Isolation and characterization of a novel recombinant human adenovirus species D

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A novel recombinant human adenovirus (HAdV) species D was isolated from the stool of a pharyngitis patient in Japan and genetic characterization was performed by sequencing variable regions between HAdV types. The nucleotide sequences of the penton base gene and loops 1 and 2 in the hexon gene showed 100% identity with that of the recently identified HAdV-56. Although we observed greatest identity for the entire hexon gene sequence with that of HAdV-56, we noted even greater similarity between the partial nucleotide sequence of the conserved region 4 and that of HAdV-37. Furthermore, the fibre gene and early region 3 sequences were completely identical to that of HAdV-37. These results suggest that the strain is a novel adenovirus related to HAdV-37 and HAdV-56.

INTRODUCTION

Human adenoviruses (HAdVs) are linear double-stranded DNA viruses belonging to the genus Mastadenovirus of the family Adenoviridae. So far, greater than 60 types of HAdV have been identified and grouped into seven species, named HAdV-A through HAdV-G (Jones et al., 2007; Aoki et al., 2008; Ishiko et al., 2008; Walsh et al., 2009, 2010, 2011; Robinson et al., 2011a; Kaneko et al., 2011b; Liu et al., 2011). HAdVs cause a range of illnesses, including pharyngoconjunctival fever (PCF), which is characterized by fever, pharyngitis and conjunctivitis. According to the national surveillance of HAdVs in Japan (Infectious Disease Surveillance Center, 2011), most HAdVs detected in PCF patients belong to the species HAdV-B and HAdV-C, with the most common type being HAdV-3 (HAdV-B) (Fujimoto et al., 2008; Enomoto et al., 2010). However, several types belonging to the species HAdV-D, which are the major cause of keratoconjunctivitis (including types HAdV-8, HAdV-19, HAdV-37, HAdV-53 and HAdV-54) (Ishiko & Aoki, 2009; Kaneko et al., 2011a; Nakamura et al., 2012), were rarely observed in the PCF patients under our surveillance, and instead were mainly detected in keratoconjunctivitis patients.

Recombination, the process by which circulating viruses exchange genes (preferentially within the same species), is important for HAdV evolution (Lukashev et al., 2008). Although serum neutralization tests with type-specific antisera are traditionally used for HAdV typing, we chose to perform genetic sequence analysis because it proved more efficient for type identification of novel HAdVs (Madisch et al., 2005; Robinson et al., 2011b). The main type-specific epitopes of HAdVs are located in loops 1 and 2 of the hexon protein (β determinant). The hexon protein is divided into four conserved (C1–C4) and three variable (V1–V3) regions and the variable regions in the hexon loops are type-specific (Ebner et al., 2005). In addition to the hexon gene, genetic analyses revealed that the penton base gene, fibre gene and the early region 3 (E3) are also type-specific (Kaneko et al., 2009; Robinson et al., 2011b). Sequence analysis of these genes has led to the recent identification of novel types of recombinant HAdV-D (Walsh et al., 2009; Robinson et al., 2011a; Kaneko et al., 2011b, 2011c; Liu et al., 2011).

Here, we genetically characterized a recombinant HAdV-D strain using combined nucleotide sequence analysis of the genes mentioned above and compared these sequences with those of other HAdV-D types. The emergence of a novel recombinant HAdV suggests that these recombination events occur relatively frequently, demonstrating that continuous surveillance is important for the identification of novel adenoviruses.

METHODS

Virus strain. A stool sample was obtained from a feverish infant patient with pharyngitis who was treated in July 2011 in Osaka, Japan, during our routine surveillance. For virus isolation, 200 μl of

Abbreviations: HAdV, human adenovirus; PCF, pharyngoconjunctival fever.

The Gen Bank (DDBJ) accession numbers for penton base, hexon, E3 and fibre gene sequences of the adenovirus strain 230272/Osaka/2011 are AB665249, AB665252, AB665253 and AB665254, respectively.

A supplementary table is available with the online version of this paper.
10 % (w/v) stool in Hank’s solution was inoculated into HEp-2 cells with 1 ml of Eagle’s minimum essential medium (Sigma) in a 24-well microplate and cultured at 34 °C. Samples (200 μl) were subcultured once a week. Typical cytopathic effect was observed in the second subculture and the isolated strain was named 230272/Osaka/2011.

**DNA sequencing.** Viral DNA extraction and nucleotide sequence analysis were conducted as previously reported (Hiroi et al., 2011). Briefly, PCR was carried out in a 25 μl reaction volume using puRe Taq Ready-To-Go PCR Beads (GE Healthcare) with 1 μl of extracted DNA using specific sets of primers and PCR protocols as described previously, targeting the C4 region (Miura-Ochiai et al., 2007), loops 1 and 2 (Madisch et al., 2005) in the hexon gene and the fibre gene (Xu et al., 2000). To analyse the penton base gene, the entire hexon gene and the E3 region, primers specific to HAdV-37 and HAdV-56 were designed (Table S1, available in JMM Online) and amplification was carried out as follows: denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s and primer extension at 72 °C for 90 s; with a final extension at 72 °C for 10 min. The PCR primers were used for the sequencing reactions and sequencing primers specific for HAdV-37 were also used to sequence the fibre gene and the E3 region. Nucleotide sequences were determined using an Applied Biosystems 3130 genetic analyser. The nucleotide sequences on both strands of the amplicons were sequenced at a mean of 3-fold coverage. Nucleotide positions are analyser. The nucleotide sequences on both strands of the amplicons sequences were determined using an Applied Biosystems 3130 genetic analyser. The nucleotide sequences on both strands of the amplicons sequences were determined using an Applied Biosystems 3130 genetic analyser.

**Phylogenetic trees.** Phylogenetic analyses were conducted using ClustalX version 3.5.1 (S. C. Ray, Johns Hopkins University) (Lole et al., 1999). Phylogenetic trees were viewed using Tree Explorer software in the molecular evolution genetic analysis (MEGA) software version 5 (Tamura et al., 2011). Sequence identities between strain 230272/Osaka/2011 and that of other types belonging to the species HAdV-D were calculated using MEGA software version 7 (Software Development). Similarity plot and bootscan analyses of the hexon gene were conducted using the default settings (window size=200, step size=20), Kimura’s two-parameter method, T=1.0) of SimPlot version 3.5.1 (S. C. Ray, Johns Hopkins University) (Lole et al., 1999).

**Nucleotide sequence accession numbers.** The sequences generated in this study were submitted to DDBJ and assigned the accession numbers AB665249 (penton base), AB665252 (hexon), AB665253 (E3) and AB665254 (fibre). The following HAdV hexon sequences were used in the analysis (GenBank accession no. in parentheses): HAdV-10 (AB330091), HAdV-13 (AB330094), HAdV-20 (AB330101), HAdV-23 (AB330104), HAdV-24 (AB330105), HAdV-25 (AB330106), HAdV-27 (AB330108), HAdV-30 (AB330111), HAdV-32 (AB330113), HAdV-33 (AB330114), HAdV-38 (AB330119), HAdV-39 (AB330120), HAdV-42 (AB330123), HAdV-43 (AB330124), HAdV-44 (AB330125), HAdV-45 (AB330126), HAdV-47 (AB330128), HAdV-51 (AB330132). The following HAdV fibre sequence was used in the analysis: HAdV-10 (AB69368). The following HAdV complete genome sequences were used in the analysis: HAdV-8 (AB448577), HAdV-9 (AB544866), HAdV-15 (AB562586), HAdV-17 (HQ910407), HAdV-19 (AB448771), HAdV-22 (FHJ04771), HAdV-26 (EF153474), HAdV-28 (EF824826), HAdV-29 (AB562587), HAdV-36 (DQ384080), HAdV-37 (AB448778), HAdV-46 (AY875648), HAdV-48 (EF153473), HAdV-49 (DQ593829), HAdV-53 (AB605244), HAdV-54 (AB333801), HAdV-56 (AB562588).

**RESULTS AND DISCUSSION**

We genetically analysed the penton base, hexon and fibre genes, as well as the E3 region of strain 230272/Osaka/2011 in order to achieve type classification. The penton base gene of the strain was nearly identical to HAdV-56 as evidenced by phylogenetic analysis (Fig. 1a). Although the loops 1 and 2 in the hexon gene of strain 230272/Osaka/2011 were nearly identical to HAdV-56 and the greatest identity for the entire hexon gene was noted with that of HAdV-56, the most significant identity for any particular sequence was noted between the partial nucleotide sequence (350 bp) of the C4 region of strain 230272/Osaka/2011 and that of HAdV-37 and HAdV-53 (Fig. 1b–e). Both the fibre gene and the E3 region of strain 230272/Osaka/2011 were located in the same cluster as that of HAdV-37 (Fig. 1f, g). While HAdV-37 and HAdV-53 have strong relationships in several regions including the second half of the hexon protein (Walsh et al., 2009), analysis of the genes displayed no such similarity to HAdV-53, except within the partial nucleotide sequence of the C4 region (Fig. 1a–g). Taken together, these findings suggest that the strain is a novel recombinant type of the species HAdV-D.

The percentage nucleotide similarities of strain 230272/Osaka/2011 genes to those of other HAdV-D types showed that the penton base gene was 100 % identical to that of HAdV-56, and both the fibre gene and the E3 region were 100 % identical to that of HAdV-37. While the entire hexon gene showed greatest similarity (99.2 %) to that of HAdV-56, the loops 1 and 2 of the hexon gene were identical (100 %) to those of HAdV-56. The partial nucleotide sequence of the C4 region within the hexon gene showed greatest similarity (98.6 %) to that of HAdV-37 and had 98.0 % similarity to HAdV-56. Furthermore, the amino acid sequence of the deduced hexon protein showed greatest similarity (99.9 %) to that of HAdV-56. The results showed that the e determinant of the novel virus was identical with that of HAdV-56, suggesting that the virus can potentially react with HAdV-15-specific antiserum in a manner similar to that previously observed with HAdV-56 (Kaneko et al., 2011b). However, as we were unable to obtain the HAdV-15- and HAdV-56-specific antiserum, we could not verify the serological features of this recombinant adenovirus, which led us to conduct our type-classification through nucleotide sequence analysis instead.

We then performed a similarity plot and bootscan analysis of the hexon gene in order to compare it with that of the HAdV-D types circulating in Japan. The full hexon gene of strain 230272/Osaka/2011 was 2826 bp long, with the first 1520 bp (including both loops 1 and 2) showing 100 % similarity to that of HAdV-56 (Fig. 2a). The analysis showed that the nucleotide sequence of the C4 region, downstream of loop 2, had similarity to the other HAdV-D types, in particular the corresponding regions of HAdV-37 and HAdV-56 (Fig. 2a, b). Accordingly, the genes of strain 230272/Osaka/2011 appeared to be related to those of either HAdV-37 or HAdV-56. Although co-infection is required to
**Fig. 1.** Phylogenetic analysis of strain 230272/Osaka/2011 penton base gene (a), loop 1 of the hexon gene (b), loop 2 of the hexon gene (c), partial hexon gene in C4 region (d), entire hexon gene (e), fibre gene (f) and E3 region (g).
Fig. 2. Simplot analysis (a) and bootscan (b) of the entire hexon gene of strain 230272/Osaka/2011 compared with other HAdV-D types.
induce natural viral recombination, we are unsure of whether such co-infection occurred in the present patient or if he was infected by the novel adenovirus. The strain was isolated from the patient’s stool but, as we did not obtain any other specimens, we were unable to verify infections of the pharynx or conjunctiva. The tropism of the novel adenovirus is unknown; however, our results showed that the fibre and penton base genes, which mediate attachment and internalization of adenoviruses (Smith et al., 2010; Robinson et al., 2011b), were completely identical to those of HAdV-37 and HAdV-56. As both HAdV-37 and HAdV-56 have been shown to cause keratoconjunctivitis (Kaneko et al., 2009, 2011b; Robinson et al., 2011a), this implies that the novel adenovirus may cause not only pharyngitis but also keratoconjunctivitis.

In this study, we reported the isolation and analysis of a novel recombinant HAdV-D strain related to HAdV-37 and HAdV-56. Although the novel adenovirus has not yet been isolated from other patients, and although the recombination events leading to its appearance remain unclear, the virus may have the potential to cause an epidemic. Further genetic analysis of the novel adenovirus is required to clarify its recombination properties.

REFERENCES


