Novel Human Adenovirus Causing Nosocomial Epidemic Keratoconjunctivitis

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In 2000, we encountered cases of nosocomial infections with epidemic keratoconjunctivitis (EKC) at a university hospital in Kobe, in the western part of Japan. Two human adenovirus (HAdV) strains, Kobe-H and Kobe-S, were isolated from patients with nosocomial EKC infection. They were untypeable by existing neutralizing antisera; however, the isolate was neutralized with homologous antisera. We then encountered several cases of EKC due to nosocomial infections in eye clinics in different parts of Japan. A total of 80 HAdVs were isolated from patients with EKC at eight different hospitals. The partial hexon gene sequences of the isolates were determined and compared to those of the prototype strains of 51 serotypes. All isolates had identical partial hexon nucleotide sequences. Phylogenetic analysis classified these isolates into species of HAdV-D. The isolates showed 93.9 to 96.7% nucleotide identity with HAdV-D prototype strains, while all 32 HAdV-D prototype strains ranged from 93.2 to 99.2% identity. The sequences of the loop 2 and fiber knob regions from the representative strain, Kobe-H, were dissimilar in all prototype strains of 51 serotypes. We believe that this virus is a novel serotype of HAdV that causes EKC.

Human adenoviruses (HAdVs), which belong to the genus Mastadenovirus of the family Adenoviridae, infect billions of people worldwide and cause various diseases, including conjunctivitis, respiratory infectious disease, diarrhea in infants and young children, and hemorrhagic cystitis (41). HAdVs initially were grouped into six subgenera (A to F) on the basis of several biochemical and biophysical criteria (8, 41). In 1999, however, a reclassification on the basis of nucleotide and deduced amino acid sequences was approved by the International Committee on Taxonomy of Viruses; under this reclassification, the 51 serotypes of HAdVs were grouped into six species, HAdV-A to HAdV-F (8).

In Japan, roughly 8,500 HAdV isolates were reported in 2003 to 2007 (Infectious Agents Surveillance Report [http://idsc.nih.go.jp/iasr/index.html]). They were obtained from patients with epidemic conjunctivitis (979; 11.5%), upper and lower respiratory tract infections (1,582; 18.6%), and gastroenteritis (348; 4.1%). Virus isolation followed by a neutralization test (NT) using a type-specific antiserum has been the standard procedure for serotyping (39). To date, the amplification of the genome by PCR, the determination of the nucleotide sequences, and phylogenetic analysis have become generalized techniques for both the classification and identification of viruses. We recently determined the partial hexon sequences of all 51 prototype strains and have developed a rapid and reliable method of molecular diagnosis based on phylogenetic analysis (25, 33). This method has successfully classified the 51 prototype strains of HAdVs into the six designated species, as approved by the International Committee on Taxonomy of Viruses. Over the last 30 years, we have applied this molecular diagnosis to the identification of hundreds of isolates and swabs without virus isolation from patients from different parts of the world with EKC and lower respiratory tract infections (5, 6, 21, 25, 33).

In 2000, we encountered cases of nosocomial EKC infection at a university hospital in Kobe, in the western part of Japan. Two HAdV strains, Kobe-H and Kobe-S, were isolated from the patients and showed identical nucleotide sequences of partial hexon genes. Adenoviral conjunctivitis is caused primarily by HAdV-3 (of the HAdV-B species), HAdV-4 (of HAdV-E), and HAdV-8, HAdV-19, and HAdV-37 (all of HAdV-D). Among these, HAdV-8, HAdV-19, and HAdV-37 cause more severe EKC than the others (3, 4, 7). The more serious outcomes occur in all age groups and can trigger highly contagious nosocomial infections (3, 7, 20, 26, 38). Kobe-H and Kobe-S were untypeable by using existing neutralizing antisera, suggesting that they represent a novel adenovirus causing EKC. After the nosocomial infections of 2000, we encountered several cases of nosocomial EKC infection, including cases at eye clinics, in different parts in Japan. Surprisingly, the 80 isolates from patients with EKC at eight different hospitals shared partial nucleotide sequences that were identical.

In this paper, we characterize the isolates from the patients with EKC. The present phylogenetic analysis classified these isolates into the HAdV-D species, and the Kobe-H strain represents only 93.9 to 96.7% nucleotide identity between the 32 prototype strains of HAdV-D, while all prototype strains of HAdV-D ranged from 93.2 to 99.2% identity. Quantitative NT and genetic analysis of the main neutralization ε determinant, loops 1 (L1) and 2 (L2) in the hexon gene, clearly indicated...
that the representative strain Kobe-H is different from previously known HAdVs. We believe that the Kobe-H strain is a novel HAdV that causes nosocomial EKC infection.

**MATERIALS AND METHODS**

**Viruses.** The isolates and conjunctival swabs were obtained from nosocomial or sporadic infections from 2000 to 2005 (Table 1). Kobe-H and -S were isolated from patients with nosocomial EKC infections at a university hospital in Kobe, Japan, in 2000. These strains were supplied from the Reference Center of Nosocomial Infection, Hokkaido University Graduate School of Medicine, Sapporo, Japan. The prototype strains of 51 HAdVs were obtained from the American Type Culture Collection and from the National Institute of Infectious Diseases, Tokyo, Japan. These viruses were used directly for DNA extraction without further propagation.

**Phylogenyp-like-based classification using a partial hexon sequence.** A partial hexon sequence of HAdV was amplified from the isolates as described previously (25, 33). In brief, viral DNA was extracted from 100 μl of virus suspension using a Sumitest EX-R&D kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) by following the manufacturer’s instructions. The DNA was dissolved in 100 μl of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). The 1,004-bp fragment of the hexon gene was amplified with 50 pmol of a pair of primers, AdTU7 (nucleotide positions 20,734 to 20,753; 5′- GGCAACCTTTCCTTCCACGTG-3′) and AdTV9 (positions 21,718 to 21,737; 5′- GTGACGGTGCCGCCGAGAA-3′). The positions of the primers for PCR were numbered according to the genome alignment in 20-nucleotide increments. We previously determined the nucleotide sequences of the partial hexon genes (916 bp) of all 32 prototype strains of HAdV-D and HAdV-E (33). In the previous study, we used the residual HAdV prototype strains, AdV-6 of HAdV-C, AdV-31 of HAdV-A, and AdV-50 of HAdV-B, to complete the database based on 350 bp of the hexon gene of HAdV; these sequences were not available from GenBank (25).

**Serological analysis.** Antisera against the Kobe-H strain were raised in rabbits by following the conventional procedure (12). Serological analyses were performed by a quantitative NT with type-specific antisera (for HAdV-8, HAdV-9, HAdV-19, and HAdV-37) purchased from Denka Seiken Co., Ltd. (Tokyo, Japan), or from the American Type Culture Collection (for HAdV-8). NTs were performed with A549 human lung cancer cells in 96-well microplates. The 50% tissue culture infectious doses of challenge virus that caused a cytopathic effect after 7 days of incubation at 37°C were used. Duplicates of the twofold serial-diluted antisera were used in the HAdV NTs (32).

**Genome typing.** Viral DNA was extracted from infected cells and placed in a 75-cm² plastic flask using 3 ml of Hirt lysis solution (10 mM Tris, 1 mM EDTA, 0.1% sodium dodecyl sulfate, pH 8.0) (18). Proteinase K was added at a final concentration of 50 μg/ml, and the samples were incubated at 37°C for 3 h. Cellular DNA was precipitated with 1 M of NaCl (final concentration) overnight at 4°C. After phenol-chloroform extraction, the supernatant was treated with a mixture of ribonucleases A (25 mg/ml) and T1 (80 U/ml) (Sigma, St. Louis, MO), and phenol-chloroform extraction was performed. Viral DNA was precipitated with isopropanol and suspended in 50 μl of TE buffer (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). One microliter of viral genomic DNA was digested with 5 U of each of the following restriction enzymes: BamHI, HindIII, and Smal (Takara Shuzo Co., Ltd., Kyoto, Japan). The digested viral DNA was loaded onto 1% agarose gels containing 1 μg/ml ethidium bromide. DNA bands were photographed with a UV transilluminator and a Polaroid camera. The migration patterns of the DNA fragments were compared to those of previously reported genome types (1, 11, 17, 31, 40).

**Nucleotide sequence accession numbers.** The GenBank accession numbers of the nucleotide sequences presented in this study are AB333801 and AB590056. GenBank sequences AB330082 to AB330132 were used to generate alignments of the hexon gene.

**RESULTS**

**Phylogenyp-like-based classification using a partial hexon sequence.** In 2000, we encountered cases of nosocomial EKC at a university hospital in Kobe, in the western part of Japan. Two HAdVs, the Kobe-H and Kobe-S strains, were isolated from the EKC patients. These strains had identical partial hexon sequences. After the Kobe episode, several studies reported an HAdV in different parts of Japan that was similar to the
Kobe-H and Kobe-S strains and that caused nosocomial EKC (Table 1). To assess the genetic constellation of the HAdV that caused the nosocomial infections, Kobe-H was selected as a representative isolate, and the alignment of the partial hexon nucleotide sequences was performed with 51 prototype strains and three HAdV-8 variants, including HAdV-8A, HAdV-8B, and HAdV-8E, using the SINCA genetic software program (34).

As shown in Fig. 1, the prototype strains were segregated into six major clusters, which corresponded well with the six newly designated HAdV species A to F (8). Kobe-H was segregated into the D cluster with 32 prototype strains and clustered with the HAdV-8 prototype strain and its genome type strains, HAdV-8A, HAdV-8B, and HAdV-8E (Fig. 1). Within this cluster, however, Kobe-H had a lineage that was different from that of the prototype HAdV-8 and the three genome type strains. The genetic relationships between Kobe-H and the HAdV-D prototype strains were further compared one by one. The nucleotide identity among the 32 HAdV-D serotypes ranged from 93.2% (between HAdV-8 and HAdV-51) to 99.2% (between HAdV-23 and HAdV-25), with an average of 97.3%, and those between the prototype HAdV-8 strain and the three genome type strains were 99.3% to 99.9%. Interestingly, Kobe-H showed only 96.7% nucleotide identity with HAdV-8 and 96.3 to 96.8% identity with the three genome type strains. These data suggest that Kobe-H is a novel serotype in the HAdV-D strains.

Serological analysis. The clinical isolates including Kobe-H formed a monophyletic cluster with the HAdV-8 prototype strain of HAdV-D. To examine the serological reactivity between the Kobe-H and HAdV-D strains, a quantitative neutralization assay was performed with the antiserum against HAdV-8 along with the antisera against the HAdV-9, HAdV-19, and HAdV-37 prototype strains, which are the causative agents of conjunctivitis. As shown in Table 2, no prototype-specific antiserum reacted with Kobe-H at a titer higher than 1:64 of homologous titer, except for the HAdV-8 antiserum. Conversely, the anti-Kobe-H serum did not react with any of the six prototype strains, except that a low-NT titer was found in the reaction mixture with HAdV-9 (Table 2). As the serotype has been defined on the basis of its immunological distinctiveness, the new serotype should show a homologous/heterologous titer ratio of >16 in either direction (39). Therefore, these results...
again suggest that Kobe-H is a novel serotype of the species HAdV-D.

**Genome typing.** To determine the genotype of Kobe-H, its genomic DNA was digested with three restriction endonucleases: BamHI, HindIII, and SmaI. Each restriction pattern was different from that of known prototype strains (Fig. 2).

**Full-length hexon and fiber knob gene nucleotide sequences.** Recently, the main neutralization ε determinant encoded in L1 and L2 in hexon genes was determined for all HAdV prototype strains, and criteria for typing based on the L1 and L2 nucleotide sequences have been proposed (24). These criteria may facilitate the identification of new HAdV prototypes, and we thus determined the full-length hexon sequence of Kobe-H as previously described by Ebner et al. (14), as well as those of the prototype strains, which were not available from GenBank. When the full-length hexon nucleotide sequence of Kobe-H was compared to those of the 51 prototype strains, the L2 nucleotide sequence of Kobe-H showed 56.9% (HAdV-41 of HAdV-F) to 85.8% (HAdV-9 of HAdV-D) identity and showed only 78.5% identity to the HAdV-8 prototype strain (Table 3). The phylogenetic analysis showed that Kobe-H does not form a monophylic cluster with any of the 51 prototype strains (Fig. 3A). Therefore, we also analyzed the L1 region because of its higher contribution to the ε determinant (24). Kobe-H showed 40.7% (HAdV-1 of HAdV-C) to 75.9% (HAdV-9 of HAdV-D) identity with the 51 prototype strains (Table 3). The phylogenetic analysis showed that Kobe-H does not form a monophylic cluster with any of the 51 prototype strains (Fig. 3B). These results clearly indicate that Kobe-H is a new HAdV prototype strain.

The inconsistent constellation of Kobe-H in the L2-based phylogeny and a partial hexon gene-based phylogeny suggest that recombination played a role in the evolution of the Kobe strains. To clarify the recombination events, we compared the complete hexon gene of the Kobe-H strain to those of all prototype strains by similarity plot analysis with a sliding window of 200 residues (Fig. 4). When Kobe-H was used as the query sequence and compared to the 51 prototype strains, the resultant 51 similarity plots indicated that the L1 and L2 regions were highly dissimilar, while 5’ and 3’ parts of the hexon gene were well conserved among some of the strains (Fig. 4). Therefore, we conclude that there was no recombination in the hexon gene between Kobe-H and the 51 prototype strains.

To date, several intermediate strains have been reported, and it is thought that they emerged due to recombination events between the hexon and fiber knob genes of the different serotype strains (15, 24, 27, 28). It is well known that the fiber knob plays an important role in HAdVs attaching to a target cell (9, 10). Therefore, we determined the nucleotide sequence of the fiber knob region of Kobe-H and the 51 prototype strains, as described previously by Madisch et al. (24). These nucleotide sequences were compared to each other in order to clarify whether Kobe-H acquired cell tropism to conjunctiva by

<table>
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<th>HAdV-4p</th>
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*The prototype strains are HAdV-3p (GB), HAdV-4p (RI-67), HAdV-8p (Trim), HAdV-9p (Hicks), and HAdV-37p (GW).

b Isolate from EKC.

**FIG. 2.** Genome type of Kobe-H. Viral genomic DNA was digested with three restriction enzymes: BamHI, HindIII, and SmaI.
recombination with known HAdVs. The comparison of the sequence of the fiber knob of Kobe-H showed 31.5 (HAdV-50 of HAdV-B) to 98.7% (HAdV-8 of HAdV-D) identity, while the three genome type strains of HAdV-8 showed 99.6 to 100% identity to the HAdV-8 prototype strain (Table 3). These results strongly support the hypothesis that Kobe-H is a novel serotype of HAdV.

DISCUSSION

To date, 51 HAdV serotypes have been identified and grouped into six species, HAdV-A to HAdV-F (8). The nine most recent serotypes, HAdV-43 to HAdV-51, were isolated from human immunodeficiency virus-infected patients, and all except HAdV-50 belong to HAdV-B (11, 17, 31). The pathogenesis of these isolates was not clear. EKC typically is caused by one of three HAdV-D serotypes, HAdV-8, HAdV-19, or HAdV-37, and these strains frequently cause nosocomial outbreaks (29, 34–36). These viruses originally were isolated from patients with EKC in 1951, 1955, and 1976 (12, 13, 19). To date, several variants of these viruses have been reported (2, 6, 37), but none of the new serotypes causes EKC. The serotype of any strain isolated from a patient is defined on the basis of its immunological distinctiveness, such as that determined by quantitative neutralization with type-specific antisera. A serotype shows a homologous/heterologous titer ratio of >16 in both directions (39). Kobe-H was shown by NT to be weakly reactive to HAdV-8 and HAdV-9, probably due to the similarity of the Kobe-H hexon gene, including the L1 and L2 regions, to that of the HAdV-8 strains. Phylogenetic analysis of the 916-bp partial hexon gene showed that Kobe-H is close to HAdV-8, although its L1 and L2 regions were different from those of HAdV-8. These results led us to perform a similarity plot analysis with the full-length hexon gene; however, no recombination event was found in the Kobe-H hexon gene. Recently, an outbreak of EKC caused by a new intermediate adenovirus, type 22/H8, was reported (15).

In conclusion, we have identified a novel HAdV on the basis of quantitative NT and the phylogenetic analysis of the hexon gene. PCR using a set of primers to amplify a partial hexon gene was found to be efficient in amplifying not only all of the prototype strains but also the novel HAdVs. We believe that the present molecular diagnosis on the basis of the partial hexon sequence will be of use not only for the rapid
diagnosis of HAdVs but also in molecular epidemiology. To date, only three HAdVs, HAdV-8, HAdV-19, and HAdV-37, are known to cause severe EKC (4). The origin and mechanism of transmission of the novel HAdV are not yet clear. Our phylogenetic analysis based on the partial hexon gene indicates that the novel HAdV had already appeared in 1995 as the causative agent of EKC (data not shown). The novel HAdV has sometimes been identified as HAdV-8 because of its weak cross-reaction with anti-HAdV-8 serum, possibly delaying its recognition as a novel HAdV. It also is possible that this virus has sometimes been identified as HAdV-8 because of its weak binding sites. J. Virol.


