Original Article

Genetic Changes of Coxsackievirus A16 and Enterovirus 71 Isolated from Hand, Foot, and Mouth Disease Patients in Toyama, Japan between 1981 and 2007

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SUMMARY: We characterized the genetic diversity of the complete VP1 region of coxsackievirus A16 (CA16) and enterovirus 71 (EV71) isolated from patients with hand, foot, and mouth disease in Toyama from 1981 to 2007 to evaluate the relationship between epidemics and genetic changes. The predominant genogroups of CA16 changed from B to C in 1995-1998, and genogroup C further changed from subgenogroup C1 to C2 around 2002, and to C3 in 2005-2007. The subgenogroups of the EV71 isolates were classified into B1, B4, C1, and C3 in 1983-1994, and into C4 in 1997-2006. However, changes of the amino acid sequences of the VP1 regions of CA16 were restored, and those of the EV71 isolates were not observed among the same subgenogroups during this survey period, indicating that the prevalence that occurred at intervals of several years seemed to depend on an accumulating number of immunologically naive children, not viral antigenic changes.

INTRODUCTION

Hand, foot, and mouth disease (HFMD) is a common febrile illness of children characterized by lesions on the skin and oral mucosa. HFMD occurs mainly in the summer in Japan, as is usually seen with enterovirus-related diseases (1,2). Coxsackievirus A16 (CA16) and enterovirus 71 (EV71) are the major causative agents of HFMD. EV71 infection is occasionally associated with severe neurological diseases such as aseptic meningitis, encephalitis, and acute flaccid paralysis (3-5). Several fatal cases have also been reported (6-9). In contrast, CA16-associated HFMD has a milder outcome (1,2,10).

EV71 has been classified into three genogroups (A, B, and C), and further divided into many subgenogroups, genotypes or lineages (B1-5, C1-5) based on the complete VP1 gene sequences (8,11-20). The VP1 region is considered to be variable and to play an important role in characterizing the antigenicity (21). Although few studies on genetic analysis have been reported on CA16 compared to EV71, CA16 has been classified into three genogroups (A, B, and C) according to the phylogenetic analysis of the VP4 or VP1 gene (22-24). While CA16 and EV71 usually co-circulate, EV71 has been predominant every 3 years since 1994 in Japan (1,2). Whereas genogroups or subgenogroups of CA16 and EV71 have been used to understand the origins of viral isolates and outbreaks (11,16), the influence of genetic variations in the VP1 region or prevalence has not been precisely investigated.

In this study, we characterized the genetic diversity of the complete VP1 region of CA16 and EV71 isolates from 1981 to 2007 to evaluate the relationship between epidemics and genetic changes.

MATERIALS AND METHODS

Patients with HFMD: The weekly numbers of patients with HFMD were reported from 21 and 29 pediatric clinics in Toyama Prefecture during 1981-1998 and 1999-2007, respectively, by sentinel surveillance. The clinical specimens of 203 patients from pediatric clinics were used for isolation of viruses. They included 185 nasopharyngeal swabs, 195 stool samples, and 99 rash swabs. Fourteen cerebrospinal fluid samples were also collected from patients with aseptic meningitis or encephalitis complication.

Viral isolation and identification: The specimens were inoculated on appropriate tissue culture cells (Vero, MA-104, LLC-MK2, RD-18S) for isolation of viruses. They included 185 nasopharyngeal swabs, 195 stool samples, and 99 rash swabs. Randomly selected isolates (40 of 177 and 26 of 143, CA16 and EV71 strains, respectively) were used for further genetic analyses.

Phylogenetic analysis of CA16 and EV71: Viral genomic RNA was extracted from 140 μL of the culture fluid of cells that appeared cytopathic using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized for 1 h at 42°C by SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA) with a random hexamer according to the manufacturer’s procedures. Polymerase chain reaction...
(PCR) was performed using an Ex Taq (TaKaRa, Otsu, Japan) to amplify the VP2/VP1/2A region of the viral genome (11, 23, 25). For CA16, we used the primers CA16F-2030 (sense 5’-AGG TAC TAC ACC CAG TGG TCA G-3’) and CA16R-3400 (antisense 5’-GCA AGG TGC CGA TTC ACT ACC CT-3’), which amplify 1371 bp corresponding to nucleotides (nt) 2030 to 3400 of G-10/South Africa/51, GenBank accession no. U05876 (23). For EV71, we used the primers 159 (sense 5’-ACY ATG AAA YTG TGC AAG G-3’, 2385-2403), 162 (antisense 5’-CCR GTA GGK GTR CAC GCR AC-3’, 2869-2850), 189 (sense 5’-CAR GCI GCI GAR ACI GGN GC-3’, 2612-2631), and 011 (antisense 5’-GCI CCI GAY TGI TGI CCR AA-3’, 3408-3389), which amplify a total of 1024 bp (nt 2385 to 3408 of BrCr/USA/70, U22521) (11, 25).

To determine the sequences of these viruses, the PCR products were directly applied for sequence analysis using an ABI Prism BigDye Terminators v3.1 cycle sequencing kit and an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, Calif., USA). The genogroups and subgenogroups were determined by comparing sequences with those of reference strains in GenBank (Table 1). The genetic relationship between the strains isolated in this study (Toyama strains) and the reference strains (8, 11-13, 15-21, 23, 24, 26-29) were analyzed by MEGA 3.1 software (30), using the genomic sequences of the complete VP1 region of 891 bp (nt 2446 to 3336 for CA16, G-10/South Africa/51; 2439 to 3329 for EV71, BrCr/USA/70). A phylogenetic tree was constructed by the neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method (31). A bootstrapping test was performed 1,000 times.

**Nucleotide sequence accession numbers**: Nucleotide sequences determined in this study were deposited in GenBank under accession no. AB465366 to AB465431.

**RESULTS**

**Causative viruses of patients with HFMD**: To determine the annual prevalence of HFMD, the numbers of patients were counted weekly at pediatric clinics from 1981-2007 (Fig. 1). As for monthly prevalence, HFMD occurred in either June to August, as typically observed in 1991 and 1997, or in August to November, as seen in 1988 and 1998. Remarkable prevalence was not observed in 1986, 1987, 1989, 1990, 1992, 1994, 1996, and 1999. The endemic was the largest in 1995, and comparatively large in 1984, 1988, 1991, 1997, 1998, and 2003, indicating that large outbreaks of HFMD occurred at an interval of several years.

The numbers of patients with HFMD, encephalitis, and aseptic meningitis from whom viruses were isolated are summarized in the lower part of Fig. 1. The numbers of patients with HFMD from whom CA16 and EV71 were detected dominated at 44.1% (79/177) and 51.4% (92/177), respectively. While CA16 was mainly isolated in 1995, 2000, and 2002, EV71 was dominantly isolated in 1983, 1991, 1993, 1997, 2003, and 2006. Therefore, the size of the annual preva-
lence did not seem to correlate with the viral serotypes.

**Phylogenetic analysis of CA16 and EV71 isolates:** To examine the genogroups of CA16 and EV71, sequences of the complete VP1 region of the CA16 and EV71 isolates were determined, and phylogenetic analysis was conducted.

Genogroups of CA16 detected in 1981-1995 and 1998-2007 were classified into two genogroups, B and C (23), respectively (Fig. 2). The isolates of genogroup C were further divided into three lineages, tentatively named subgenogroups C1, C2, and C3 in this paper. The representative strains of each cluster were 260/Toyama/2002, 261/Toyama/2002, and 459/Toyama/2007, respectively. Differences of nucleotide sequences (total 891 nucleotides) among C1, C2, and C3 ranged from 3.7 to 8.0%. Remarkable differences in sampling areas, symptoms and ages of the subjects between C1 and C2 in 2002 were not observed, except the time-detected isolates when the former were in June to the beginning of July, and the latter at the end of July. Recent isolates in 2005-2007 were classified into subgenogroup C3, including 355/Toyama/2005, which was detected from a patient with aseptic meningitis. The scale bar denotes the genetic distance (nucleotide substitution per site).

**Fig. 2.** Phylogenetic tree for CA16 using 891 nt, the complete VP1 region by the neighbor-joining method with isolates in Toyama and reference strain. Filled circles (○) and squares (●) indicate isolates in Toyama derived from patients with HFMD and aseptic meningitis, respectively. Isolates and reference strains were represented as strain's name_accession number. The scale bar denotes the genetic distance (nucleotide substitution per site).
malignancy complications.

The EV71 isolates were classified into five subgenogroups of B1, B4, C1, C3, and C4 (Fig. 3) (8,11,13,16,17,23). The isolates of EV71 in 1983, 1989, and 1994 were classified into B1, C1, and C3, respectively. 185/Toyama/2000 detected from patients with encephalitis belonged to subgenogroup B4. The isolates from patients with HFMD in 1997, 2003, and 2006 including 763/Toyama/1997 and 818/Toyama/1997 detected from patients with aseptic meningitis were classified into subgenogroup C4.

It thus appeared that the CA16 isolated in Toyama had predominantly changed from genogroup B to C in 1995 - 1998, and the genogroup C changed from C1 to C2 around 2002, and to C3 in 2005 - 2007. The subgenogroups of the EV71 isolates were classified into B1, B4, C1, C3 in 1983 - 1994, and C4 in 1997 - 2006.

Sequence diversities of CA16 and EV71 isolates: We compared nucleotide and amino acid sequences of the VP1 region to investigate diversities among Toyama strains. The differences in nucleotide sequences (total 891 nucleotides) of CA16 between genogroups B and C ranged from 5.8 to 11.0%, and those in the same subgenogroups of C1, C2, and C3 were 3.40% to 3.55%.

Fig. 3. Phylogenetic tree for EV71 using 891 nt, the complete VP1 region by the neighbor-joining method with isolates in Toyama and reference strain. Filled circles (●), triangle (▲), and squares (■) indicate isolates in Toyama derived from patients with HFMD, encephalitis, and aseptic meningitis, respectively. Isolates and reference strains were represented as strain’s name _accession number. The scale bar denotes the genetic distance (nucleotide substitution per site).
DISCUSSION

In this study, we summarized the prevalence of HFMD in Toyama from 1981 to 2007. Large outbreaks of HFMD occurred at intervals of several years, which is consistent with the report by National Epidemiological Surveillance of Infectious Diseases in Japan showing that CA16 and EV71 are detected every year, and EV71 has been predominant every 3 years since 1994 (1,2). Since there are some patients with HFMD in winter, CA16 and EV71 seem to exist year round in the community in Toyama.

As for recent CA16 isolates in Asia, genogroup B was detected in 1999-2000, 1998-2000, and 1987-1998 and genogroup C was isolated in 1999-2004, 1997-2005, and 1995-2003 in China (23), Malaysia (24), and Fukushima (22), respectively. Moreover, the strains detected in Hiroshima from 2000-2001 and the strain of Aichi in 2005 were respectively classified as subgenogroups C1 and C2. As for EV71, while recent isolates in Toyama were classified as C4, C4 was also found in Yamagata in 2003 (16), China in 1998-2004 (23), and Vietnam in 2005 (20). It thus appeared that recent genogroups of CA16 and EV71 in Toyama were similar to those detected in these countries.

There are several factors influencing the large prevalence of HFMD occurring at intervals of several years. HFMD is prevalent mainly in young children aged 0 to 6 years old. A seroepidemiological study by Hagiwara et al. has shown that whether EV71 causes an endemic or an epidemic depends on the seropositive rate among children (32,33). They reported that the age distribution possessing the antibody against EV71 gradually shifted to a higher age until the next prevalence occurred. Therefore, once children are immunized by viruses, it may take several years to have another sensitive generation.

We found that there were only small amino acid variations of the VP1 region that correlate to the serotype of enteroviruses and play an important role in characterizing antigenicity (21) among the Toyama strains, since the variations were restored in CA16, and were not observed among the same subgenogroups of EV71. Only one variation of E or D at 164 between genogroups B and C of EV71 was detected in neutralization epitopes of the VP1 region (amino acid residues 162-177 and 208-222) (34), and no variation in the extended BC loop (residues 92-107), which is predicted to be immunogenic (35). These facts suggest that the VP1 region has not easily altered its antigenicity to escape immune pressure. Consistently, antibodies against EV71 have reportedly
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