Recombination analysis of intermediate human adenovirus type 53 in Japan by complete genome sequence

Hisatoshi Kaneko,1 Koki Aoki,2 Susumu Ishida,2 Shigeaki Ohno,3 Nobuyoshi Kitaichi,3,4 Hiroaki Ishiko,5 Tsuguto Fujimoto,6 Yoshifumi Ikeda,7 Masako Nakamura,8 Gabriel Gonzalez,9 Kanako O. Koyanagi,9 Hidemi Watanabe9 and Tatsuo Suzutani1

Correspondence
Hisatoshi Kaneko
h-kane@chive.ocn.ne.jp

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1Department of Microbiology, Fukushima Medical University School of Medicine, Fukushima, Japan
2Department of Ophthalmology, Hokkaido University Graduate School of Medicine, Sapporo, Japan
3Department of Ocular Inflammation and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan
4Department of Ophthalmology, Health Sciences University of Hokkaido, Sapporo, Japan
5Host Defense Laboratory, Mitsubishi Chemical Medience Corporation, Tokyo, Japan
6National Institute of Infectious Diseases, Tokyo, Japan
7Hiroshima City Institute of Public Health, Hiroshima, Japan
8Fukui Prefectural Institute of Public Health and Environmental Science, Fukui, Japan
9Laboratory of Genome Sciences, Research Groups of Bioinformatics, Division of Bioengineering and Bioinformatics, Hokkaido University Graduate School of Information Science and Technology, Sapporo, Japan

Human adenovirus type 53 (HAdV-53) has commonly been detected in samples from epidemic keratoconjunctivitis (EKC) patients in Japan since 1996. HAdV-53 is an intermediate virus, containing hexon-chimeric, penton base and fiber structures similar to HAdV-22 and -37, HAdV-37 and HAdV-8, respectively. HAdV-53-like intermediate strains were first isolated from EKC samples in Japan in the 1980s. Here, the complete genome sequences of three such HAdV-53-like intermediate strains (870006C, 880249C and 890357C) and four HAdV-53 strains were determined, and their relationships were analysed. The seven HAdV strains were classified into three groups, 870006C/880249C, 890357C and the four HAdV-53 strains, on the basis of phylogenetic analyses of the partial and complete genome sequences. HAdV strains within the same group showed the highest nucleotide identities (99.87–100.00 %). Like HAdV-53, the hexon loop 1 and 2 regions of 870006C, 880249C and 890357C showed the highest identity with HAdV-22. However, these strains did not show a hexon-chimeric structure similar to HAdV-22 and -37, or a penton base similar to HAdV-37. The fiber genes of 870006C and 880249C were identical to that of HAdV-37, but not HAdV-8. Thus, the three intermediate HAdVs isolated in the 1980s were similar to each other but not to HAdV-53. The recombination breakpoints were inferred by the Recombination Detection Program (RDP) using whole-genome sequences of these seven HAdV and of 12 HAdV-D strains from GenBank. HAdV-53 may have evolved from intermediate HAdVs circulating in the 1980s, and from HAdV-8, -22 and -37, by recombination of sections cut at the putative breakpoints.

INTRODUCTION

Adenoviruses are non-enveloped, dsDNA viruses with icosahedral capsids (Swenson et al., 2003). Human adenoviruses (HAdVs) belong to the genus Mastadenovirus of the family Adenoviridae and infect billions of people
HAdV-53 is an intermediate virus formed by recombination among different HAdV types. The hexon gene in HAdV-53 shows a chimeric structure, and the loop region, which contains the neutralization epitope, and the 3′-conserved region 4 (C4) show the highest nucleotide identity with HAdV-22 and -37, respectively. HAdV-53 is also neutralized by HAdV-22 antisera (Walsh et al., 2009; Aoki et al., 2011). In contrast, the penton base and fiber genes in HAdV-53 show the highest identity with HAdV-37 and -8, respectively. HAdV types are traditionally numbered by neutralization test (NT). However, according to analysis of the complete genome sequences, HAdV-53 is identical to HAdV-22 in only the hexon loop regions, with other regions differing in sequence from those of HAdV-22. HAdV-53 was therefore numbered as a new HAdV type on the basis of recombination and bioinformatics analyses of the complete genome sequence. To date, HAdV-53 has only been isolated from EKC patients in Japan and Germany (Engelmann et al., 2006; Aoki et al., 2008; Walsh et al., 2009). In Japan, HAdV-53 was reported in samples from EKC patients in different cities between 2003 and 2004 (Aoki et al., 2008). We redetermined the HAdV type in a number of conjunctival samples obtained from EKC patients before 2000 in Japan (Kaneko et al., 2011a). Our results showed that HAdV-53 had already been isolated from EKC patients in 1996, although most HAdV-53 isolates were mistyped as other HAdV types, such as HAdV-8, -22 or -37, on the basis of NT or molecular analysis of the partial genome. HAdV-53 has been isolated in a number of cities since 1996 and has recently become the third most commonly detected strain in EKC patients in Japan behind HAdV-37 and -54 (Aoki et al., 2008; Kaneko et al., 2011a).

A remarkable characteristic of HAdV-53 is that only the loop region of the hexon gene shows identity to HAdV-22, which rarely causes EKC. In the 1980s, two intermediate recombinant HAdVs were isolated from EKC patients in Hiroshima, in the western part of Japan, and reported as HAdV-22/H10,19,37 and -22/H8,9 on the basis of an NT and haemagglutination inhibition (HI) test (Noda et al., 1991). These isolates were neutralized by HAdV-22 antisera and, as with HAdV-53, contained regions similar to those of HAdV-8 or -37 in the genome. Thus, these intermediate HAdVs isolated in the 1980s were similar to HAdV-53.

In this study, we determined the complete genome sequences of three HAdV-53-like intermediate HAdVs isolated in the 1980s and four HAdV-53 strains currently in circulation in Japan. Through recombination-event searches and bioinformatics analyses using complete genome sequences, the genomic relationships among these HAdVs were evaluated.

Results

General properties and organization of the complete genome of the seven intermediate HAdV strains

The genome size and G+C content of the seven HAdV strains are shown in Table 1. The genome size and percentage G+C content were found to be almost identical among the seven strains. The genome organization of the seven strains was determined by analysis of the ORFs and alignment of their nucleotide sequences. The layout of the early and late regions in the complete sequences of these strains is shown in Fig. 1(a). The number of genes and their genome organization for each of the seven strains were identical to those of recently determined HAdV-D sequences (Robinson et al., 2008; Kaneko et al., 2009, 2011b; Walsh et al., 2009), although the size of some ORFs varied slightly between strains or types (data not shown).

Phylogenetic analysis of identities among the complete nucleotide sequences of the seven intermediate HAdV and the other HAdV-D strains

Phylogenetic analyses of the complete genome sequences were carried out using the seven HAdVs described above and 12 fully sequenced HAdV-D strains from GenBank. The seven recombinant HAdVs in this study formed a single cluster and did not form a cluster with any other HAdV-D type (Fig. 2a). Three subclusters were formed within the single cluster. The 870006C and 880249C strains showed the highest identity (99.89 %) and formed a single subcluster. Similarly, the four HAdV-53 strains also showed the highest identities (99.87–100.00 %) with each other and formed a second subcluster. The 890357C strain alone formed a separate subcluster. Thus, the seven strains were classified into three groups containing 870006C/880249C, 890357C and the four HAdV-53 strains, respectively. The nucleotide identities among HAdV strains in the different groups were 96.99–98.55 %. The identities of the complete genome sequences among the seven recombinant HAdVs and other HAdV-D strains were even lower at 91.63–94.79 %.
Table 1. Genome size, G+C content and HAdV type in five regions of the seven intermediate HAdVs

<table>
<thead>
<tr>
<th>Strain/place/year</th>
<th>Genome size</th>
<th>G+C content (mol%)</th>
<th>HAdV type detected by phylogenetic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Penton base</td>
</tr>
<tr>
<td>870006C/Hiroshima/1987</td>
<td>35048</td>
<td>56.76</td>
<td>ND</td>
</tr>
<tr>
<td>880249C/Hiroshima/1988</td>
<td>35045</td>
<td>56.75</td>
<td>ND</td>
</tr>
<tr>
<td>890357C/Hiroshima/1989</td>
<td>35118</td>
<td>56.44</td>
<td>ND</td>
</tr>
<tr>
<td>960528C/Hiroshima/1996 (HAdV-53)</td>
<td>35137</td>
<td>56.39</td>
<td>HAdV-37</td>
</tr>
<tr>
<td>C075/Matsuyama/2003 (HAdV-53)</td>
<td>35137</td>
<td>56.37</td>
<td>HAdV-37</td>
</tr>
<tr>
<td>FS161/Fukui/2004 (HAdV-53)</td>
<td>35116</td>
<td>56.76</td>
<td>HAdV-37</td>
</tr>
<tr>
<td>FS165/Fukui/2004 (HAdV-53)</td>
<td>35116</td>
<td>56.76</td>
<td>HAdV-37</td>
</tr>
</tbody>
</table>

Phylogenetic analysis of the hexon, penton base, fiber gene and E3 region sequences

The HAdV genome contains four highly variable regions, the hexon, penton base, fiber genes and the E3 region (Kaneko et al., 2009); phylogenetic analyses of these regions were carried out using the seven HAdV strains in the study as well as other HAdV-D types. The entire hexon, penton base and fiber genes and the E3 region sequences of 32, 14, 15 and 13 HAdV-D types, respectively, were obtained from GenBank and used for the analyses. In the hexon gene, phylogenetic analyses of loops 1 and 2 and C4 were carried out.

Phylogenetic analysis of loops 1 and 2 in the hexon gene showed that all seven HAdV strains, including HAdV-53, in this study formed a cluster with HAdV-22 (Fig. 2b, c). In contrast, phylogenetic analysis of C4 in the hexon gene (Madisch et al., 2005) revealed that the four HAdV-53 strains formed a monophyletic cluster with HAdV-37, whereas the other three strains did not form a monophyletic cluster with any HAdV-D strain (Fig. 2d). Thus, the three recombinant viruses isolated in the 1980s, 870006C, 880249C and 890357C, did not show a hexon-chimeric structure of HAdV-22 and -37.

Phylogenetic analysis of the penton base gene revealed that the four HAdV-53 strains formed a monophyletic cluster with HAdV-37, whereas the other three strains did not form a monophyletic cluster with any HAdV-D strain (Fig. 2e). Phylogenetic analysis of the E3 region revealed that all seven strains formed a single cluster but did not form a monophyletic cluster with any other HAdV-D strain (Fig. 2f). Phylogenetic analysis of the fiber gene showed that all four HAdV-53 strains, as well as 890357C, formed a cluster with HAdV-8, and that the other two HAdV strains, 870006C and 880249C, formed a cluster with HAdV-37 (Fig. 2g). The HAdV type of each region in the seven recombinant HAdV strains is summarized in Table 1.

Similarity plot analysis of the complete genome sequences of HAdV-8, -22 and -37 and the seven intermediate HAdV strains

Similarity plots were performed on the seven HAdV strains in this study, which were classified into three groups (870006C/880249C, 890357C and HAdV-53 strains) by phylogenetic analysis of complete genome sequence (Fig. 2a). The identities among strains within the same groups were high, with little variability over the entire genome (Fig. 1c). Identity over the entire genome among the 870006C and 890357C strains was examined and found to be low in late region 5 (L5) and E4, with that of the fiber gene in L5 being particularly low (Fig. 1d). Similarly, identity among the 870006C and HAdV-53 strains was also low in L5 and E4. In addition, the identities for the sequence located between the pTP gene in E2B and the 100 kDa protein in L4, except for the hexon loop region, among these strains were a little lower than those of 870006C and 890357C. In contrast, the identities of E1A and E1B among the 870006C and HAdV-53 strains were higher than those of 870006C and 890357C (Fig. 1e). The identities among 890357C and the HAdV-53 strains were shown to be lower for the sequence located between the pTP gene in E2B and the 100 kDa protein in L4, except for the hexon loop region, as shown among the 870006C and HAdV-53 strains. The identities of the other regions over the entire genome, including L5 and E4, were high (Fig. 1f).

Similarity plot analyses of HAdV-8, -22 and -37 and the seven intermediate HAdV strains were also carried out (Fig. 1g–i). HAdV-8 showed a high identity in L5 and E4 with the 890357C and HAdV-53 strains, and HAdV-22 showed a high identity for just the loop region in the hexon gene of all seven HAdVs. The regions with a high identity for HAdV-37 were E1A and E1B in the 870006C and HAdV-53 strains. The identities among the 870006C and HAdV-53 strains were shown to be lower for the sequence located between the pTP gene in E2B and the 100 kDa protein in L4, except for the hexon loop region, as shown among the 870006C and HAdV-53 strains. The identities of the other regions over the entire genome, including L5 and E4, were high (Fig. 1f).

Phylogenetic analysis of sections cut at the putative recombination breakpoints

We inferred the recombination regions by comparing the whole genomes of the seven HAdV strains in this study and 12 fully sequenced HAdV-D strains (HAdV-8, -9, -17, -19, -22, -26, -28, -37, -46, -48, -49 and -54) from GenBank.
Fig. 1. Genome organization of the seven complete HAdV genomes. (a) The grey horizontal bar in the centre shows the linear double-stranded HAdV genome with each vertical line representing 5000 bp. Transcription units are shown by black arrows relative to their position and orientation in the HAdV genome. Grey arrows show the positions of the penton, hexon and fiber genes. (b) Twelve sections cut at the putative recombinant breakpoints. The solid areas indicate short sections of <1000 bp in length that were excluded from the analyses. (c–i) Similarity plots of the complete genome sequence between C075/Matsuyama/2003 vs FS181/Fukui/2004.
Recombination analysis of HAdV-53 in Japan

Matsuyama/2003 (HAdV-53) and FS161/Fukui/2004 (HAdV-53) (c), 870006C/Hiroshima/1987 and 890357C/Hiroshima/1989 (d), 870006C/Hiroshima/1987 and C075/Matsuyama/2003 (HAdV-53) (e), 890357C/Hiroshima/1989 and C075/Matsuyama/2003 (HAdV-53) (f), 870006C/Hiroshima/1987 and HAdV-B, -22 and -37 (g), 890357C/Hiroshima/1989 and HAdV-B, -22 and -37 (h), and C075/Matsuyama/2003 (HAdV-53) and HAdV-B, -22 and -37 (i), generated using SimPlot 3.5.1. The vertical axis indicates the nucleotide identity, expressed as a decimal. The horizontal axis indicates the nucleotide position of the complete sequences. HAdV-B, -22 and -37 are shown as blue, green and red lines, respectively, in (g), (h) and (i). Double-headed arrows in (g), (h) and (i) show the regions with the highest nucleotide identity with HAdV-8 (red), -22 (green) and -37 (blue).

using RDP (Martin et al., 2005b). As a result, 15 putative recombination breakpoints were identified, thereby cutting the HAdV genomes into 16 sections. Among these sections, four shorter sections of <1000 bp were excluded from the analyses. Phylogenetic analyses were performed on the 12 sections that were >1000 bp in length (Fig. 1b), and the type among the 12 HAdV-Ds closest to each of the 12 sections of the seven HAdV strains is shown in Table 2. The analyses revealed that the closest HAdV type for our seven HAdV strains was different for each section due to frequent recombination events. In section 1, only 890357C was clustered with HAdV-37. In section 5, which includes the region encoding the loops of the hexon gene, all seven of our HAdV strains formed a cluster that was closely related to HAdV-22. In contrast, in sections 3, 4, 6 and 7, which include the regions encoding the penton base gene and C4 of the hexon gene, the four HAdV-53 strains, but not the other three HAdV strains, were clustered with HAdV-37. In sections 11 and 12, which include the fiber gene, 870006C and 880249C were clustered with HAdV-37, whereas 890357C and the four HAdV-53s were closely related to HAdV-8. In all other sections, the seven HAdV strains did not cluster with any of the other 12 HAdV-D strains, and the closest HAdV types could not be detected.

DISCUSSION

HAdV recombination can occur within the same species more readily than between species, and several intermediate recombinant HAdVs have been reported (Adrian et al., 1985; Noda et al., 1991; Engelmann et al., 2006; Aoki et al., 2008; Lukashev et al., 2008; Kaneko et al., 2009, 2011b; Walsh et al., 2009, 2010; Yang et al., 2009). Previous reports have already demonstrated the characteristics of the HAdV-53 genome by recombination among HAdV-D strains (Engelmann et al., 2006; Aoki et al., 2008; Walsh et al., 2009). The identities of the complete genome sequences of all four HAdV-53 strains in this study were high, and the strains formed a single phylogenetic cluster. We compared the complete genome sequences of HAdV-53 strains isolated in Germany with those from Japan (data not shown) and found that all HAdV-53 strains, including those from Germany, formed a single phylogenetic cluster showing high identities (>99.17 %). Thus, it was confirmed that all strains were the same HAdV type, HAdV-53. The identities of the complete genome sequences of the German and Japanese HAdV-53 strains were high (99.17–99.24 %) but were slightly lower than the identities among the Japanese HAdV-53 strains (99.87–100.00 %). The FS161 and FS165 strains, which were isolated in Fukui, were closer to the German strains than the other two Japanese HAdV-53 strains.

In contrast, analyses of the complete genome sequences of three intermediate HAdVs isolated in the 1980s revealed that these did not form a single cluster with the four HAdV-53 strains and showed only low nucleotide sequence identities. The three HAdV strains isolated in the 1980s were neutralized by HAdV-22 antisera (Noda et al., 1991) and showed high nucleotide sequence identity with HAdV-22 at the loop regions in the hexon gene, as observed in the HAdV-53 strains. The identity of the E3 region in these strains and the HAdV-53 strains was also high; however, the three HAdVs from the 1980s did not show hexon-chimeric structures similar to those of HAdV-22 and -37, or a penton base structure similar to that of HAdV-37. Moreover, 870006C and 880249C showed a fiber structure comparable to that of HAdV-37 but not to that of HAdV-8. These three HAdV strains were HAdV-53-like intermediate viruses, but were not HAdV-53. We propose that the 870006C/880249C and 890357C strains are intermediate to HAdV-22/H37 and -22/H8, respectively. Eight HAdV-22/H8 and seven HAdV-22/H37 strains were detected in Japan prior to 1989. However, none of these strains has been isolated since 1990. Many HAdV-53 strains have been isolated from EKC samples since 1996 (Aoki et al., 2011; Kaneko et al., 2011a), suggesting that HAdV-53 evolved from the intermediate HAdVs circulating in the 1980s, and from HAdV-8, -22 and -37, by recombination of sections cut at the putative breakpoints. The origins of the regions for which the HAdV type could not be detected are unknown (Table 2). These sequences might be unique to HAdV-53.

HAdV-53 is serologically related to HAdV-22, which rarely causes EKC. However, HAdV-53 has recently caused epidemic and nosocomial EKC infections in Japan (Kaneko et al., 2011a). It also caused an EKC outbreak in Germany in 2005 (Engelmann et al., 2006). An in vivo experiment has shown that HAdV-53, but not HAdV-22, can induce corneal inflammation in a mouse model of corneal infection (Walsh et al., 2009). HAdV-53 obtained its fiber gene, which contains functions related to cell tropism, from HAdV-8 by recombination. Two intermediate HAdV strains isolated in the 1980s, HAdV-22/H37 and -22/H8, also contained the fiber gene from HAdV-37 or -8, respectively, which are the main aetiological agents of...
EKC. These intermediate strains were isolated between 1986 and 1989 in Japan but did not cause EKC epidemics. HAdV-53 obtained not only the fiber gene from HAdV-8 but also the penton base gene, which is related to cell entry, from HAdV-37 by recombination. This combination of recombination events is thought to have afforded HAdV-53 the ability to infect the eye easily, leading to conjunctivitis.

Fig. 2. Phylogenetic analysis of the nucleotide sequences of the complete genome (a), loop 1 (b), loop 2 (c) and C4 (d) in the hexon gene, the penton base gene (e), the E3 region (f) and the fiber gene (g).
Table 2. HAdV type on the basis of phylogenetic analysis of 12 sections cut at the putative recombinant breakpoints

<table>
<thead>
<tr>
<th>Section (nt position*)</th>
<th>Closest HAdV type by phylogenetic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>870006C/880249C</td>
</tr>
<tr>
<td>1 (1–4311)</td>
<td>ND</td>
</tr>
<tr>
<td>2 (4312–11146)</td>
<td>ND</td>
</tr>
<tr>
<td>3 (11201–16151)</td>
<td>ND</td>
</tr>
<tr>
<td>4 (16390–18181)</td>
<td>HAdV-22</td>
</tr>
<tr>
<td>5 (18182–19357)</td>
<td>HAdV-22</td>
</tr>
<tr>
<td>6 (19581–21371)</td>
<td>ND</td>
</tr>
<tr>
<td>7 (21372–26447)</td>
<td>ND</td>
</tr>
<tr>
<td>8 (26448–28608)</td>
<td>ND</td>
</tr>
<tr>
<td>9 (28609–30091)</td>
<td>ND</td>
</tr>
<tr>
<td>10 (30092–31117)</td>
<td>ND</td>
</tr>
<tr>
<td>11 (31118–32189)</td>
<td>HAdV-37</td>
</tr>
<tr>
<td>12 (32190–35050)</td>
<td>HAdV-37</td>
</tr>
</tbody>
</table>

*The numbering of the positions of the 12 sections corresponds to that of the total sequence of C075/Matsuyama/2003 (HAdV-53).

ND, Not detected.
In conclusion, the complete genome sequence analyses suggested not only the genome organization but also the putative recombination breakpoints and evolutionary processes of the intermediate HAdV-53. Novel HAdVs, including intermediate HAdVs, often cause disease epidemics (Jones et al., 2007; Aoki et al., 2008; Ishiko & Aoki, 2009; Yang et al., 2009; Kaneko et al., 2011a, b). Analyses of both the genes and recombination events in the genome might help predict the appearance of novel HAdVs and disclose the relationships between novel HAdVs and epidemics in the future.

**METHODS**

**Virus strains.** Seven clinical intermediate HAdV strains, obtained from EKC patients in Japan, were used in this study (Table 1). Four strains, 870006C, 880249C, 890357C and 960528C, were collected in Hiroshima in 1987, 1988, 1989 and 1996, respectively. The C075 strain was collected in Matsuyama in 2003, and both FS161 and FS165 strains were collected in Fukuji in 2004. The strains 870006C/880249C and 890357C were identified previously as HAdV-22/H10,19,37 and HAdV-22/H8,9, respectively, on the basis of NT and HI tests (Noda et al., 1991), whilst the remaining four strains, 960528C, C075, FS161 and FS165, were reported as HAdV-53 on the basis of sequence analyses of the entire hexon and fiber genes (Aoki & Tagawa, 2002; Kaneko et al., 2011a). Viral isolation was propagated in A549 cells. Cell cultures were maintained in Dulbecco’s modified Eagle’s medium containing 50 μg gentamicin ml⁻¹, 0.5 μg fungizone ml⁻¹ and 10% FCS.

**DNA extraction.** Viral DNA was extracted from 100 μl of each virus solution using a QiAamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions. The extracted DNA was then suspended in 100 μl TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA].

**DNA sequencing.** We sequenced the complete genome of HAdV using a PCR/direct sequencing method, as described previously (Kaneko et al., 2009). The primers for PCR and sequencing were designed with reference to the complete genome sequences of other previously deposited HAdV-D strains in GenBank. PCR amplification was performed in 50 μl reaction mixtures containing 10 mM Tris/HCl (pH 8.2), 50 mM KCl, 1.5 mM MgCl₂, 200 μM each dNTP, 500 nM each primer and 0.5 U Taq DNA polymerase. PCR products were purified with a QIAquick PCR purification kit (Qiagen) and used as templates for DNA sequencing reactions (Kaneko et al., 2005). To determine the sequences of both ends of the genome of each strain, we extracted whole viral DNA from HAdV-infected cells (Shinagawa et al., 1983) and performed a sequencing reaction using the extracted viral DNA as template (Kaneko et al., 2009). Nucleotide sequencing was performed using an autosequencer (ABI Prism 3100 Genetic Analyzer) and a commercial kit (BigDye; Applied Biosystems). The nucleotide sequences were determined on both strands of the amplicons by sequencing to a mean value of greater than threefold coverage of the target regions.

**Sequence analysis.** Multiple alignments were performed and analysed using CLUSTAL W software (Thompson et al., 1994). Unrooted phylogenetic trees were constructed using the neighbour-joining (NJ) method, and visualization and edited using MEGA4 software (Saitou & Nei, 1987; Walsh et al., 2009). The evolutionary distances were estimated using the Kimura two-parameter method (Kimura, 1980), and unrooted phylogenetic trees were constructed using the NJ method (Saitou & Nei, 1987). Bootstrap analyses were performed with 1000 resamplings of the datasets (Felsenstein, 1985). Similarity plot analyses were generated using SimPlot version 3.5.1 (Lole et al., 1999). Similarities among the complete genomes of the HAdV-D strains were calculated in each window of 200 nt by the Kimura two-parameter method (Kimura, 1980) with a transition : transversion ratio of 2.0. The window was advanced successively along the genome alignment in 20 nt increments.

**Identification of putative recombination breakpoints and phylogenetic analysis of each section.** To identify recombination breakpoints, the multiple-alignment results were input into the Recombination Detection Program (RDP) (Martin et al., 2005b), which simultaneously applied the following algorithms: RDP (Martin & Rybicki, 2000), Chimaera (Posada & Crandall, 2001), Bootscan (Martin et al., 2005a), GENECONV (Padidam et al., 1999), MaxChi (Smith, 1992) and SISCAN (Gibbs et al., 2000). It was used for the complete genome sequences of the seven HAdV strains in this study and 12 HAdV-D strains from GenBank. For the RDP algorithm, the reference sequence parameter was ‘Internal and External References’, as recommended in the manual, based on the fact that some of the sequences were closely related, whereas others were less closely related. The multiple-aligned sequences were cut into sections at the positions where the putative recombination breakpoints were identified. Among these, sections >1000 bp in length were identified and phylogenetic analyses were carried out by MEGA4 (Tamura et al., 2007). Shorter sections of <1000 bp in length were excluded from the analyses.

**Nucleotide sequence accession numbers.** The following complete genome HAdV-D sequences, already available in GenBank, were used: HAdV-8 (AB448767), -9 (AJ854486), -17 (AF108105), -19 (AB448771), -22 (FJ404771), -26 (EF153474), -28 (FJ824826), -37 (AB448775), -46 (AY875648), -48 (EF153473), -49 (DQ393829) and -54 (AB448770).

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