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Genome-wide linear B-cell epitopes of enterovirus 71 in a hand, foot and mouth disease (HFMD) population

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ABSTRACT

Background: Enteroviruses cause hand, foot and mouth disease (HFMD). The host B-cells recognize the viral proteins and provoke humoral responses. Deciphering the B-cell responses to the viral epitopes helps diagnosis and vaccine development.

Objectives: The objective of the present study was to investigate for the first time the landscape of genome-wide linear B-cell epitopes of enterovirus 71 in HFMD population.

Study design: The peptides encompassing the entire coding region of EV71 were chemically synthesized and displayed on a microarray. The peptide microarray was used to screen serum samples from an HFMD population, including EV71-, CAV10-, CAV16- and CAV6-infected patients. We identified the dominant epitope-containing-peptides (DECPs) that react with the sera of more than 20% of the HFMD population and the common DECPs that cross-react with the sera from other enteroviruses-infected population.

Results: Ten DECPs reacting with IgM and 9 DECPs reacting with IgG antibodies were identified, of which, 6 IgM and 5 IgG common DECPs cross-reacted with the sera from other enteroviruses. Some DECPs preferentially reacted with IgG or IgM antibodies and some epitope-antibody interactions correlated with the severity of HFMD.

Conclusions: We uncovered the DECPs and the common DECPs among a group of enteroviruses in HFMD population and found that some epitope-antibody reactions were associated with the outcome of HFMD. These data may guide developing vaccines against the enteroviruses and help the diagnosis and prognosis of HFMD.

1. Background

Enteroviruses, such as enterovirus 71 (EV71), coxasckievirus A6 (CAV6) [1], coxasckievirus A16 (CAV16) and coxasckievirus A10 (CAV10) [2] cause Hand, foot and mouth disease (HFMD) that is associated with herpangina, severe neurological complications and even fatalities in infants and young children worldwide [3–9]. EV71 is one of the major agents and can cause severe complications and death in children [3].

EV71 is a member of the family *Picornaviridae*. Its 7.4kb positive-sense genome encodes a single open reading frame, which is translated into a polypeptide. The translated polypeptide is cleaved into at least 11 individual proteins in the following protein order: N′-VP4-VP2-VP3-VP1-2A-2B-2C-3 A-3B-3C-3D. The VP1, VP2, VP3 and VP4 are structural proteins [10,11]. The non-structural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D participate in genome amplification and play important roles in virion assembly [12–14].

The humoral immunity against the viral proteins plays an important role in controlling virus infection. The landscape of the humoral immune responses to the viral proteins in the HFMD patients at a population level may help uncover the dominant humoral immune responses in the HFMD patients. Identifying the dominant epitopes eliciting

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rigorous humoral immune responses in the most HFMD population may help developing vaccines against the enteroviruses.

2. Objectives

Although there are accumulated data about EV71 linear epitopes including neutralizing linear epitopes [15–17] and non-neutralizing epitopes [18–21], the landscape of genome-wide linear B-cell epitopes of enterovirus 71 in HFMD population is lacking. In addition, the cross-reactive antibody responses in the HFMD population are poorly understood.

To investigate the landscape of genome-wide linear B-cell epitopes of enterovirus 71 in HFMD population, by using a genome-wide-EV71 peptide array, we conducted a serum screening in a HFMD population including 447 patients infected with EV71, CAV10, CAV16 and CAV6.

3. Study design

3.1. Serum samples

The sera were collected from 447 patients (278 male, 169 female; age range, 2 months to 12 years old; average age, 2.7 years) diagnosed with HFMD by the Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine from May 2009 to October 2013. The diagnoses are according to the diagnostic criteria defined by the National Health and Family Planning commission of the People's Republic of China (http://www.nhfpc.gov.cn/yzygj/s3593g/201306/6d935c0f43cd4a1fb46f8f71acf8e245.shtml). The serum samples were collected within 17 days (average: 5.9 days) after onset of HFMD. All samples were kept frozen at -20℃ prior to analysis. The patients were further diagnosed by Real time-PCR detection of the nasopharyngeal swabs and stool specimens. Primers and probes are described in Supplementary Table S5. Among them, 353, 26, 35 and 33 patients were diagnosed as infected with EV71, CAV10, CAV16 and CAV6, respectively. This study was approved by the Ethics Committee of Shanghai Xinhua Hospital, and the procedures were carried out in accordance with approved guidelines. Informed consent was obtained from the subjects' parents or guardians.

3.2. EV71 peptides library

The 20-amino acid peptides covering the entire EV71 protein region were chemically synthesized according to the sequence of strain Hubei-XF/CHN/2010 (Total 219 peptides) with the exception that the last peptide contains 13 amino acids (Fig. 1A). The peptides overlap by 10-amino acid with 10-amino acid offset.

3.3. Reagents

15 × 15 mm^2 polymer coated initiator integrated poly (dimethysiloxane) membrane (iPDMS) was purchased from Epitope-Bio (Suzhou, China). N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were both obtained from Medpep (Shanghai, China). EV71 peptides were chemically synthesized by GL Biochem (shanghai, China). Human IgG (H-IgG) and Human IgM (H-IgM) were purchased from DGCS-Bio (Beijing, China) and Millipore Corporation (USA) respectively. Horseradish peroxidase-labeled goat anti-human IgG (HRP-IgG) and IgM (HRP-IgM) was obtained from ZSGB-Bio (Beijing, China) and Sigma (USA) respectively. Peroxidase Conjugate Stabilizer/Diluent and Super Signal ELISA Femto Maximum Sensitivity Substrate for Chemiluminescence were obtained from Thermo Fischer (USA).

3.4. Peptide microarray and serum screening

Preparation of peptide microarray and serum screening were essentially as previously reported [22]. Peptides were printed onto the activated iPDMS membranes by Smart 48 (Capitalbio, Beijing, China) to form 9 × 9 × 4 microarrays (Fig. 1B). In each sub-array there are four positive controls printed with H-IgG or H-IgM at the concentration of 100 μg/ml and one negative control printed with printing buffer (Fig. 1B, left). Two-microliter serum was diluted and incubated with peptide microarray and analyzed as previously reported.

3.5. Data analysis

The chemiluminescence intensity of each dot was converted to signal-to-noise ratio (SNR) by subtracting the background intensity averaged from the intensity from eight blank dots and calculated as: (signal intensity − background intensity)/(background intensity). SNR equal or higher than 2 is considered as positive. The heat map was prepared with Excel. To identify the common epitopes among EV71, CAV16, CAV10 and CAV6 the "Three mode analysis" was conducted by R version 3.1.0 (R Foundation for Statistical computing, Vienna, Austria, ISBN 3-900051-07-0, URLhttp://www.R-project.org) using "samr" package as reported [22]. Briefly, each three successively overlapped-epitopes were analyzed. A peptide with SNR < 2 was defined as 0 and a peptide with SNR ≥ 2 was defined as 1. The modes 010, 011/110 and 111 indicate a seropositive to the middle peptide in the three successively overlapped-epitopes.

3.6. Epitopes blocking test

The peptides were first dissolved with 30% acetonitrile solution (v/v, in pure water) to 2 mg/ml and then diluted to 0.5 mg/ml. The sera were diluted at 1:100 and mixed with equal volume of the diluted peptide solution and incubated on a shaker for 30 min at room temperature. The mixed peptide and serum were then used in the serum screening by peptide microarrays as described above.

3.7. Statistical analysis

Student's *t*-test and repeated measures of ANOVA were used for analysis between the before and after epitopes blocking test. Specific tests are described in the figure legends.

4. Results

4.1. Serum screening of a HFMD population by a genome-wide peptide microarray of EV71

A total of 219 overlapped 20-aa peptides (Supplementary Table S1) spanning the whole coding region of EV71 were chemically synthesized and printed onto a microarray chip (Fig. 1B, left). The peptide microarrays were incubated with the sera from HFMD patients who were diagnosed as infecgted by EV71, CAV16, CAV10 and CAV6. The bound IgG and IgM were detected by HRP-conjugated secondary antibody. Representative negative and positive results are shown in Fig. 1B. To verify the specificity of the epitope-antibody reactions, peptides P060 and P061 were used in epitope-blocking experiment. After peptide blocking, the binding of P060 to No. 349 serum sample and P061 to No. 123 serum sample was significantly decreased as evidenced by the reduction of SNR (Fig. 1C), suggesting the specificity of the epitope-antibody binding.

Then, using this peptide microarray, we carried out serum screening for a total of 447 serum samples, including the sera from 353 EV71-, 26 CAV10-, 35 CAV16- and 33 CAV6-infected patients. Each serum sample was incubated with two peptide microarrays. In summary, a total of 343 and 353 peptide microarrays displayed valid signals for EV71 IgM and IgG antibodies, respectively. The peptide number with valid signals per peptide microarray in the IgM antibody screening ranges from 0 to 62, with an average of 11; and in the IgG antibody screening it ranges from 0 to 33, with an average of 7 (Fig. 1D).