor for CV-A16 is PSGL1 and SCARB2 [44, 45], the receptor for CV-A10 is KREMEN1 [46–48], and the receptors for CV-A6 are PSGL1 and KR [45]. However, interestingly, although it is mainly an EV-A71 receptor, SCARB2 is also recognized by CV-A16 [44, 45], while PSGL1, a receptor of CV-A16, is also recognized by CV-A6 and EV-A71 [45, 49].

According to these findings, the major pathogenic viruses that cause HFMD in children, as observed in clinical research, can recognize a class of cell surface receptors with certain similar structures because of the similarity of the viral structures, especially the common receptor-binding "canyon-like" trench on the viral surface. However, due to the conformational changes in the canyon-like channel structure endowed by the respective encoded protein sequences, these viruses show affinity for specific receptors [29, 46, 47, 50–52].

Therefore, through the study of infection biology, we found that the propensity of HFMD-causing viruses to bind receptors differs. That is, although several viruses exhibit crossover binding to a certain extent, in general, they tend to bind only receptors in a class identified by a limited set of similar structures. That is, our analysis of the virus receptors has revealed an interesting pattern by which virus receptors can be classified.

First, the EV-A71-binding scavenger receptor group B member 2 (SCARB2) protein is a type III transmembrane protein belonging to the family of immune cells expressing the CD36 surface marker [43, 53]. Second, PSGL1, an important immunomodulatory transmembrane-like adsorption protein residing on a variety of immune cells, serves an important signaling receptor [49, 54]. Moreover, CV-A10-binding KREMEN1 is a transmembrane protein with an extracellular characteristic Kringle domain and is a soluble gatekeeper receptor [46, 48, 55].

The HFMD viral receptors exhibit both unique characteristics and similar structures, and the four aforementioned HFMD viruses show confirmed structural similarity. We comprehensively compared the structures of these viruses, especially the structural features of the receptor-binding sites, and the binding characteristics of viruses with their host receptors (Fig. 5.3).

We found that the regional structures where the four viruses bound to their corresponding receptors and the structures where the four receptors bound to each virus show high degrees of similarity. These findings indicate that these viruses can bind to their receptors in varying combinations during the process of infection. That is, viruses specifically bind to receptors with similar or corresponding reaction kinetics and structural fit and bind several receptors in different combinations. Clearly, the analysis and understanding of the biological process of infection by

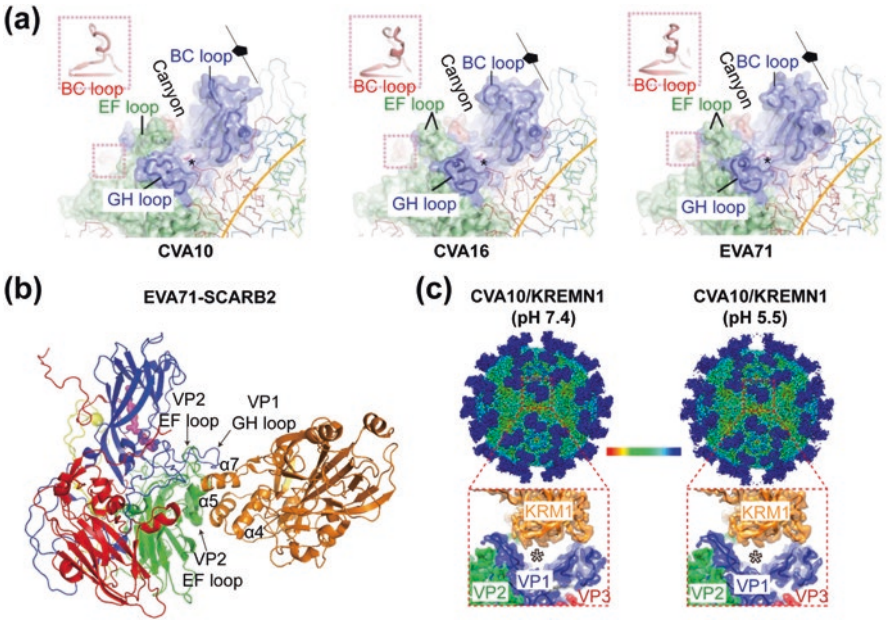

Fig. 5.3 The structural features of the receptor-binding sites in picornavirus virions [29, 45, 46]. (**a**) A canyon-like trench structure in picornavirus virions; (**b**) the receptor-binding sites in picornavirus virions. Reused with permission from [29, 45, 46]

these different pathogenic HFMD viruses in children, as well as the pathological processes with common clinical features, indicate corresponding molecular biological significance.

3 Physiological and Pathological Significance of the Major Pathogenic Viruses Binding to Receptors

The binding of viruses to receptors, a key biological event in infection, is the basic step through which viruses infect the body, which leads to clinical and pathological outcomes. Notably, no direct connection of the macromolecular binding response based on characteristic structural recognition by viruses with the pathological process by which a clinical disease is caused by a virus infecting a host has been described. However, in-depth research via molecular and cell biology has suggested the significance of virus-receptor binding and pathology; for example, [1] the relationship between the body and pathological stimuli has been observed at the microscopic, tissue, and body levels; [2] pathogenic antigens stimulate the body's systemic immune response to prevent clinical disease; and [3] the macromolecular reaction

between viruses and receptors based on structural affinity influences the pathology of infectious diseases and host immune responses.

Significance of the Physiological Characteristics of the Interaction Between Receptors and Different Viruses

Virus binding to receptors, the premise of viral infection in a host, determines virus-induced pathological outcomes and clinical manifestations. Although little data support the understanding of the biological relationship of the structural interaction of viral proteins and cellular receptors or the clinical pathological processes this interaction induces, some independent studies into the molecular mechanism of viral protein interactions with host cellular proteins and subsequent pathological events and immunological responses in vivo have suggested a link throughout the interaction of viruses and receptors. Specifically, the connection is based on etiological stimulation of the host innate immune response and local inflammatory response, which leads to pathological features and immune response phenotypes.

The Physiological Significance of Virus Binding to Receptors

According to the data obtained from a previous basic study on viruses infection of cells via specific receptor binding, a cell surface structure is recognized and bound by a virus, which leverages it to enter cells. Notably, this may be a specific receptor by which the virus infects the host. It is the interaction finally formed through the structural adaptation of protein macromolecules during the long-term interaction between the virus and the human host [21–25]. This means that the so-called viral receptors on the surface of human cells are surface proteins utilized by viruses and have their own specific cellular biological functions. Most of the important cellular molecules that communicate with molecules in the extracellular space are involved in various types of signaling, transmitted in the form of molecular structure changes, to activate the downstream intracellular molecules through associated signaling pathways, and this process of signal transduction is usually initiated after an extracellular ligand binds its corresponding receptor on a cell. Thus, an external signal carried by the ligand molecule is transmitted into the cell, and subsequently, a series of downstream molecules are modified by structural changes and modifications. The most common approach to modifying these molecules, which promotes the expression or suppression of genes that encode surface molecules, includes phosphorylation and acetylation [56–59], which ultimately regulate the transcription of the cellular genome and forming a new functional state of the cell (Fig. 5.4).

Considering these mechanisms, it becomes clear that the process by which the virus specifically binds to a corresponding receptor on the cell surface to induce infection needs to simulate a process by which a ligand in the extracellular

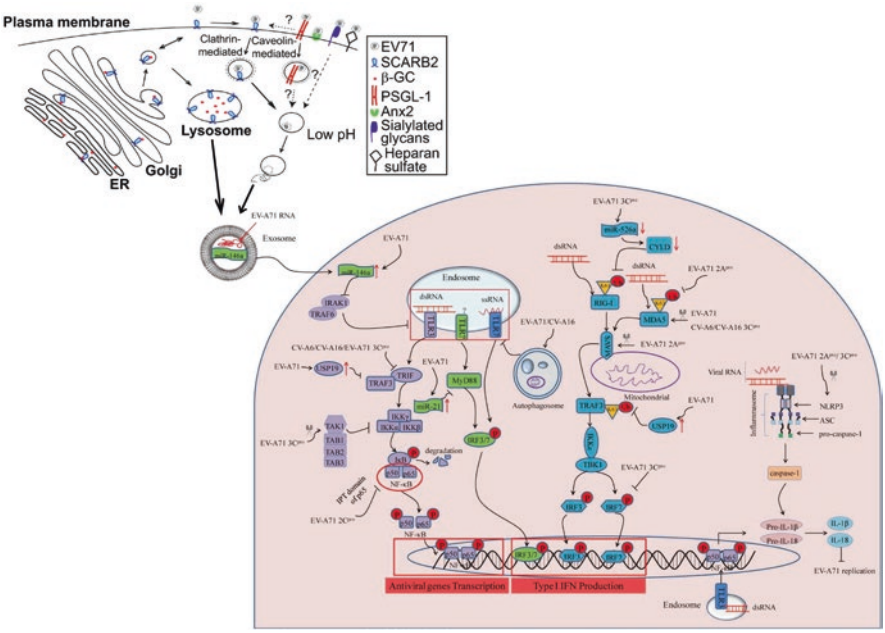

Fig. 5.4 The associated signaling pathways during viruses infection [23, 60]. Reused with permission from [23, 60]

environment binds the receptor and transmits a signal into the cell. The signal transduced by a virus may differ from that of the natural ligand to some extent, but both a virus and cognate ligand trigger dynamic changes in receptor structure upon physiological binding.

The Signal Transduction After Virus Binding to Receptors

The subsequent questions are very clear: Can the macromolecular reaction of virus-bound receptors induce the flow of information into the cell in a manner similar to the natural ligand? If the answer is yes, what are the functional changes in the information flow in cells? How does the intracellular environment that is functionally changed by these changes affect viral proliferation and replication after cell entry? What effect do functional changes exert on the normal function of the innate immune response in cells, including TLR, NLR, and other signal receptor systems that recognize viral pathogen-associated molecular patterns (PAMPs)? Obviously, these questions lead to a great expansion in possible research directions. For many of these questions, the answers remain unclear.

Although these questions suggest that similar structures are involved in similar processes, as indicated by several similar patterns of HFMD infection observed in children because virus binding to receptors causes similar pathological changes,

and that viruses with different structural protein sequences can bind similar proteins. Moreover, the cellular responses elicited by receptors with different signaling functions, including innate immune responses, local stress responses, and inflammatory responses, clearly influence the common pathological process observed in children with HFMD who show similar clinical manifestations of the disease.

As indicated by an in-depth study, the clinical characteristics of children with HFMD are generally mild; that is, patients with cold symptoms, including mild fever, fatigue, and other symptoms, present with typical hand, foot, and mouth lesions. However, a few children with typical HFMD lesions present with moderate or severe clinical manifestations, including a moderately high or high fever. Moreover, the clinical symptoms can involve the heart and brain organs, such as neurogenic pulmonary edema, heart failure, and various types of nervous system damage (Table 5.1). To distinguish between these clinical outcomes, we obtained clues and evidence from studies into the molecular biological processes and mechanisms of virus binding to receptors.

Table 5.1 HFMD clinical manifestations

	Age (years)	Typical clinical manifestations	Recovery
Light	All	1. Fever (>38.5 °C) with malaise associated with a maculopapular rash or blisters on the hands, soles, and buttocks 2. Painful ulcerative lesions of the throat, mouth, and tongue (hemorrhagic or purpuric lesions, an eruption similar to Gianotti–Crosti disease, generalized vesicular exanthema) 3. Atypical cutaneous manifestations can occur 4. Some cases may be accompanied by cough, vomiting, diarrhea, runny nose, loss of appetite, and other symptoms	1. Fever usually subsides within 48 h 2. Cutaneous and mucosal lesions disappear in no more than 7–10 days
Mild	<5 years	1. Most symptoms are similar to those of light symptoms (above) 2. Atypical cutaneous manifestations can occur	Similar to those of light symptoms
Serve	<5 years	1. Persistent high fever, increased neutrophil count, and absence of mouth and skin lesions (atypical cutaneous manifestations can occur) 2. Aseptic meningitis, acute flaccid paralysis, and encephalomyelitis with or without muscle weakness 3. A number of proinflammatory cytokines and chemokines have been detected in significant concentrations in the plasma and/or cerebrospinal fluid (CSF)	Survivors develop neurological sequelae such as cognitive and motor disorders
Death	<5 years	1. A typical cutaneous manifestations can occur 2. Autonomic dysregulation, pulmonary oedema, and myocardial impairment	Death

Molecular Biology Analysis of EV-A71/CV-A16 Infection of Cells

Among several viruses that cause HFMD in children, EV-A71 has shown to cause severe cases/death with symptoms of neurological/cardiopulmonary damage. Studies on the receptor of EV-A71, SCARB2, have shown that this pattern recognition receptor (PRR) is a transmembrane protein and belongs to the CD36 family [43, 53]. Therefore, from a theoretical perspective, after EV-A71 binds to the receptor SCARB2, information flow into a cell is commenced.

Similarly, the receptor PSGL1, to which CV-A16 binds, is an immunomodulatory protein receptor [49, 54], and it triggers information flow into a cell after binding by a virus. In the cases of EV-A71 and CV-A16, the information flows into leukocytes, but the cellular response and mechanism of viral proliferation remain unclear.

First, through an in-depth analysis, we observed that in EV-A71-infected epithelial cells, only a few genes (IFN-stimulated gene factors, known as ISGs), which are related to the interferon gene signaling pathway, are expressed [61–63]. However, after infection with CV-A16, the expression of ISG-related genes was significantly higher than in EV-A71 infection [61]. In fact, EV-A71 inhibited IFN production through the targeted binding of its encoded protein 3C to cellular RIG1 [64] or via cleavage of IRF7 or TRIF [65, 66].

Although no direct relationship between a cellular event and the protease activity of the 3C protein has been reported after CV-A16 or EV-A71 infection [64], the 3C protein of other enteroviruses, such as poliovirus, has been shown to degrade RIG1 [67]. The different events in the same types of cells indicate that the EV-A71 3C protein is affected by environmental factors to inhibit interferon production.

Other studies have shown that the 2A protease of EV-A71 can reduce the level of IFNAR1 in some cells [68, 69] and, together with the 3C protein, reduce the phosphorylation levels of JAK1 and TYK2 in cells by reducing the level of JAK1 [70, 71]; however, in this case, the mRNA levels of JAK1 were unchanged [72]. In addition, these studies suggested that the 3C protein of EV-A71 enzymatically degraded the IRF3, IRF7, and IRF9 proteins in the IFN response network [64, 66, 71]. Interestingly IRF9 is an important component of the IFN signaling pathway and has been shown to inhibit interferon stimulated response element (ISRE) promoter activity in the viral genome [71, 73].

Notably, these observations have not been fully validated via experiments. However, these studies suggested that, although EV-A71 inhibits most components of the IFN-related response gene network to induce the degradation of KPNA1 mediated through caspase-3 or through the competitive binding of phosphorylated/unphosphorylated STAT3/STAT1 to reduce the KPNA1 level, KPNA1 degradation is not the cause of inhibited P-STAT1 or KPNA1 responses [71, 74].

Certainly, these results need to be further explored, but the inhibition of the expression of various genes related of the IFN response induced by the EV-A71 infection of cells is obviously different from the effect of other pathogens causing

HFMD in children and certain other enteroviruses. Notably, studies have shown that CV-A16 infects cells by binding to SCARB2 [45, 75], but its effect on the IFN response system is significantly different from that of EV-A71 [76]. This finding indicated that EV-A71 and CV-A16 not only interact with cells differently during infection but also induce different cellular response upon binding to their respective receptor.

EV-A71 triggers the innate immune response system, which is mediated by the IFN response network during cell infection, and its effects on the inflammatory response are specific and closely related to the innate immune response system. Data reveal that EV-A71 infection can induce the production of intracellular reactive oxygen species (ROS) by increasing the oxidative stress system in cells [77–80]. This increased production of intracellular ROS in mitochondria is caused by EV-A71 infection. In addition, EV-A71 infection may be involved in the activation of certain kinases, such as p38, leading to a range of cellular abnormalities [81, 82], including the untimely increase in JNK1/2, ERK1/2, and NF-KB activity [73, 83, 84]. In addition, infections increase the cellular inflammatory stress response capacity, and therefore, the activity of a variety of inflammatory factors and cell signaling factors will show an increasing trend.

However, interestingly, the aforementioned results are considered to be the combined outcomes of the 3C protein encoded by EV-A71 acting on TAK1/TAB1/TAB2/TAB2 complexes, which are involved in the transcription of certain proinflammatory factor genes [85]. Different experimental observations seemed to suggest that EV-A71 induces a specific pattern of action by the fast-acting innate immune system in cells, as it elicited different biological responses in experiments with different members of the cellular environment. Additional experiments have also shown that after entering cells, EV-A71 acts in different ways on RIG-I receptors and signaling pathways [63, 86–89], the MAVS signaling pathway [90, 91], TLRs, the NLR receptor system, and related signaling pathways [92–95].

Therefore, infection by EV-A71 inhibits the antiviral effects initiated by the innate immune response system at the cellular level, but the manifestations of these effects on different cells seem to vary. Most of the preliminary research on the EV-A71 virus has been performed with nonhuman primate Vero cells and human rhabdomyosarcoma cells. Therefore, the results may be different from EV-A71 virus infection of normal human epithelial cells, especially respiratory and intestinal epithelial cells.

From a comparative study in which EV-A71 and CV-A16 were used to infect human embryonic lung diploid cells, EV-A71 and CV-A16 viruses infected human bronchial epithelial cells to induce the transcriptional expression of cellular innate immune response, and inflammatory response, and related immune regulatory molecules, and the effect of the two viruses was clearly different (Fig. 5.5a).

These differences were clearly manifested in the IFN system, which regulates and activates the body's antiviral immune response and related signaling pathway molecules, as well as certain local inflammatory response-related molecules, including IFNs and related signaling molecules involved in immune system activation. In another study analyzing the influence of the structural protein VP1 of the two viruses

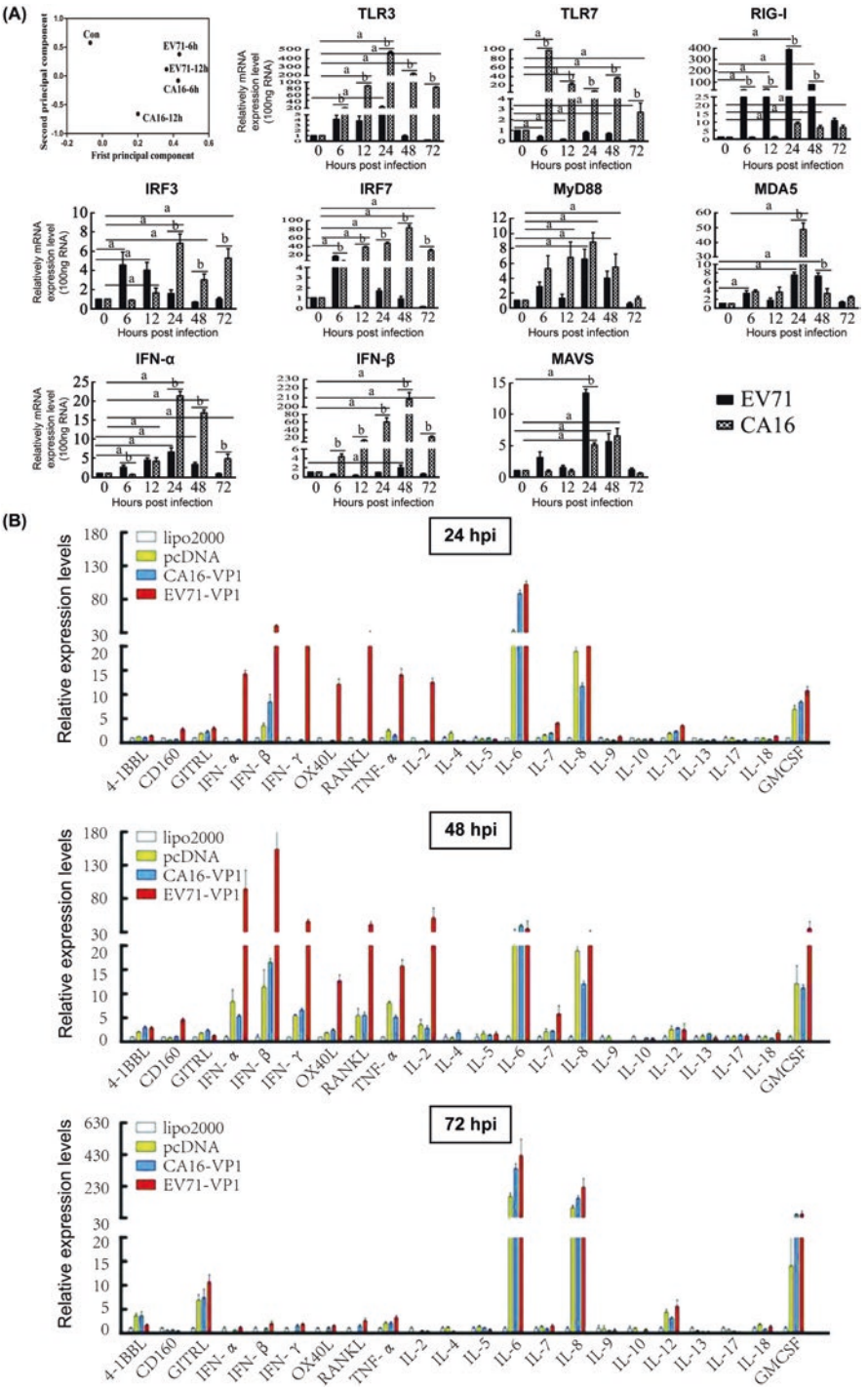

Fig. 5.5 Comparing the transcriptional expression of genes associated with immune response during EV-A71 and CV-A16 infected human bronchial epithelial cells [20, 76, 96]. (**a**) Changes in the

on different signal responses in cells, the VP1-encoding genes of EV-A71 and CV-A16 introduced to human respiratory epithelial cells using eukaryotic expression vectors induced the same cellular changes (Fig. 5.5b). Notably, these studies mainly focused on important molecules, such as IFN-γ, TNF-α, and IL-2, that guide the innate immune and inflammatory systems, but other molecules show immune response regulation significance, such as OX40L and RANKL, and infection with EV-A71 and CV-A16 led to obvious differences in their activities. The main difference was reported to be that in the EV-A71-encoded VP1-transfected group, these molecules showed a time-dependent and substantial increase, while in the CV-A16-encoded VP1 group, no stimulatory effect was observed.

In summary, EV-A71 and CV-A16 exhibit differences in their effects on the body's innate immune system/inflammatory response system in the early stage of human infection, that is, when infecting human respiratory/gastrointestinal epithelial cells. These differences theoretically cause the subsequent pathological process of infection and the formation of the antiviral immune response in the body. From this perspective, the two viruses infect cells based on their structural differences and differences in binding receptors, including receptors on the cell surface and inside the cell (TLR, NLR, etc.). The pathological effects of the two viruses on the body and the different immune responses may be thus explained. However, differences in receptor activity induced by these viruses may not have a decisive impact at the tissue or body level.

Molecular Biology Analysis of the Effects of EV-A71 and CV-A16 on Histopathology

No strict definition has been applied to identify differences in the clinical diagnosis of HFMD in children that is frequently caused by the major pathogens EV-A71 and CV-A16 because the common symptoms of these patients, regardless of which virus infected them are mild fever, fatigue, and other cold-like syndromes and the typical skin-mucous membrane lesions on the hand, foot, and mouth. To determine which virus caused an infection, pathogenic or serological testing must be performed. Interestingly, differences in the pathology of HFMD caused by the two viruses were

Fig. 5.5 (continued) pathway of IFN-I production related molecules subjected to EV-A71 and CV-A16 infections in CD1c+DC from rhesus monkey; (**b**) Expression of natural immunity-associated signal molecules induced by expressions of EV-A71- and CV-A16-VP1 in 16HBE cells. Reused with permission from [20, 76, 96]

evident in a large-scale population statistical analysis; that is, EV-A71 caused severe cases in 1–3% of all affected populations. The main manifestations of severe disease were nervous system damage and subsequent cardiopulmonary dysfunction, especially neurogenic pulmonary edema, for which the mortality range was found to be 2–5%, but the proportion of patients with CV-A16 infection and nervous system damage was relatively low. Additionally, according to different statistical data reports, the percentage of severe cases caused by CV-A16 infection were lower than 0.1%, and the mortality rate is much lower.

Although the reasons for these differences have not been revealed in research performed thus far, recent data have provided some clues for the study of the pathogenic differences between the two viruses. Previous pathophysiological studies have suggested that both EV-A71 and CV-A16 virus enter the loose tissue of the subepithelial layer by infecting respiratory epithelial cells and CD141+ and CD14+/CD11+ labeled dendritic cells (DCs) and subsequently proliferate in them [97–99]. Importantly, while the proliferation of the two viruses exhibited different kinetics, subtle differences in the expression of the two types of surface markers in DCs were identified; specifically, these cells produced different inflammatory factors and exhibited different transcriptional expression profiles of immune signaling molecules (Fig. 5.6).

Differences in transcriptional profiles imply a variety of trends in innate immune responses and inflammatory responses in tissues. These differential trends may reveal the basic background of the corresponding infected tissues of the body, and

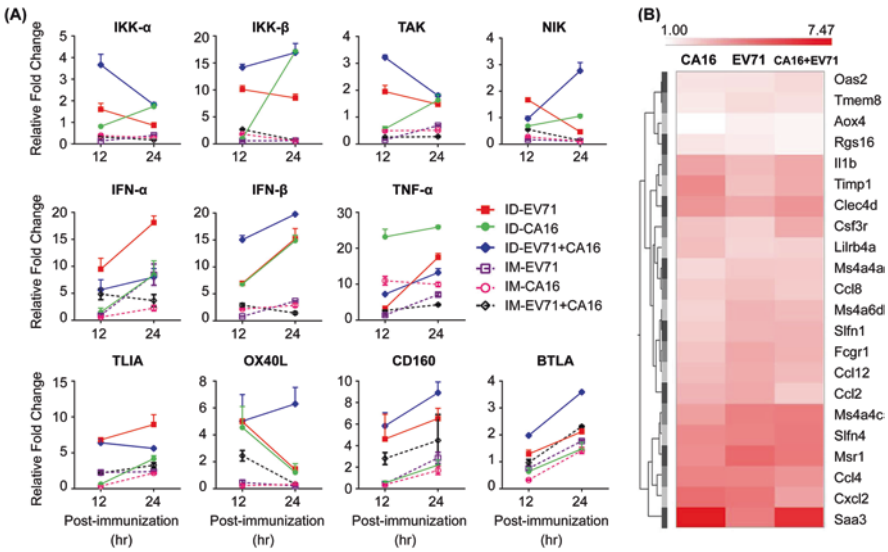

Fig. 5.6 Different transcriptional expression profiles of immune signaling molecules induced by EV-A71 and CV-A16 infection [98]. (**a**) Expression profiles of signaling molecules in peripheral tissues; (**b**) mRNA expression profiles of skin from each group at 12 h post-inoculation. Reused with permission from [98]

therefore, when the two viruses infect DCs or enter the blood circulatory system and reach the nervous system, differential responses are triggered in nervous tissues.

Our previous study demonstrated that EV-A71 first enters the central nervous system by infecting vascular endothelial cells in the brain parenchyma after it reaches the blood–brain barrier through the blood circulatory system [100]. Subsequently, EV-A71 infects astrocytes in neural tissue and induces the production cytokines and neurotransmitters, including IL-6, IL-8, and norepinephrine, in the cells that perform immune functions in nerve tissue; these immune molecules can act on brainstem nerve cells to activate the brainstem–hypothalamus–adrenal gland axis, resulting in a rapid increase in blood epinephrine and norepinephrine levels, followed by pulmonary edema development that eventually contributes to spasmodic contraction of pulmonary arterioles and continuous aggravation of cardiac function impairment [101].

During this pathological process, specifically EV-A71 infection of astrocytes that elicits innate immunity, as well as subsequent tissue inflammatory responses, the pathophysiological process of infection may be profoundly impacted. Considering the EV-A71 and CV-A16 infection patterns in DCs, clinical reports of CV-A16 virus isolated from the central nervous system of individual patients [102, 103], and observations of rare severe neurological damage, we can speculate that differences in the effects of EV-A71 and CV-A16 on the innate immune cells, which correspond to those in the innate immune receptor system, largely determine the pathophysiology and direction of viral infection.

Moreover, an interesting finding on the basic pathological process of HFMD caused by several viral pathogens that results in herpetic lesions of the skin and mucous membranes at specific sites has raised a specific question on how we understand the molecular biological relationship of viruses and humans. That is, the current understanding is based on the interaction between infectious viruses and body systems that has been observed to induce the same pathological and immunological responses, even though the viruses causing the disease exhibit different structures and interact with different cells.

Although the recent evidence has been realized through many detailed analyses of the molecular mechanisms of viruses and cells, the relationship between the basic pathological features of HFMD and pathogens remains unclear. Considering previous studies on the infection of rhesus monkey models with EV-A71 and CV-A16, we specifically observed the pathological structure of animal mucosa/skin lesions that developed after viral infection. Microstructural observations revealed that the lesion was surrounded by several layers of virus-infected hyperdifferentiated epithelial cells, in which a number of typical mononuclear inflammatory cells could be seen (Fig. 5.7). In addition, herpes caused by EV-A71 infection or CV-A16 infection showed the same herpes structure.

Although no accurate or detailed molecular biological mechanism analysis of the formation of these pathological structures was performed, based on the infectious properties of EV-A71 and CV-A16 in DCs, together with the evidence showing that the CV-A16 virus can be transferred to different tissues of the body through the

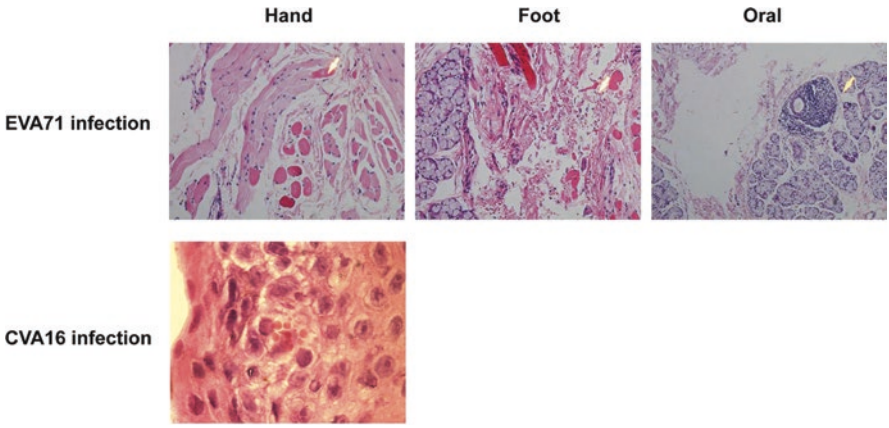

Fig. 5.7 The pathological structure of animal mucosa/skin lesions during EV-A71 and CV-A16 infection. Reused with permission from [104, 105]

lymph/blood circulatory systems and that the epithelial cell system can elicit innate immune response and inflammatory responses, we infer that the process of both EV-A71 and CV-A16 infection and their proliferation in DCs and the subsequently generated PAMPs stimulate the PRR system in DCs, which leads to specific activation states of the DCs.

Thereafter, infected DCs flow through the small blood vessels of the skin and mucous membranes, which are fed by the lymph/blood circulatory systems, and the activation phenotype remains in the local area of the skin and mucous membranes due to the increase in cell surface adhesion molecules. This local inflammatory response through the inflammatory factors and chemokines produced by the activated DCs leads to the proliferation and further differentiation of corresponding epithelial cells. This inference is supported by an increase in the transcript levels of certain inflammatory factors and chemokines in the tissues with lesions.

Since this inference is based on the actual pathological process of these two viruses that infect the body, the same clinicopathological symptoms are likely attributed by the characteristics of similar interactions between EV-A71 or CV-A16 and DCs, even though these interactions have been shown to be subtly mechanistically different and lead to specific differences in subsequent pathological manifestations. This presumption that the viruses share common characteristics and exhibit specific differences seems to imply that HFMD multi-pathogen infection largely depends on the similar structural and biological characteristics of the pathogens. The structural and biological determinants act on corresponding cell structures and tissues by binding to similar receptors and in similar patterns upon infection of the body, thereby causing a class of diseases with common clinicopathological processes and clinical symptoms, such that clinicians consider each disease in the class to be the same based on their clinical manifestations.

Analysis of the Effects of EV-A71 and CV-A16 Interactions with the Immune System

Obtained through previous studies on enterovirus immunology, substantial data have led to an interesting finding: There is a large difference in the specific antiviral immune responses induced by infection with various members of this viral family and induced by immunizing the body with antigens, regardless of the similarities in viral structure and characteristics of the pathogen. For example, pathogens of diseases that have fully been controlled through efficient vaccination—poliovirus and hepatitis A virus—exhibit similar picornavirus structures and infect people via the intestinal route [106–108]. However, the antiviral immune responses induced by the two viruses exhibit remarkable phenotypic differences, especially in people infected by the virus or immunized with the corresponding vaccine, and the titers of their serum-neutralizing antibodies vary greatly.

For hepatitis A, only seroconversion of antibodies, including binding antibodies, is likely to confer effective protection against infection [109, 110]. However, a higher titer of the serum-neutralizing antibody after poliovirus infection seems to be required for significant protection of a population [107, 111]. Although this interpretation is easy to understand based on the pathological patterns of infection by these two viruses, the differences suggest differences in their effects on the immune systems despite their similar structures. For children with HFMD, the confirmed pathogens are all enteroviruses, and their tendency to bind cellular receptors, undergo tissue tropism, and pathologies are very similar, leading to common clinical symptoms. Nevertheless, for each specific pathogen, the cellular innate immune response system signaling is greatly influenced by either the binding process of the virus to the receptor, which is a result of differences in the surface structure or the binding process of PRRs in the infected cells, which can be attributed to the differential structural and nonstructural proteins.

These effects may eventually lead to obvious phenotypic differences derived through a series of signaling cascades, including signal transduction, such as the cascade triggered by signals transmitted from innate immune cells to acquired immune response cells; the regulatory transcription response cascade; and cell activation and proliferation signaling cascades. In this regard, we made these conclusions by simply comparing the consequences of EV-A71 and CV-A16 virus infection in individuals and populations.

According to a serological analysis of EV-A71-infected individuals and populations, the gradual elevation in neutralizing antibodies in convalescent sera taken long after infection has been identified, and certain serum-neutralizing antibody levels have been reported to be maintained (at least in the range of 1:8–1:256) for at least 2 years [112] in the vast majority of rehabilitated patients.

Rare clinical reports have indicated that some EV-A71-infected individuals are reinfected by the same virus, causing HFMD. More reports have indicated

reinfection or multiple infections in HFMD patients [113–116] caused by viruses other than EV-A71 [117].

An observation of CV-A16-infected populations revealed that the serum-neutralizing antibody levels were generally low [118–121]. One study with hundreds of samples confirmed that the neutralizing-antibody geometric mean titer (GMT) of the CV-A16-infected children with HFMD was low; it was only 4.18 within 28–56 days after clinical recovery (Fig. 5.8).

A comparative study of the immune response characteristics of CV-A16 and EV-A71 infection in rhesus macaque models suggested that the immune responses in terms of typical neutralizing-antibody response and specific cytotoxic T-lymphocyte (CTL) response elicited by EV-A71 infection conferred a protective effect against viral reinfection, while a weaker immune response was elicited by the CV-A16 infection, which was characterized by same clinical manifestations and pathological features after subsequent reinfections with CV-A16 [104].

Taken together, these results suggested large differences in the dynamic profiles of EV-A71 and CV-A16 interactions with the cellular receptors and the innate immune system in cells, innate immune cells in tissues, and the corresponding links with the immune response system. Specifically, EV-A71 might contribute to distinct differences in the final immunological phenotype via signaling cascades, which leads to the greater differences in recent research findings and has implications for the development of EV-A71 and CV-A16 vaccines.

To some extent, the specific structural characteristics of other enteroviruses that cause HFMD, as well as differences in the viral interactions with physiological molecules, including cellular receptors and various cellular components of the immune system in various host proteins within the cell, directly contribute to subtle differences in pathological processes and corresponding immune characteristics frequently associated with these disease pathogens.

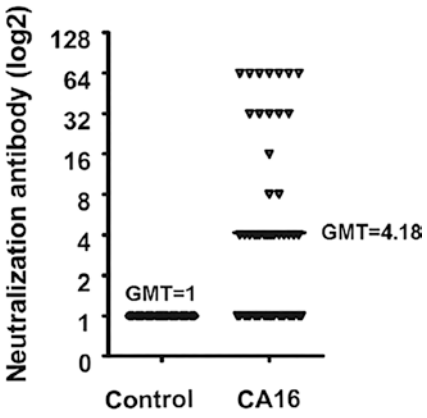

Fig. 5.8 The neutralizing-antibody geometric mean titer (GMT) of the CV-A16-infected children with HFMD

4 Immunological Study of a Multivalent Vaccine for HFMD Caused by Two or More Pathogens

Research on different pathogens of HFMD in children, especially similarities and differences in pathological infection mechanisms or in characteristics of immune responses, has been focused on the development of therapeutic drugs and vaccines. We have not obtained sufficient data on several other pathogens that cause HFMD in children in addition to that for EV-A71 and CV-A16. Therefore, obtained mainly via EV-A71- and CV-A16-related research, the data obtained to date can provide a basic understanding of the design and application of multivalent vaccines against HFMD for children. Therefore, further exploration through research into and development of multivalent vaccines for HFMD is needed; however, the previous work on the research and development of EV-A71 and CV-A16 bivalent vaccines has demonstrated promising results, enabling their entry into clinical trials [122, 123].

Study on the Immunological Mechanisms of Different Types of HFMD Vaccines

According to previous studies on the preparation of the inactivated EV-A71 vaccine and its immunology, our experimental results indicated that the EV-A71 vaccine antigen elicits a clear immune response in animals and humans after primary and booster immunization. That is, clear seroconversion and increases in neutralizing antibodies and sustainable specific IFN-γ and IL-4 dependent CTL responses were found to be sustained for several years.

Importantly, the immune response elicited by the inactivated-EV-A71 vaccine showed remarkable clinical protective efficacy in both susceptible animals and human populations [112, 124–128]. Simultaneously, the inactivated-CV-A16 vaccine prepared using the same technology failed to induce an effective immune response, as indicated by not only low neutralizing-antibody seroconversion and GMT levels but also lack of a protective effect against virus infection in sensitive animal models.

Further studies exploring the associated mechanism will contribute to the development of a CV-A16 vaccine in the future. Difficulties with activating an overall innate immune response by the corresponding RNA molecules generated during the replication of structural antigens of viral PAMPs in cells, including VP1, VP2, and VP3 proteins, must be overcome. From this perspective, CV-A16 vaccine development with the conventional vaccine antigen formulation seems to be ineffective. Thus, a logical hypothesis is proposed: as a complete viral pathogen that can proliferate in host cells as well as encode specific related antigens, the deficiency and insufficiency of CV-A16 to activate the innate immune system might limit the elicitation of an effective immune response through only a vaccine antigen designed to

stimulate the immune system, but a CV-A16 vaccine might be developed to trigger the following pathway: The whole innate immune response →specific antigen presentation → adaptive immune response.

If a corresponding adjustment and correct supplement can be made to the antigen-stimulating innate immune response system, the activation of the innate immune response might be theoretically increased. Additionally, the effective activation of the innate immune response system by EV-A71, as an essential component of the HFMD multivalent vaccine, might produce an effective adjuvant effect. Therefore, a bivalent vaccine formulated with these two viral components serving as mutual adjuvants might be developed using another approach. The possible enhancement in activation of the innate immune system may make this formulation an innovative alternative for vaccine development.

Considering this possibility, we developed a new EV-A71/CV-A16 bivalent vaccine formulation by employing an intradermal immunization approach to profoundly increase the antigen stimulation threshold. That is, we developed an optimal dose formulation with inactivated EV-A71 and CV-A16 viral antigens for intradermal immunization in sensitive animals. We analyzed the innate immune response and followed adaptive immune response and the possible associated mechanism.

The experimental observations demonstrated that the phagocytosis rate of the two antigens by antigen-presenting cells, such as DCs and Langerhans cells (LCs), in the animals immunized with this inactivated EV-A71/CV-A16 bivalent vaccine via intradermal injection was much higher than that administered via intramuscular injection (Fig. 5.9). Specifically, the analysis of various immune regulatory protein transcripts associated with innate immune responses in local immune tissues revealed remarkable innate immune signaling and effector network activation potentially induced by this bivalent vaccine administered via intradermal injection (Fig. 5.6).

In summary, employing certain immunological regulation approaches can effectively increase the activation level of the innate immune response system elicited by

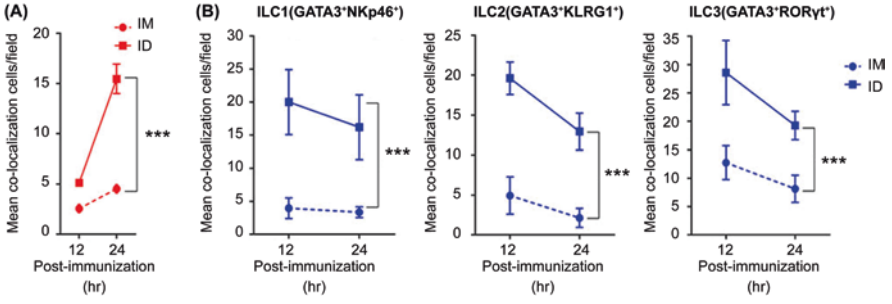

Fig. 5.9 Relationship between the viral antigen and ILCs or DCs in tissues [98]. (**a**) Colocalization rates of DCs after intradermal (ID) or intramuscular (IM) inoculation; (**b**) Colocalization rates of ILC1, ILC2, and ILC3 cells after intradermal (ID) or intramuscular (IM) inoculation. Reused with permission from [98]

the CV-A16 antigen. This regulatory approach is necessary because the interaction of CV-A16 with the immune system, potentially at the initial stage when the virus binds receptors, fails to generate sufficient stimulation to reach the activation threshold, or the stimulation CV-A16 induces is blocked through in an unknown mechanism.

Theoretical and Technical Analysis for the Research and Development of Multivalent HFMD Vaccines

HFMD in children is an infectious disease caused by enteroviruses with similar structures and pathological processes. The research on preventative vaccines is of theoretical and technical significance because it can satisfy the need for treatment and prevention strategies for public health initiatives.

In the research on vaccines for effectively preventing HFMD caused by major pathogens, we encounter a series of immunological theories and challenges in vaccinology. Specifically, we need to understand the interactions of a class of viruses with similar structures but different antigenic traits, which respond to the immune system through diverse mechanisms and reaction dynamics mediated by the structure of viral particles binding to cell surface receptors. Additionally, we need to comprehend the entire process of signal stimulation by viral antigen molecules and innate immune receptor molecules, as well as characterize the elicitation of immune responses with phenotypic differences and their clinical or pathological manifestations, all of which are necessary for vaccine development.

First, in the process of disease development, multiple viral infections induce a common pathophysiological process at the organismal level, which is characterized by a similar inflammatory response process in tissues. What is the specific association between viral infections, the innate immune system, the acquired immune system, and the overall response process? The conventional theory of infectious diseases suggests that the pathology caused by pathogens is attributable to the total effects of pathological injury to the body, tissues, or cells and the immune response mechanisms, especially the innate immune and inflammatory response mechanisms. If HFMD in children is mainly attributed to a process by which the inflammatory response is elicited via multiple pathogenic infections, how can we identify a variety of immune system responses to different pathogen infections with similar clinical manifestations?

Clearly, greater understanding of these theoretical issues will broaden our view of the pathophysiology of infectious diseases. With an increase in understanding, the development of technological system for vaccine research, through which issues related to the variety of immune responses attributed to multiple pathogen interactions with immune molecules can be resolved, is possible, and this new technology will be critical for the practical prevention and treatment of HFMD in children.

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Chapter 6
Research and Development of HFMD Vaccines

Heng Zhao

Abstract HFMD is a viral contagious disease that occurs most often in children under 5 years old (Zhang et al. Emerg Microbes Infect 4(2):e12, 2015). It is a self-limiting disease with common clinical manifestations, including sores in the mouth, rash with blisters on the hands and/or feet, herpangina, and fever. It has a good prognosis, and some patients with severe cases may further develop neurological diseases (He et al., Epidemiol Infect 145(9):1865–1874, 2017). Substantial molecular etiological and epidemiological studies have demonstrated that the common pathogens that cause HFMD are enterovirus A 71 (EV-A71), coxsackie virus A 16 (CV-A16), A10 (CV-A10), and A6 (CV-A6), and coxsackie virus B 3 (CV-B3) and B5 (CV-B5). EV-A71 and CV-A16 infections are the most common, with the identified cases accounting for over 60% of all HFMD cases (Koh et al., Pediatr Infect Dis J 35(10):285–300, 2016; Chang et al., Pediatrics 109(6):e88, 2002). Furthermore, EV-A71 is recognized as the major pathogen for fatal cases (Wong et al., Epidemiol Infect 138(8):1071–1089, 2010).

Keywords Hand and foot mouth disease · Vaccine · Research progress

1 Introduction

Severe and fatal disease caused by EV-A71 infection have been greatly eliminated since the licensure for the use of an inactivated EV-A71 vaccine, and EV-A71 has gradually been replaced by other enteroviruses, such as CV-A16, CV-A10, CV-A6, and CV-B5 [1], which are becoming the dominant circulating strains. However,

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coinfection with multiple enteroviruses has been observed in HFMD patients, making it more complex and difficult to control and treat HFMD [2]. For a viral infectious disease such as HFMD, there is no doubt that preventive vaccines are the optimal tool for controlling epidemics. In this chapter, the research and development of HFMD vaccines are briefly described.

It is well known that there are a variety of pathogens that cause HFMD, and the interactions and pathogenic mechanisms between different viruses and host cells tend to be different [3]. The enterovirus capsid consists of the structural proteins VP1–VP4, which are assembled to form an icosahedral shell. VP1, VP2, and VP3 are located on the surface of the capsid, making them good antigenic epitopes with good immunogenicity, while VP4 is located inside the capsid [4]. A substantial number of studies have shown that VP1 retains strong immunogenicity and can elicit strong immune responses [5]. In addition to VP1 and other structural proteins, nonstructural proteins, such as 3CD, exhibit a certain immunogenicity [6]. Aw-Yong et al. [7] reviewed and summarized the studies on the B-cell and T-cell epitopes of different enterovirus proteins, providing a good reference for the design and development of HFMD vaccines.

With HFMD epidemics being reported frequently worldwide, as well as the detailed studies of the causative pathogens of HFMD, the design and development of HFMD vaccines have been initiated based on a large number of different aspects, and some results of HFMD vaccine have been reported. Currently, the major types of HFMD vaccines include inactivated vaccines, live attenuated vaccines, virus-like particle vaccines, subunit vaccines, viral vector vaccines, DNA vaccines, and RNA vaccines.

2 Inactivated Vaccines

Inactivated vaccines are produced by a conventional technology that has widely been used in the licensed production of inactivated poliovirus vaccines. The real-world data for the administration of these vaccines in large populations for several decades have sufficiently demonstrated the safety and effectiveness of such vaccines. Importantly, inactivated vaccines contain almost all the protein components of the virus, including the extensive antigen components; theoretically, such a vaccine should be capable of eliciting a great variety of antiviral immune responses. Hence, inactivated vaccine design and technology have become a priority for developing HFMD vaccines. The numerous inactivated vaccines are further classified as monovalent vaccines (containing only 1 virus component) and multivalent vaccines (containing 2 or more virus components).

Monovalent Inactivated Vaccines

EV-A71 Monovalent Inactivated Vaccine

In 2008, large HFMD outbreaks occurred in Fuyang, Anhui Province, China, with a substantial number of severe and fatal cases [8]. EV-A71 was recognized as the common causative pathogen [9]. Thereafter, the research and development of inactivated EV-A71 vaccines was initiated by many institutions and enterprises in Mainland China. Fortunately, the inactivated EV-A71 vaccines developed by the Institute of Medical Biology, Chinese Academy of Medical Sciences, Sinovac and Beijing Weigu Biological Pharmaceutical Co., Ltd., were subsequently approved for licensure by the Chinese Regulatory Authority in 2015 [10]. These three individual inactivated EV-A71 vaccines were prepared from C4 genotype EV-A71 by cell culture, inactivated by formaldehyde and purified, and formulated with aluminum hydroxide. Phase III clinical trials revealed that these three vaccines exhibited good safety and efficacy, with protective efficacies of over 90% against C4 genotype EV-A71 infection, and good protection against infections caused by different genotypes of EV-A71 but no cross-protection against other enteroviruses [11]. However, the research and development of EV-A71 and other enterovirus inactivated vaccines have continued. In et al. [12] prepared an inactivated vaccine from genotype C4a EV-A71 cultivated in RD and Vero cells followed by formaldehyde inactivation. The 4 prepared vaccine [vaccine+AL(OH)3,vaccine+MPLA,vaccine+poly I:C,vaccine+(AL(OH)3+MPLA+poly I:C)] formulations with 3 different adjuvants (AL(OH)3, MPLA, poly I:C and (AL(OH)3+MPLA+poly I:C) were used to immunize a mouse model via intramuscular injection. The results showed that both the vaccine with 1 adjuvant and the vaccine with 3 adjuvants had the capacity to elicit both humoral and cellular immune responses, but the vaccine with 3 different adjuvants elicited a stronger immune response and Th1 and Th2 cellular responses, with cross-protection against infections caused by other genotypes of EV17, whereas the vaccine with only 1 adjuvant merely elicited a certain immune response and a Th1-cellular response. However, no virus challenge results have been obtained in such an animal model. Lin et al. [13] also developed an inactivated EV-A71 antigen with a CpG (C type) adjuvant and immunized the mice via the intranasal route. The results showed that this candidate vaccine-elicited good humoral and cellular immune responses in terms of Th1 and Th2 responses, as well as the production of IgA. Overall, this candidate vaccine had the capacity to activate the mucosal immune response to elicit a neutralization antibody for cross-protection against infections caused by different genotypes of EV-A71, such as C2, B4, and B5. Furthermore, a protective efficacy study in the SCARB2 humanized transgenic mouse model revealed that the immune response elicited by this candidate vaccine could protect the mice against lethal EV-A71 challenge.

CV-A16 Monovalent Inactivated Vaccine

Li et al. [14] prepared a CV-A16 inactivated vaccine with an aluminum hydroxide adjuvant from the CV-A16 virus cultivated in Vero cells and inactivated by formaldehyde or β-propiolactone. The results of mouse immunization with the vaccine showed neutralization antibodies against different genotype CV-A16 viruses, and viral challenge in the suckling mice of the filial generation further demonstrated the protective efficacy against CV-A16 virus challenge.

CV-A10 Monovalent Inactivated Vaccine

Zhang et al. [15] prepared a CV-A10 inactivated vaccine with Freund's adjuvant by using CV-A10 virus cultured in RD cells and inactivated by formaldehyde. The antisera collected from the mice immunized with the vaccine via intramuscular injection could passively offer good protection in suckling mice that were 5 days old. Similarly, Gao et al. [16] reported a CV-A10 inactivated vaccine prepared from a clinically isolated CV10-25 viral strain cultivated in Vero cells followed by formaldehyde inactivation and aluminum hydroxide formulation for immunizing BALB/c mice via intraperitoneal injection. The highest neutralization antibody titer elicited by the vaccine was 1:512, and the maternally transferred antibody of the immunized mice could protect the suckling mice against CV-A10 infection.

In summary, the abovementioned studies of monovalent inactivated vaccine formulations with different viruses and adjuvants characterized the immune responses in mice immunized via different routes, providing some data for further development of inactivated EV-A71, CV-A16, and CV-A10 vaccines.

Multivalent Inactivated Vaccines

EV-A71/CV-A16 Bivalent Inactivated Vaccine

It is well understood that HFMD is caused by one or multiple enterovirus infections [17]. Currently, the licensed inactivated EV-A71 vaccine exhibits weak cross-protection or no cross-protection against other enteroviruses, which makes it essential to develop multivalent inactivated enterovirus vaccines. Cai et al. [18] prepared an EV-A71/CV-A16 inactivated vaccine from the EV-A71 (EV-A71/FY573) and CV-A16 (CV-A16/G08) viruses cultivated in Vero cells, followed by β-propiolactone activation and aluminum hydroxide formulation for immunizing the mice via intraperitoneal injection. Titers of the anti-EV-A71 and CV-A16 virus-specific neutralization antibodies as high as 1:1024 were elicited and maintained until 9 weeks post-immunization, suggesting good durability of the neutralizing antibody.

Additionally, the challenge test in suckling mice of the filial generation revealed good protection against EV-A71 and CV-A16 virus infection, while in contrast, no protection was noticed against other enterovirus infections. Fan et al. [19] prepared inactivated antigens from EV-A71 and CV-A16 viruses with an aluminum hydroxide adjuvant for immunizing mice via intradermal injection. The anti-EV-A71 and CV-A16 virus-specific neutralizing antibodies, as well as the specific cellular responses, were elicited, and the anti-EV-A71 virus-specific antibody titer was higher than that of the anti-CV-A16-specific antibody. The virus challenge in suckling mice of the filial generation illustrated good protective efficacy against lethal EV-A71 and CV-A16 virus infection. Furthermore, the bivalent vaccine was used to immunize rhesus macaques via intradermal injection, and again, good humoral and cellular responses were elicited in the animal model. The protective efficacy was further evidenced in the rhesus macaque model by subsequent virus challenge. This study describes the improvement in the immunogenicity of the CV-A16 inactivated vaccine by altering the immunization route and the details of the associated mechanism, suggesting the essential function of intradermal injection in activating the immune response and its related mechanism and shedding light on the selection of the immunization route in vaccine research and development. Sun et al. [20] also prepared an EV-A71/CV-A16 bivalent inactivated vaccine in RD cells for immunizing mice via intraperitoneal injection. Although the anti-EV-A71 and CV-A16 virus-specific antibodies were elicited, there were differences in the titers, characterized by the obviously higher anti-EV-A71 virus-specific antibody titer than the anti-CV-A16 virus-specific antibody titer. Further challenge tests in suckling mice of the filial generation indicated complete protection against lethal EV-A71 or CV-A16 virus challenge.

Unlike the abovementioned preparation approaches, Yang et al. [21] successfully constructed an infectious chimeric EV-A71 virus by replacing the EV-A71 VP1/210-225 sites with the corresponding CV-A16 VP1 sites, which still maintained similar infectivity and proliferation features of the prototype virus. A special EV-A71/CV-A16 bivalent inactivated vaccine was prepared from this chimeric virus in cell culture, followed by formaldehyde inactivation and aluminum hydroxide formulation for immunizing the mice via intraperitoneal injection. The results indicated that the anti-EV-A71 and CV-A16 virus antibodies were elicited, with higher anti-EV-A71 virus-specific antibody titers than anti-CV-A16-specific antibody titers. Interestingly, these elicited immune responses could protect suckling mice against lethal EV-A71 and CV-A16 virus challenge. This innovative concept for chimeric vaccine design not only simplifies the culture, purification, inactivation, and other complicated production processes of conventional multivalent inactivated vaccines but also had the same efficacy offered by multivalent vaccines. Unfortunately, the cellular immune response deemed one of the critical indicators for assessing vaccine efficacy was not examined in this study, and the genetic stability of such a chimeric virus constructed by a gene editing approach awaits further exploration.

CV-A6/CV-A10 Bivalent Inactivated Vaccine

Zhang et al. [15] prepared a CV-A6/CV-A10 bivalent inactivated vaccine from Vero cells by formaldehyde inactivation and sucrose density gradient centrifugation purification with an aluminum hydroxide formulation for immunizing mice via intradermal injection. The results revealed the activation of the immune response. The active immunization with the bivalent vaccine indicated a protective efficacy of approximately 80% against CV-A6 or CV-A10 infection, which was similar to the efficacy (80–90%) of single CV-A6 or CV-A10 vaccine immunization.

Trivalent Inactivated Vaccines CV-A6, CV-A10, and CV-A16

To achieve extensive immune protection, the development of trivalent and tetravalent vaccines, or even more multivalent vaccines, might be an ideal scenario. Liu et al. [22] prepared a trivalent inactivated vaccine from CV-A6, CV-A10, and CV-A16 viruses cultivated in Vero cells and inactivated by formaldehyde or β-propiolactone with an aluminum hydroxide formulation. The vaccine had the capacity to elicit anti-CV-A6, CV-A10, and CV-A16 specific neutralization antibodies in mice immunized via intramuscular injection, but the anti-CV-A6 and CV-A10 virus-specific antibody titers were higher than the anti-CV-A16 virus antibody titer. Furthermore, the antibody titers elicited by the vaccines prepared with formaldehyde or β-propiolactone inactivation tended to be different, with the anti-CV-A10 and CV-A16 virus-specific antibody titers being higher in the β-propiolactone-inactivated group than in the formaldehyde-inactivated group, and there was no significant difference in anti-CV-A6-specific antibody titers in either group. Additionally, the cellular immune response analysis indicated a clearly elicited Th1 and Th2 cellular response, with a significant increase in the levels of the cytokines IFN-γ, IL-2, IL-6, and IL-10 in the sera of the immunized mice compared with the control group. The cellular response in the β-propiolactone inactivated group was stronger than that in the formaldehyde group. Further virus challenge tests showed protective efficacies of 96.2%, 100%, and 59.1% against lethal CV-A6, CV-A10, and CV-A16 virus infection, respectively, in suckling mice of the filial generation. CV-A6 has difficulties adapting to cell culture, which has become a bottleneck for the development of inactivated CV-A6 vaccines [23]. In this regard, the approach of using CV-A6 virus culture in Vero cells described in this study helps to provide valuable data for the preparation of inactivated CV-A6 vaccines. Furthermore, the protective efficacies of the inactivated vaccines with either formaldehyde or β-propiolactone inactivation were compared, shedding light on the selection of an inactivation approach in CV-A6 vaccine development.

CV-A6, EV-A71, and CV-A16 Trivalent Inactivated Vaccines

Elizabeth A et al. [24] prepared CV-A6, EV-A71, and CV-A16 trivalent inactivated vaccines from Vero and RD cell cultures, followed by formaldehyde and ethyleneimine (EI) inactivation and aluminum hydroxide formulation. The vaccine-elicited

anti-CV-A6, EV-A71, and CV-A16 virus-specific neutralization antibody titers of 1:485 (EV-A71), 1:285 (CV-A16), and 1:1436 (CV-A6) in mice immunized via intramuscular injection. Further virus challenge in the mice revealed complete protection against lethal CV-A6, EV-A71, or CV-A16 virus challenge. However, a variety of neutralization antibody titers were observed, and whether this variety resulted from an imbalance in the immune response awaits further study. Although ideal protection was observed in the mouse model, protection in rhesus macaques still requires further verification.

3 Live Attenuated Vaccines

Live attenuated vaccines are made by sufficiently attenuating the virulence and pathogenicity of the pathogen to maintain immunogenicity to induce a specific antipathogen immune response or by protection through physical, chemical, or genetic engineering approaches. Live attenuated vaccines have been widely used for over 100 years since the invention of the cowpox vaccine. To date, multiple live attenuated bacterial and viral vaccines have been successfully developed and licensed, such as the mumps live attenuated vaccine, poliomyelitis live attenuated vaccine, hepatitis A live attenuated vaccine, varicella live attenuated vaccine, and Bacillus Calmette Guerin (BCG) and typhoid live attenuated vaccine [25]. As live attenuated vaccines have the capacity to mimic the natural infection process, they can elicit strong humoral and cellular immune responses, while in contrast, conventional inactivated vaccines can merely elicit humoral immune responses and weak cellular responses [26]. Furthermore, live attenuated vaccines replicate in the human body and stimulate memory T-cell and B-cell formation, which contributes to eliciting a long-term immune response. Generally, one inoculation is adequate to elicit a long-term immune response [27]. Although live attenuated vaccines have many advantages, finding a balance between their safety and immunogenicity remains an essential issue. Ideally, live attenuated vaccines should be made by attenuating virulence and pathogenicity as much as possible while effectively maintaining replication capacity and immunogenicity. The major conventional techniques for preparing live attenuated vaccines include animal passage, cell passage, chemical mutagenesis, and nutritional mutagenesis.

Based on the advantages of live attenuated vaccines, many studies on the development and evaluation of HFMD live attenuated vaccines have been reported. Zhang et al. [28] prepared an attenuated CV-A16 strain from 6 passage cultures in Vero cells. The gene sequencing indicated that there were mutations at seven nucleotide sites: 1434 (C to U), 2744 (A to G), 2747 (A to G), 3161 (G to A), 3182 (A to G), 4968 (C to U), and 6064 (C to U). These mutations led to the alteration of 6 amino acids of the viral proteins: VP2-T161M, VP1-N102D, PV1-T103A, VP1-E241K, VP1-T248A, and 2C-S297F. The virulence study of the 1st generation (P1), 6th generation (P6), and purified 6th generation (PP) showed that all BALB/c mice infected at 1 day of age died 11 days post-P1 infection, whereas over 80% of the mice survived post P6 and PP infections at a high dose. Further amino acid sequencing and virulence analysis of P1 and P6 revealed that the amino acid alterations

VP1-N102D, VP1-E241K, and 2C-S297F were associated with weak virulence of P6. This study described the preparation of an attenuated CV-A16 strain via limited passages in Vero cells, and the virulence analysis in 1-day-old mice suggested good safety, which made this viral strain a potential candidate for preparing a live attenuated CV-A16 vaccine. Unfortunately, the genetic stability and immunogenicity of this viral strain were not examined and need to be further explored.

Chua et al. [29] prepared an attenuated EV-A71 strain from serial passage cultures (40 generations) in Vero cells by the cold-adaptation approach. The neurovirulence test in rhesus macaques revealed very weak infectious virulence, good safety and passage stability, with no obvious clinical manifestation and tissue injury noticed in the infected monkeys, no virus isolated from the monkey tissues, and no virulence recovery after the culture temperature was increased. This virus was found to elicit a great variety of neutralization antibodies of the anti-71 virus with different genotypes in monkeys immunized via intravenous inoculation. This study described the preparation of an attenuated EV17 strain via a cold-adaptation approach, which made this viral strain a potential candidate for preparing a live attenuated EV-A71 vaccine and provided good guidance for preparing other live attenuated HFMD vaccines.

According to reports, Yang et al. [30] prepared attenuated CV-A16 and EV-A71 strains from human diploid cells (KMB17) after serial passage for 50 generations. Strong humoral and cellular immune responses were elicited in rhesus macaques by combined immunization with these viral strains. The anti-EV-A71 and anti-CV-A16 virus-specific neutralization antibody titers were 1:4096 and 1:256, respectively. The virulence test indicated good safety in terms of the absence of obvious pathological changes in brain, spinal cord, lung, and liver tissues of the immunized monkeys. This study described the feasibility of preparing a bivalent live attenuated HFMD vaccine, shedding light on the preparation of multiple live attenuated HFMD vaccines.

Although it is possible to prepare live attenuated vaccines by the serial cell passage method, it indeed is unpredictable, takes substantial time and resources, and carries the potential risk for virulence recovery in the prepared vaccines. For example, oral live attenuated poliovirus vaccine administration may lead to the potential occurrence of vaccine-associated paralytic poliomyelitis (VAPP) and vaccine-derived poliovirus (VDPV) development. Hence, preparation of live attenuated vaccines with good safety and efficacy is the target for future research. With the rapid development of genetic engineering technology, site-directed mutagenesis, reverse genetics, etc., these techniques are gradually being applied for the development of live attenuated vaccines, which will greatly help to improve the safety, genetic stability, and immunogenicity of live attenuated vaccines.

Arita et al. [31] prepared an attenuated EV-A71 strain from a normal-virulence EV-A71 viral genome cloned with a partial temperature-sensitive attenuated EV-A71 viral genome and attenuated poliovirus type I genes by a site-directed

mutagenesis approach. Gene analysis showed mutations in the 5′-UTR, 3′-UTR and 3D regions compared with the parent strain. The neurovirulence test in rhesus macaques indicated substantial attenuation of neurovirulence after intravenous injection. More importantly, this viral strain was observed to elicit high levels of neutralization antibody (GMT: 1:1024-1:2048) in rhesus macaques. Further challenge studies in rhesus macaques indicated no development of clinical symptoms and no presence of live virus in swabs, but there were certain neurological system injuries in the tissue examination. This study describes an attenuated EV-A71 strain prepared by a genetic engineering approach, which was shown to exhibit a good attenuation effect for potential preparation of live attenuated EV-A71 vaccines. Unfortunately, this study failed to analyze the cellular immune response, virulence recovery and genetic stability, which require further exploration.

According to another report, Yee et al. [32] prepared an attenuated EV-A71 strain by a genetic engineering approach, i.e., two miRNA binding sequences of let-7a and miR-124a were inserted into the EV-A71 genome, site-directed mutagenesis was conducted in the 3D region, and 11 bp fragments were deleted in the 5′-UTR. The genetic stability analysis showed that the viral genome remained very stable without virulence recovery after 20 serial passages in RD cells. This viral strain was observed to elicit strong humoral and cellular immune responses in mice immunized via intraperitoneal inoculation and to prevent limb paralysis completely.

Most amino acids can be encoded by multiple codons. Theoretically, the codons are equally used to transcribe, but codon optimization is frequently applied in practice [33], when the common codons are replaced with noncommon codons, i.e., under codon deoptimization, causing viral replication and protein expression to typically be abated [34]. Additionally, there is great variety in RNA replication due to poor polymerase fidelity, which may lead to virulence instability or virulence recovery. Thus, increasing RNA polymerase fidelity would substantially decrease the viral mutation frequency [35]. On the basis of these theories and approaches, Tsai et al. [36] prepared an attenuated EV-A71 viral strain via codon deoptimization and increased RNA polymerase fidelity in the VP1 protein. The virus was used to immunize ICR and C57BL/6 mice that were 2–3 days old via intraperitoneal inoculation. The results revealed a mild clinical manifestation, lower viral loads in tissues, and increased survival rate compared with the control group with the wild virus infection, which is most likely to suggest good effects and safety of live attenuated vaccines. Furthermore, neutralization antibodies with high titers (1:1280–1:10240) were elicited in the mice, and the cross-neutralization assay on the sera showed good cross-protection against EV-A71 viruses with different genotypes. The viral genome was stable, with only 2–3 mutation sites being identified after 10 serial passages. Hence, codon deoptimization and increasing RNA polymerase fidelity might be deemed an effective approach for rapid preparation of live attenuated viral strains with promising evidence of immunogenicity, genetic stability, and virulence abatement.

4 Virus-Like Particle Vaccines

Inactivated vaccines are well known for being well developed and stable, but they have some disadvantages, such as the limited number of cell lines for virus culture. Currently, only a few cell lines are approved for vaccine production, such as Vero cells and KMB17 cells, and it is difficult to cultivate many of the viruses, such as CV-A6, in these cells. These disadvantages greatly contribute to restricting the research and development of inactivated vaccines. Virus-like particle (VLP) vaccines are defined as vaccines containing effective viral antigen components without the viral genome or infectious and proliferative capacities. VLP vaccines have many advantages, including complete antigen components, good safety, no limitation of viral culture, easy large-scale production by fermentation, and few requirements for production conditions and biosafety levels. More importantly, VLP vaccines, such as HPV, have successfully been approved for licensure. In light of this aspect, VLP vaccines are becoming increasingly favored by researchers, and a substantial number of studies of HFMD VLP vaccines have been initiated. It has been reported that the P1 of EV-A71 and other viruses could be proteolyzed by 3CD proteinase to be VP0 (VP2+VP4), with VP1 and VP3 simultaneously aggregating and forming the VLP protein after being expressed in a baculovirus-insect cell expression system. Thus, the research and development of several HFMD VLP vaccines have been conducted based upon this understanding.

Monovalent VLP Vaccines

CV-A5 Monovalent VLP Vaccine

Zhang et al. [37] constructed the recombinant expression vector pOET5-CVB5-P1-3CD by cloning the CV-A5 P1 and 3CD genes into the pOET5 vector and transfected them into Sf9 cells for the expression of P1 and 3CD proteins, followed by 3CD protein proteolysis, modification, and aggregation for a VLP with a diameter of approximately 30 nm correctly expressed in Sf9 cells. The VLP vaccine was formulated by mixing with an equal volume of Freund adjuvant and used to immunize the mice. The results indicated that neutralization antibodies with high titers (1:128–1:192) were elicited at levels significantly higher than those elicited by the CV-A5 inactivated vaccine antigen. The protection study in suckling mice of the filial generation revealed effective protection against lethal CV-A5 virus challenge. Unfortunately, this study failed to show results for the cellular immune response, which requires further exploration.

CV-A6 Monovalent VLP Vaccine

Shen et al. [38] cloned the recombinant expression vector pFBD-CV-A6-P1/3CD by cloning the CV-A6 P1 and 3CD genes into the baculovirus vector pFastBac and transfected them into Sf9 cells for the expression of the P1 and 3CD proteins, followed by P1 protein proteolysis to obtain a VLP with a diameter of approximately 30 nm. The VLP vaccine was formulated with aluminum hydroxide and used to immunize the mice via intraperitoneal injection. A good humoral immune response, as well as good protection against infections of CV-A6 virus with the same or different genotypes, was observed. This study provides proof-of-concept evidence for the development of a CV-A6 VLP vaccine, providing good reference data. However, whether such a VLP vaccine could elicit a cellular immune response awaits further exploration.

Unlike the abovementioned approach, Zhou et al. [39] successfully expressed another kind of CV-A6 VLP by using the Pichia pastoris expression system, i.e., by constructing the recombinant expression vector YCV-A6-003 via cloning of the CV-A6 P1 and 3CD genes into the expression vector for transfection and expression in Pichia pastoris. The results showed correct expression of the recombinant expression vector in Pichia pastoris, confirmed by the correct expression of the VP0, VP1, and VP3 proteins, as determined by Western blotting. Additionally, a spheroidal particle with a diameter of 30 nm was observed by transmission electron microscopy, suggesting the expression of CV-A6 VLP from the recombinant expression vector in Pichia pastoris. This CV-A6 VLP was formulated with aluminum hydroxide to immunize mice, and subsequently, a strong anti-CV-A6 virus IgG humoral immune response was elicited. Further passive immune protection studies in suckling mice revealed that 7-day-old mice injected with the anti-sera could be protected from the lethal challenge of CV-A6 virus, suggesting good protective efficacy. This study describes the successful expression of CV-A6 VLP in a new expression system, providing some data for further development of CV-A6 VLP vaccines. However, the neutralization antibody levels and cellular immune response elicited by the CV-A6 VLP vaccine still require further investigation.

CV-A10 Monovalent VLP Vaccine

By using the same approach, Zhou et al. [40] constructed the CV-A10 VLP recombinant expression vector CV-A10-003 for transfection and expression in Pichia pastoris, followed by CV-A10 VLP formation. This CV-A10 VLP was formulated with aluminum hydroxide and used to immunize mice. The results showed that a high level of IgG antibody was elicited in the mice and that suckling mice injected with the anti-sera could be protected from the lethal challenge of CV-A10 virus. Unfortunately, the neutralization antibody levels and cellular immune response were not described in this study.

Unlike the previous description, Dai et al. [41] constructed the fusion protein expression vector pET28-HBc-P28 by inserting the CV-A10 virus VP2-P28 into the hepatitis B virus core antigen (Hbc) for transfection and expression in E. coli to form VLP with a diameter of 30 nm. This VLP was formulated with aluminum hydroxide and used to immunize BALB/c mice via intraperitoneal inoculation. The results indicated that a neutralization antibody titer of approximately 1:16 was elicited in the mice and that the anti-sera passively injected into the ICR mice could induce an obvious protective efficacy of 92% compared with the control group. However, the data of the cellular immune response were not reported in this study.

CV-A16 Monovalent VLP Vaccine

Feng et al. [42] constructed a CV-A16 virus VLP with a diameter of approximately 30 nm by cloning the P1 and 3CD genes of CV-A16 virus into the Pichia pastoris system for expression. The VLP was formulated with Freund's adjuvant for immunizing BALB/c mice. The results demonstrated that a specific humoral and cellular immune response was elicited. The neutralization antibody titer peaked at 1:512 2 weeks post-immunization, which was higher than the titer elicited by an inactivated CV-A16 vaccine. Furthermore, VLPs were shown to elicit the expression of the cytokine IL-2, IL-6, IL-10, etc. The CV-A16 VLP-elicited maternal antibody could protect suckling mice of the filial generation against the lethal challenge of CV-A16 virus, which was consistent with the results of passive immunization, indicating good in vivo protective efficacy. This study describes the overall evaluation of the humoral and cellular immune responses and protective efficacy elicited by CV-A16 VLP in mice, which sheds light on the further development of CV-A16 VLP vaccines.

EV-A71 Monovalent VLP Vaccine

In addition to the development of coxsackie virus VLP vaccines, many studies on EV-A71 VLP vaccines have been published. Lin et al. [43] constructed an EV-A71 VLP recombinant expression vector by cloning the P1 and 3CD genes of EV-A71 virus into the baculovirus system for the expression of VLPs with a diameter of 33 nm. The VLP vaccine was used to immunize BALB/c mice, and an anti-EV-A71 virus-specific neutralization antibody was elicited. The maternal antibody protected suckling mice of the filial generation against the lethal challenge of EV-A71 virus. The obvious protective efficacy was better than that elicited by the same dose of inactivated vaccine. To improve the expression capacity of the EV-A71 VLP vaccine in the baculovirus system, Kim et al. [44] introduced a gp41 promoter into the EV-A71 VLP expression vector to construct the new recombinant expression vector baculo-P1-3CD-gp41. After the recombinant expression vector was transfected into Sf9 cells, the VLP production capacity was significantly increased to 11.3

mg/L. Further protective efficacy studies showed that the VLP vaccine-elicited antibody could neutralize EV-A71 viruses with the same or different genotypes and that the durability of the neutralization antibody was good. These study data provide a good and practical reference for the large-scale production of the EV-A71 VLP vaccine.

Unlike the results of previous study, Wang et al. [45] constructed an innovative recombinant EV-A71 VLP expression vector expressing VP1, VP2, and VP3 proteins rather than VP4. The spherical VLP was formed after the vector was transfected into Sf9 cells. The VLP was formulated with aluminum hydroxide for immunizing the ICR mice via intraperitoneal inoculation. An anti-EV-A71-specific neutralization antibody was elicited with no significant difference in the titers compared with the titer elicited by a complete EV-A71 VLP (containing VP4). Similar to the protective efficacy resulting from complete EV-A71 VLP vaccine immunization, the maternal antibody protected suckling mice of the filial generation against the lethal challenge of EV-A71 virus. In parallel, this study detailed the mechanisms of virus and cellular receptor binding and neutralization, protein structure formation, etc., which were potentially inhibited by the anti-EV-A71 virus-specific antibody that was elicited by the VLP vaccine, which provided reference and immune mechanism data for further development of the EV-A71 VLP vaccine.

Zhijian Yang et al. [46] successfully constructed the recombinant expression vector P13C-pPEXZ by cloning the P1 and 3CD genes of EV-A71 into the vector for the expression and formation of VLPs in the Pichia pastoris system. The VLP was formulated with aluminum hydroxide to immunize BALB/c mice. An anti-EV-A71-specific neutralization antibody was elicited with a titer of 1:1024, which was significantly higher than that (1:512) elicited by an inactivated EV-A71 vaccine. Furthermore, similar to the protective efficacy of the inactivated EV-A71 vaccine, the maternal antibody protected suckling mice of the filial generation against lethal challenge with the EV-A71 virus. Additionally, this study described the improvement in VLP production capacity by optimizing the codons and expression conditions of the recombinant expression vector, which provides some data for production process development for the large-scale production of EV-A71 VLP vaccines.

Yueh-Liang Tsou et al. [47] constructed the recombinant adenovirus vector Ad-EVVLP by inserting the P1 and 3CD genes of EV-A71 into an adenoviral genome with E1/E3 deficiency and transfected them into HEK-293A cells for successful expression and formation of a spherical VLP with a diameter of approximately 30 nm. This VLP did not contain the VP2 protein, unlike other EV-A71 VLPs, but could still elicit a specific humoral immune response and balanced cellular immune response (Th1/Th2) in BALB/c mice. When the VLP vaccine was used to immunize hSCARB2 transgenic mice, the mice were shown to be protected against lethal challenges with EV-A71 or CV-A16 viruses, while in contrast, the inactivated EV-A71 vaccine protected the mice against only EV-A71 infection, and no obvious protection against CV-A16 was observed. Furthermore, although VLP immunization failed to elicit an anti-CV-A16 virus-specific neutralization antibody in the mice, it did induce 3C-specific CD4+ and CD8+/IFN-γ T-cell immune

responses, which was presumed to lead to cross-protection against CV-A16 virus infection. This study described the construction of an EV-A71 VLP vaccine based upon the adenovirus vector that exhibited good immunogenicity and efficacy and certain cross-protection against CV-A16 virus infection. Whether it has the capacity for cross-protection against other coxsackie viruses awaits further exploration.

To prepare a VLP by using the major immune epitope is another good alternative for direct and effective antigen delivery to T cells, B cells, and DCs and for subsequent stimulation of the immune response. Liu et al. [48] constructed a VLP with a diameter of approximately 25–40 nm by expression in the *E. coli* (BL21) system of the shortened fusion proteins VP1 (131 amino acids at the C-terminus), VP2 (180 amino acids at the N-terminus), and VP3 (120 amino acids at the N-terminus). The VLP was formulated with aluminum hydroxide to immunize BALB/c mice. A high-level and durable specific neutralization antibody was elicited and reached 1:128 4 weeks post-immunization, which was similar to that elicited by an inactivated vaccine. Furthermore, the neutralization antibody titer was maintained at more than 1:64 6 weeks post-immunization. The maternal antibody protected suckling mice of the filial generation against nonlethal challenge with the EV-A71 virus. When the mice were immunized with high-dose (10 μg) VLP vaccine, the protective efficacy was shown to be 87% in suckling mice. Additionally, good protective efficacy was noticed in 1-day-old neonatal mice immunized with two doses of VLP followed by a subsequent nonlethal challenge with the EV-A71 virus, which showed no significant difference from the inactivated vaccine.

Multivalent VLP Vaccines

EV-A71/Nov Bivalent VLP Vaccine

Xiaoli Wang et al. [49] prepared an EV-A71 (P1+3CD) and Norovirus (VP1) bivalent VLP vaccine in the baculovirus system. This bivalent VLP formulated with aluminum hydroxide was used to immunize BALB/c mice, and a high level of anti-EV-A71-specific neutralization antibody (1:3649) and inhibitory capacity of norovirus binding to porcine gastric mucosa (PGM) were demonstrated.

CV-A6/CV-A10/CV-A16/EV-A71 Tetravalent VLP Vaccine

Zhang et al. [50] prepared CV-A6, CV-A10, CV-A16, and EV-A71 virus tetravalent VLP vaccines by the expression of the target proteins P1 and 3CD of each single virus in the baculovirus system and subsequently formulated them with aluminum hydroxide. This VLP vaccine was used to immunize BALB/c mice via intraperitoneal inoculation, and anti-CV-A6-, CV-A10-, CV-A16-, and EV-A71-specific neutralization antibodies with GMT titers of1: 28, 1:362, 1:4598, and 1:1825,

respectively, were elicited, and no difference observed when compared with the control group immunized with single monovalent VLPs. Further passive protection studies revealed that the anti-sera had the capacity to protect newborn ICR mice against single or combined lethal challenges with the CV-A6, CV-A10, CV-A16, and EV-A71 viruses. In contrast, the monovalent VLPs could only protect the mice against challenge with the same genotype of the virus without any cross-protection. This study demonstrated the feasibility of preparing a HFMD multivalent VLP vaccine in terms of the immunogenicity and protective efficacy elicited by the tetravalent VLP vaccine. However, the 4 VLPs elicited a variety of immunogenicities, with higher titers of anti-CV-A16 and EV-A71 virus-specific antibodies than of anti-CV-A6 and CV-A10 virus-specific antibodies, but the underlying reason and immune response mechanism remain to be further studied.

5 Subunit Vaccines

Compared with inactivated vaccines and VLP vaccines, subunit vaccines are well known for their advantages of simple preparation, easy large-scale production, good safety, etc. For viruses with difficulties in adapting for growth in cell culture and forming VLPs, developing a subunit vaccine has become a good alternative. To date, several subunit vaccines have been approved by the regulatory authority for licensure, such as the hepatitis B vaccine made by recombinant DNA techniques in yeast and the hepatitis B vaccine made by recombinant DNA techniques in CHO cells [51]. Some studies for developing HFMD subunit vaccines have been conducted. VP1 is the major immunogen associated with HFMD, exhibiting good immunogenicity, and is a major epitope for determining neutralization [52]. Thus, VP1 is frequently used as the target antigen for preparing the subunit vaccine and subsequently evaluating its immunogenicity and protective efficacy.

Wang et al. [53] prepared an EV-A71-VP1 subunit vaccine by cloning the VP1 gene of EV-A71 into the pPICZaA plasmid to construct the recombinant expression plasmid PICZaA-VP1, which was transfected into Pichia pastoris for the expression of the VP1 protein, followed by purification and formulation with Freund's adjuvant. The vaccine was used to immunize BALB/c mice via intramuscular injection, and an anti-EV-A71-specific antibody with a titer of approximately 1:32 was elicited. Further passive protective efficacy study revealed that the vaccine could protect 3-day-old newborn mice against lethal challenge with EV-A71. This protective efficacy was better than that of the single VP1 (without Freund's adjuvant) immunization group. Additionally, this subunit vaccine was shown to elicit the cellular immune response, with substantial proliferation of CD4+ and CD8+ cells and expression of the cytokines IFN-γ and IFN-α.

Similarly, Zhang et al. [54] prepared an EV-A71-VP1 subunit vaccine by cloning the VP1 gene of EV-A71 into the ET30a plasmid to construct the recombinant expression plasmid ET30a-VP1, which was transfected into BL21 (DE3) cells for

expression of the VP1 protein, which was then formulated with chitosan adjuvant. The vaccine was used to immunize the ICR mice via intragastric administration, and the anti-EV-A71 specific neutralization antibody with titers of 1:4–1:8, as well as mixed Th1/Th2/Th3 cellular immune responses, was elicited. Additionally, the vaccine had the capacity to elicit a mucosal immune response, with upregulation of IgA secretion in the vagina and respiratory tract of the mice. Further passive protective efficacy studies in newborn ICR mice indicated an efficacy of approximately 30%. This study describes the preparation of an EV-A71-VP1 subunit vaccine formulation with chitosan adjuvant and intragastric injection for immunization, and the results suggest the activation of a unique immune response, such as the elicitation of a mucosal immune response, which provides some data and references for the design, immunization route, and adjuvant selection for the development of innovative EV-A71 subunit vaccines.

The lack of protein protection in subunit vaccines may lead to the degradation or loss of activity via a variety of enzymes. Thus, improving the efficacy is deemed to be critical in the successful development of subunit vaccines. Currently, it is understood that nanoparticle-like subunit vaccines might help to solve this problem, as this kind of vaccine is prepared by coating the antigen and adjuvant in a nanoparticle to protect the antigen from being degraded by enzymes and deliver it directly to the lymph nodes for better activation of lymphocytes. Furthermore, this system could enhance APC intake and antigen delivery capacity for activating the corresponding T cells [55]. On the basis of this understanding, Qiao et al. [56] prepared an EV-A71-VP1 nanoparticle with a diameter of 93–130 nm by coating the VP1 protein and adjuvants (CpG or TNF) with chitosan and heparin sodium. This subunit vaccine was used to immunize the mice via subcutaneous injection. Specific humoral and cellular immune responses were elicited, and there was no significant difference in antibody titers compared with those elicited by an inactivated EV-A71 vaccine. However, the neutralization antibody levels elicited by the EV-A71-VP1 nanoparticle vaccine with TNF adjuvant were higher than those with CpG adjuvant. Th1 and Th2 immune responses were elicited by the nanoparticle vaccine with different adjuvants, whereas only the Th2 response was elicited by the inactivated vaccine. A high level of IgA antibody was elicited by the gastric-specific IgA assay, suggesting that the nanoparticle-like vaccine could induce a mucosal immune response that might have the potential to prevent early EV-A71 infection. The passive protective efficacy indicated that the vaccine could completely protect the mice against lethal challenge with EV-A71. The active protective efficacy assay also revealed that the vaccine could protect 7-day-old gerbils against lethal challenge with EV-A71. Unlike other technologies for preparing conventional subunit vaccines, this study described a new approach for preparing nanoparticle-like subunit vaccines, and the results demonstrated good immunogenicity and protective efficacy, shedding light on the technique and providing reference data for the development of innovative EV-A71 subunit vaccines.

6 Viral Vector Vaccines

Viral vector vaccines are innovative vaccines that use live viruses to carry encoded target antigens into human cells for expression to elicit an immune response. Generally, viral vectors have the following features: broad-spectrum infection, infecting multiple cells or tissues; nonpathogenicity; large capacity for antigen delivery to the viral surface; weak antigen of the vector itself, which fails to elicit a strong immune response. Currently, the common vectors used for vaccine research and development include adenovirus, respiratory syncytial virus, and flu virus vectors [57]. Adenovirus is well known for being able to infect multiple cells and tissues, and the recombinant adenovirus genome has a large exogenous gene capacity and low toxicity and can express exogenous proteins on the viral surface to stimulate human cells and elicit a strong immune response. Hence, several COVID-19 adenovirus vector vaccines have been developed during the COVID-19 pandemic, such as the recombinant COVID-19 vaccine (adenovirus type 5 vector) developed by Chinese researchers and the recombinant COVID-19 vaccine (adenovirus type 5 and 25 vectors) developed by Russian researchers, which have been approved for licensure [58]. It is understood that other adenoviruses, such as the chimpanzee adenovirus type 1 vector, could also be used as vectors for vaccine development [59].

Some researchers have prepared HFMD vaccines by using adenovirus vectors. Zhang et al. [60] prepared the bivalent EV-A71/CV-A16 adenovirus vector vaccine AdC68VP1-Hx-PEP71(R1)-sSP70(R2) by cloning the VP1 genes of EV-A71 and EV-A16 viruses into the hypervariable regions 1 and 2 of adenovirus type 68-vector. VP1 protein and adenovirus particles were successfully expressed in HEK293 cells infected with the vaccine. The vaccine was used to immunize BALB/c mice via intramuscular injection, and anti-EV-A71 and CV-A16 virus-specific neutralization antibodies and cellular immune responses were elicited at anti-EV-A71 and CV-A16 virus antibody levels of approximately 1:80 and 1:16, respectively, with the activation of Th1 and Th2 cellular immune responses. Nevertheless, the Th1 and Th2 cellular immune responses elicited by each antigen tended to vary in a pattern of strong Th1 response elicited by CV-A16 VP1 versus similar Th1 and Th2 responses elicited by EV-A71 VP1. Further passive immune protection studies using the antisera indicated 100% versus 80% protection against the lethal challenge of EV-A71 virus versus CV-A16 virus in ICR mice that were 2 days old. This study described the successful preparation of a bivalent EV-A71-CV-A16 adenovirus vector vaccine with immunogenicity and protective efficacy, which suggests the potential feasibility of preparing monovalent or multivalent HFMD adenovirus vector vaccines and provides important data for preparing effective HFMD viral vector vaccines in the future.

7 DNA Vaccines

DNA vaccines are innovative vaccines that have undergone rapid development in the past several decades. It is made by cloning an antigen epitope gene into a constructed plasmid for the expression of the target antigen in humans. The DNA vaccine has the following advantages: well developed and simple preparation and purification process; low costs for large-scale production; elicitation of a durable immune response; no requirement for adjuvant due to the potential adjuvant function of the plasmid itself; good stability and ease of storage and transport of the lyophilized formulation; suitability for viruses with difficulties in adapting for growth in cells, due to simple chemical synthesis of DNA sequences of the target antigen at the gene level [61].

Currently, DNA vaccines of multiple viruses have been reported, such as SARS-CoV-2, avian flu virus, and enterovirus [62]. Tung et al. [63] prepared a VP1-DNA vaccine by inserting VP1-encoding genes into the pVAX1 plasmid to construct the recombinant expression plasmid pVAX1/VP1, which was transfected in vitro in Vero cells for correct expression of the VP1 protein. The vaccine was used to immunize BALB/c mice via intramuscular injection, and anti-EV-A71-specific IgG and neutralization antibodies were elicited. Interestingly, the IgG antibody level in sera decreased after booster immunization; the anti-EV-A71 specific neutralization antibody titer was approximately 1:32 at 14 days post-immunization, and no increase was noticed after booster immunization. This study describes the successful preparation of an EV-A71-VP1 DNA vaccine with evident immunogenicity, implying the feasibility of developing an EV-A71 DNA vaccine in terms of concept and approach and providing good data for further development of other HFMD DNA vaccines, especially for those enteroviruses with difficulty growing in cells, such as CV-A6.

Andreashenke et al. [64] prepared 4 different kinds of CVB3 protein expression plasmids (PCMV/VP1, PCMV/VP4-1, PCMV/VP4-2, and PCMV/VP3-1) by cloning 4 different CVB3-encoding genes into the PCMV plasmid. The in vitro results indicated clear expression of viral proteins from these 4 DNA plasmids. Specific IgG antibodies were elicited in BALB/c mice immunized with these 4 DNA vaccines. The virus challenge study of these 4 DNA vaccines (PCMV/VP1, PCMV/VP4-1, PCMV/VP4-2, and PCMV/VP3-1) showed protective efficacies of 72.2%, 23.1%, 30.8%, and 14.3%, respectively, against CVB3 virus infection, and the DNA vaccine prepared from the VP1 target gene demonstrated the highest protective efficacy, which might make it a candidate for CVB3 DNA vaccine. Similarly, Kim et al. [65] prepared 4 different VCB3 DNA vaccines (PCA-VP1, PCA-VP3, PCA-VP1-1 (partial VP1 sequences), and PCA-VP1-2 (remaining VP1 sequences)) by using plasmids with high expression in mammalian cells. Specific IgG antibodies were elicited in BALB/c mice immunized with these 4 DNA vaccines via electroporation injection. Interestingly, none of the neutralization antibodies was detected. The virus challenge study in the immunized mice showed protective efficacies of 42.9%, 75%, 12.5%, and 50%, respectively, and the PCA-VP3 DNA vaccine demonstrated the highest protective efficacy of 75%. The study described a DNA vaccine prepared from the target gene of VP3 with high protective efficacy, suggesting the

potential use of VP3 as a candidate target antigen for the development of a CVB3 DNA vaccine.

To improve vaccine efficacy, some CVB3 DNA vaccine-associated immunization programs and adjuvants have been studied. Lan et al. [66] compared the efficacies of the CVB3-VP1 DNA vaccine with two doses of intramuscular injection, CVB3 subunit VP1 protein with two doses of intraperitoneal injection and CVB3-VP1 DNA prime and VP1 protein booster. The results revealed the specific humoral immune response and CTL response in the immunized BALBC/c mice elicited by these three different programs, the neutralization antibody titers for which were 1:23.71, 1:42.66, and 1:70.79, respectively. The highest level (1:70.79) was elicited by the DNA prime and VP1 booster immunization program, but there was no difference in the CTL response noticed in the different immunization programs. The virus challenge study showed protective efficacies of 8.23%, 33.3%, and 75% for these 3 different immunization programs. Xu et al. [67] conducted a study of coimmunizing BALB/c mice with chitosan adjuvant and CVB3 DNA vaccine (pcDNA3-VP1). Compared with the control group without the adjuvant, the pcDNA3-VP1 vaccine with chitosan adjuvant could elicit stronger humoral and cellular immune responses, as well as mucosal immune responses with the sIgA antibody. The virus challenge study showed an efficacy of 42.9% for the pcDNA3-VP1 vaccine with chitosan and 16.7% for the control group without the adjuvant. Yin et al. [68] conducted a study to improve the immunogenicity and protective efficacy of the CVB3-VP1 DNA vaccine in BALB/c mice by using the absent in melanoma 2 (AIM2), i.e., coimmunization with the AIM2 DNA plasmid (pAIM2) and CVB3-VP1 DNA vaccine (VP1), which elicited a strong and durable cellular immune response (CTL) and activated memory CD8+ T cells. The virus challenge results indicated that the protective efficacy (75%) of pAIM2 and pVP1 coimmunization was higher than that (40%) of single pVP1 immunization.

The abovementioned studies reported the comprehensive results of CVB3 DNA vaccine development from the aspects of antigen selection, immunization programs, heterologous/homologous immunization, adjuvant selection, etc. The findings suggest that the VP1 and VP3 proteins of CVB3 might be potential target genes for the development of CVB3 DNA vaccines due to their good immunogenicity. Of course, as this is an innovative vaccine without evidence for use in large human populations, many questions require further exploration, such as whether the vaccine DNA can be integrated into the host cell genome, safety concerns, the interaction between DNA vaccines and the host immune system, and the efficacy and durability of DNA vaccines.

8 RNA Vaccines

RNA vaccines are made by preparing mRNA via in vitro transcription, capping, and tailing of target antigen genes and then formulated with a specific delivery system for the expression of target antigens, eliciting an immune response in the human

body [69]. mRNA vaccines have the following advantages: first, the preparation is simple and quick with low costs, especially for emerging infectious diseases; second, the immune efficacy is good, eliciting both humoral and cellular immune responses, resulting from direct expression of antigen proteins to ensure correct modification and folding; third, no large-scale cell culture is required due to direct synthesis in vitro, especially for viruses with difficulty in growing in cells; and the potential to produce multivalent vaccines. It is promising that two COVID-19 mRNA vaccines, mRNA-1273 (Moderna) and BNT162b2 (Pfizer-BioNTech), have successfully been licensed, which has greatly promoted the rapid development of mRNA vaccines. The available data from clinical studies and the real world have demonstrated that these two mRNA vaccines elicit strong cellular and humoral immune responses and subsequently contribute to reducing the severe and fatal cases of COVID-19 [70]. However, only a few datasets on HFMD mRNA vaccine development have been reported.

Hunziker et al. [71] prepared a CVB3 RNA vaccine. The gene sequence analysis of this mRNA vaccine indicated a site epitope mutation in the joint of 2A and 2B. The in vitro transcription results showed the expression of mature polyproteins. However, the viral protein and neutralization antibody were not detected in the C57BL/6 mice immunized with this RNA vaccine. Surprisingly, the vaccine protected the mice against challenge with live attenuated or wild CVB3 viruses with an efficacy of approximately 50%. Although no data on the cellular immune response elicited by the mRNA vaccine were reported, the study somewhat showed the feasibility of preparing an HFMD RNA vaccine and provided a reference for the development of other HFMD RNA vaccines.

With the development of RNA vaccine technology in recent years, the key bottleneck issues of instability, low transfection efficacy, low expression efficacy, etc., of RNA vaccines are being addressed, and RNA vaccines for disease prevention and treatment have rapidly been developed. In particular, with successful licensing of the two COVID-19 mRNA vaccines, more innovative approaches have continuously emerged; for example, codon optimization and nucleotide substitution have been shown to greatly improve the stability and expression efficacy of RNA vaccines. Additionally, innovating a new delivery system, such as the development of nanoparticle liposomes, will help to improve the stability and immune efficacy of RNA vaccines and maintain a more durable immune response than that of inactivated vaccines and conventional vaccines. This has fully been evidenced by the clinical data of these 2 mRNA vaccine trials [72]. As far as the development of HFMD vaccines is concerned, preparing other HFMD RNA vaccines might become another effective way of protecting against HFMD given the dynamic circulation change of HFMD pathogens since the application of the inactivated EV-A71 vaccine.

9 Conclusion

To date, the research and development of HFMD vaccines have achieved promising results. Although the administration of the licensed inactivated EV-A71 vaccine contributes to effective prevention against EV-A71 infection and HFMD outbreaks, it frequently changes the background circulating strains to CV-A6, CV-A10, CV-A5, and CV-A16, which may potentially lead to mixed infection and HFMD outbreaks [73]. Hence, the development of multivalent HFMD vaccines is becoming a priority for future research. Based on the substantial amount of reported data from HFMD vaccine development, the type of vaccine, route of immunization, dose and interval of immunization, and selection of adjuvants were identified as affecting vaccine efficacy. In terms of vaccine type, both the conventional inactivated vaccines and live attenuated vaccines and the innovative pseudovirus particle-like vaccines, protein vaccines, and nucleic acid vaccines have their own advantages and disadvantages, but overall, the conventional vaccines have the irreplaceable advantages of safety and efficacy. In contrast, innovative vaccines have advantages in production processes, costs, and development speeds. Further exploration is required to understand the safety and interaction with the human body of innovative vaccines, especially nucleic acid vaccines prepared by new technologies. In terms of vaccine immunization routes, intramuscular injection is recognized as the most common route. However, an increasing number of studies have found that intradermal injection could elicit a strong innate immune response and enhance the subsequent acquired immune response to ultimately enhance immunogenicity. The nasal drop route receives high attention due to the elicitation of a mucosal immune response. Further study and more clinical data of intradermal and nasal drop immunization are required to promote their extensive use. In terms of the immunization program dose and interval, an interval of 28 days for 2 doses is known to be most common, but this program can be improved for some vaccines prepared by special production processes. For example, the heterologous and homologous booster immunization strategy has been promoted for the COVID-19 vaccine based upon previous experiences. This experience contributes to the design of special immunization programs for improving immunogenicity and efficacy prior to guaranteeing safety. Currently, the major adjuvants, including aluminum hydroxide, squalene-in-water emulsions (MF59), and MPL/alum (MPLA), are approved for use. The abovementioned adjuvants MPLA, poly I:C, CpG (type C), chitosan, etc., have exhibited potential advantages in different candidate vaccines. The selection of adjuvants plays an essential role in improving vaccine immunogenicity, and the variety of immune responses elicited by different antigen formulations with adjuvants is becoming another important aspect in the research and development of HFMD vaccines.

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