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Dissecting complicated viral spreading of enterovirus 71 using in situ bioorthogonal fluorescent labeling

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ABSTRACT

Enterovirus 71 (EV71), the major pathogen of hand-foot-and-mouth disease (HFDM), can cause severe neurological and respiratory manifestations in young children. Viral spread route and tissue tropism are key factors contributing to different pathogenicity of EV71, however it remains a challenge to dynamically visualize EV71 infection in vivo. The present study applies an in situ bioorthogonal fluorescent labeling strategy to track clinically isolated EV71 strains with different pathogenicity in neonatal mice. The results show that the in situ labeling strategy effectively captures EV71 viruses through in vivo bioorthogonal reaction in multiple infected organs without interfering viral spread and tissue tropism. More importantly, the in situ labeling reveals different viral dynamics, dissemination, and tissue tropism of severe case EV71 (SC-EV71) and mild case EV71 (MC-EV71), consistent with their different pathogenicity in HFDM patients. Compared with MC-EV71, SC-EV71 not only enters the blood circulation and spreads out more quickly, but also shows more significant neuronal and respiratory tropism, which certainly contribute severe neurological complications and clinical manifestations in the patient. Hence, the in situ bioorthogonal fluorescent labeling is a plausible strategy to dissect complicated process of EV71 viral spread in the early stage of infection, thereby offering great opportunities to understand its pathogenesis and develop anti-viral drugs.

1. Introduction

Enterovirus 71 (EV71) is a member of the *Picornaviridae* family, causing hand-foot-and-mouth disease (HFMD) in the Asia-Pacific region. The typical clinical manifestations of HFMD are fever, rash, or herpes on hand, foot, and mouth, which are generally mild [1–3]. However, some EV71 strains can cause severe complications, such as encephalitis, acute flaccid paralysis, pulmonary edema, especially in children under 5 years of age [4–6]. The pathogenesis of EV71 can be complicated and attributable to numerous genetic variations [7], which subsequently affect viral spread and tissue tropism [8]. For example, the neurotropism of EV71 can be associated with amino acid substitutions in capsid protein VP1, which are essential for viral invasion and tissue tropism [9,10]. Till data, many genetic variations have been identified to be related with the virulence and pathogenesis of EV71, it remains a challenge to visualize EV71 dissemination and tissue tropism in vivo due to the lack of appropriate techniques.

Fluorescent imaging offers great opportunities to explore viral invasion, dissemination, and virus-host interaction [11,12]. Reporter-expressing viruses with reporter genes, such as luciferase and fluorescent proteins, has been widely applied to monitor virus invasion, replication, and dissemination [13,14]. However, the genetic modification of live viruses required special expertise and is usually time consuming. Moreover, the integration of reporter genes into viral genome often affect genetic stability and infectivity of viruses [15] Direct fluorescence labeling is another important strategy to track in

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vitro and in vivo virus infection [16,17]. To achieve successful virus labeling, the fluorescent tags must be conjugated to viruses through covalent or noncovalent reaction, which unfortunately often dampens viral infectivity because of the chemical modification of viral binding sites [18]. In the past ten years, the bioorthogonal chemical reporter strategy has emerged as a promising alternative to label living organisms with great efficacy, specificity, and simplicity [19,20]. This approach involves the incorporation of a bioorthogonal chemical reporter, such as dibenzocyclooctyl (DBCO), azide, tetrazine and bicycle [6.1.0] nonyne (BCN) motifs, into target biomolecules in a living system [21]. An exogenous fluorescent probe is then covalently linked to the chemical reporter through a bioorthogonal reaction, thereby allowing imaging living organisms, such as viruses [22], cells [23], tumor [24,25] and bacterial [26], etc.

Conventionally, living organisms must be pre-labeled with fluorescent tags in vitro before subjecting to the in vivo imaging [11,17]. However the pre-labeled fluorescence often causes biological perturbation and biases our observations. We recently developed an in situ bioorthogonal fluorescent labeling strategy (so called “in situ labeling”, which successfully tracked pseudo virus infection in lung tissues [27]. However, whether this strategy would be able to track EV71 infection with complicated spread routes is unknown. Herein, we applied the in situ labeling strategy and in vivo imaging to dynamically monitor viral dissemination and tissue tropism of two clinically isolated EV71 strains with different pathogenicity (Scheme 1). The results showed that the in situ labeling strategy not only effectively captured EV71 viruses in multiple infected organs through an in vivo bioorthogonal reaction, but also preserved viral infectivity without significantly interfering viral dissemination and tissue tropism. More importantly, the in vivo imaging of the in situ labeled viruses clearly demonstrated different dynamics, dissemination, and tissue tropism of two EV71 strains, consistent with their different pathogenicity in HFDM patients.

2. Materials and methods

2.1. Materials

Human rhabdomyosarcoma (RD) cells and African green monkey kidney epithelial cells (Vero line) provided by Guangdong Center for disease control and prevention were used for EV71 isolation. RD and Vero cells were maintained in DMEM supplemented with 10% FBS (GIBCO, USA) at 37 °C in a humidified atmosphere with 5% CO_2. The mouse anti-EV71 monoclonal antibody was obtained from Abcam (USA). Dibenzocyclooctyl (DBCO) ester and azide conjugated dye (N_3-Cy5.5) were purchased from Click Chemistry Tools (Scottsdale, AZ, USA). ICR mice were purchased from the Laboratory Animal Center of Guangdong province and maintained in the animal facility of Shenzhen Center for Disease Control and Prevention. All experiments were conducted according to the guidelines and protocols approved by IACUC of Shenzhen Center for Disease Control and Prevention.

2.2. EV71 patients and virus isolation

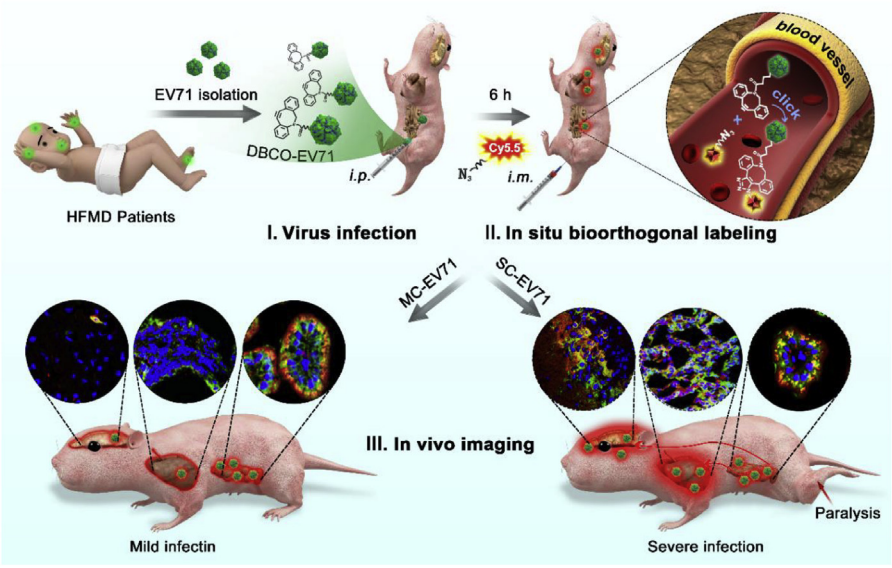
Two HFMD patients were diagnosed in Shenzhen Guangming People's hospital. The mild case patient was a two-year-old boy with mild symptoms, such as fever, oral ulcers, and vesicular on the hand. The severe case patient was a 20-month-old boy, who developed CNS associated symptoms, such as severe vomiting, lethargy, muscle twitching, and aseptic encephalitis, and then died in a few days after hospitalization (see supporting information). Two EV71 strains were isolated from patient stool samples as previously reported [28], and defined as the MC-EV71 strain (EV71/SZ17/CHN/2013) and SC-EV71 strain (EV71/SZ08/CHN/2013), respectively.

2.3. Genomic sequencing of EV71 virus

Viral RNA was extracted using the High Pure viral RNA kit (Roche, Germany), followed by RT-PCR using a real-time RT-PCR kit (ZHONGSHAN DAAN Genomics, Inc., China). A pair of universal primers EVA-F16 (5′-TTAAAACAGCCTGTGGGTTGCACCCACTC-3′) and EVA-R13 (5′-TTTTTTTTTTTTTTTTTTTTTTTTGCTATTCT-3′) was designed to amplify the whole genome of EV71 strain using a Primescript one Step RT-PCR kit version 2 (Takara, Japan). Amplified DNA products was sequenced by a commercial corporation (Takara, Japan) using a primer walking method, and contigs were assembled using sequencer version 4.9. The raw genome sequences were examined by using BioEdit Version 7.2.5 before submission to Genbank. The nucleotide sequences of SC-EV71 and MC-EV71 were submitted to the Genbank database under accession numbers MF405075, MF431793 for complete genome sequences.

2.4. Phylogenetic analysis

The alignment of the two EV71 strains and reference sequences were conducted using Bioedit software (Version 7.2.5). Phylogenetic and molecular evolutionary analyses were conducted using the



Scheme 1. In situ bioorthogonal labeling for dynamically dissecting EV71 spread in vivo. Severe case EV71 (SC-EV71) and mild case EV71 (MC-EV71) were clinically isolated from two HFMD patients, and chemically modified by DBCO motifs to obtain DBCO-EV71. (I-II) ICR neonatal mouse was intraperitoneally (i. p.) infected with DBCO-EV71 followed by intramuscularly (i. m.) injection with N_3-Cy5.5 after 6 h infection to achieve in situ bioorthogonal fluorescent conjugation in vivo. (III) Viral dissemination and tissue tropism were dynamically monitored by in vivo imaging from day 1 to day 5 post infection. Two EV71 strains not only caused different symptoms, but also demonstrated distinct dissemination and tissue tropism in the intestine, brain and lung.