Short Communication

Molecular Epidemiology of Human Adenoviruses D Associated with Epidemic Keratoconjunctivitis in Osaka, Japan, 2001–2010

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SUMMARY: Isolation of novel types of human adenovirus D (HAdV-53, -54, and -56) from keratoconjunctivitis patients has been reported since 2008. We examined the molecular epidemiology of HAdV-D strains using 39 field isolates collected from epidemic keratoconjunctivitis (EKC) patients from 2001 to 2010 in the province of Osaka, Japan. The molecular types were analyzed by sequencing partial penton base gene, loop 1 in the hexon, and complete fiber genes. Of the 39 isolates, the majority were HAdV-19 (14/39, 35.9%) and -37 (13/39, 33.3%), followed by -53 (4/39, 10.3%) and -54 (8/39, 20.5%). Analysis of annual distribution showed that HAdV-19 and -37 were detected before 2004, whereas HAdV-53 and -54 were first identified in 2001 and 2003, respectively, and persistently detected during the study period. It is noted that both HAdV-53 and -54 isolates were misclassified by the serological method. Altogether, the molecular analysis elucidated the epidemiology of HAdV-D and presence of novel types from the early 2000s in Osaka. Further genetic analysis of serologically classified HAdV-D isolates may provide insights into the epidemiology of EKC.

Human adenoviruses (HAdVs) belong to the genus *Mastadenovirus* of the family *Adenoviridae*, and are grouped into species A (HAdV-A) to G (1,2). The major epitopes specific to each HAdV type are located in the hexon protein loops 1 and 2, which have been utilized to classify HAdV by serological methods. Advancement of genetic analyses clarified that type-specificity lies in the genes encoding penton base, hexon, fiber, and early region 3 (3,4). Therefore, genetic analysis offers an alternative and accurate method to classify HAdV and provides an opportunity to identify novel HAdV types. Recently, field isolates of novel HAdV types have been documented bearing unique recombination of their genome, and the genetic analysis revealed that majority of these genotypes belong to HAdV-D (4–11).

HAdV causes a wide range of illnesses, including respiratory infections, gastroenteritis, and keratoconjunctivitis (1). HAdV-D types, namely HAdV-8, -19, and -37, are major causes of epidemic keratoconjunctivitis (EKC). In addition to these original types, novel HAdV-D strains associated with EKC have been reported in recent years, including HAdV-53, -54, and -56 (7–11). Moreover, the analysis of genome organization clarified that HAdV-19 associated with EKC was a recombinant virus and distinct from prototype HAdV-19p (12,13). In our surveillance, we employed neutralization tests to determine the type of HAdV-D isolates acquired before 2008. To clarify the molecular epidemiology and correlation between serotyping and molecular typing of HAdV-D, previously acquired isolates from

our surveillance of EKC from 2001 to 2010 were analyzed genetically.

A total of 39 HAdV-D strains were isolated from conjunctival swabs collected from 2001 to 2010 using HEP-2 or A549 cells. These specimens were obtained from patients with sporadic EKC during our routine infectious disease surveillance. Molecular typing of 8 HAdV-54 strains was documented previously (14). Viral DNA was extracted from tissue culture supernatants containing virions, and viral genes were amplified by polymerase chain reaction (PCR) targeting the penton base gene covering RGD motif, loop 1 in hexon, and complete fiber genes using puRe Taq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, UK) with the method previously described (14–17). The amplicons were purified using NucleoSpin Gel and PCR Clean-up kits (Macherey-Nagel, Duren, Germany). The nucleotide sequences on both strands of the amplicons were determined using the Applied Biosystems 3130 genetic analyzer (Applied Biosystems, Foster City, Calif., USA). Sequence analyses of the penton base, hexon, and fiber genes (nucleotide positions 14320–15033, 18181–18733, and 31055–32152, respectively, according to the reference strain HAdV-19a; GenBank accession no. AB448772) were performed using the BLAST at National Center for Biotechnology Information (NCBI) and Molecular Evolutionary Genetics Analysis software (MEGA5; Arizona State University, Tempe, Ariz., USA) (18). The sequences generated in this study were submitted to the DNA database of Japan (DDBJ) and assigned the following accession numbers: HAdV-19 (AB761319–AB761346 and AB765595–AB765608), HAdV-37 (AB761347–AB761372 and AB765609–AB765621), and HAdV-53 (AB761373–AB761376, AB761378–AB761382, and AB765622–AB765625). For reference, we employed a strain of HAdV-37 (GenBank accession no. AB448778) and a strain of HAdV-53

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Fig. 1. Type distribution of HAdV-D associated with EKC in Osaka from 2001 to 2010.

(GenBank accession no. AB605244).

BLAST analysis of penton base and loop 1 in the hexon and complete fiber genes showed that the 31 strains consisted of HAdV-19 (14 isolates), -37 (13 isolates), and -53 (4 isolates). Genetic analysis revealed that the loop 1 in hexon and fiber genes of the HAdV-19 strains had 100% identity to the referral HAdV-19a sequence. The partial penton base gene of 7 HAdV-19 strains shared 99.9% identity (1-base pair substitution), and 2 strains shared 100% identity to the referral HAdV-19a sequence. All gene sequences of the HAdV-37 and -53 isolates were identical to those of the reference strains.

The annual and type distributions of a total of 39 HAdV-D strains were then analyzed (Fig. 1). The largest number was isolated in 2002, decreased after 2003, and increased in 2010. HAdV-19 was isolated only in 2002, and HAdV-37 was isolated during 2001 to 2004, and both were not detected from 2005 onward. HAdV-53 and -54 were detected in 2001 and 2003, respectively. Overall, HAdV-19 and -37 were dominant before 2004, and novel types, namely HAdV-53 and -54, were prevalent since 2006. HAdV-8 and -56 were not isolated during these years.

The present study clarified the prevalence and endemic transition of HAdV-D molecular types associated with EKC in Osaka, Japan over a decade (2001–2010). HAdV-19 was detected the most; however, it was isolated only in 2002 due to an endemic caused by HAdV-19 in that year. A recent report described that the genome and tropism of HAdV-19 associated with EKC was different from those of HAdV-19p and proposed a new strain HAdV-64, a recombinant of HAdV-19p, -22, and -37 (12). This recombinant form of HAdV isolates (HAdV-19a) from EKC patients has been previously reported in Japan (13), and in the present study, our data suggested that HAdV-19 strains were likely to be the recombinant virus.

HAdV-D is the most common among the novel HAdVs. Genetic recombinations among HAdV occur within the same species (19), which could be important for the evolution of HAdV. Unfortunately, because HAdV-D isolates before 2000 were not preserved in our laboratory, we were unable to evaluate the beginning of the spread of novel HAdV-D types in Osaka. However, isolation of novel HAdV-Ds has been documented in many parts of Japan (20–22), and novel types have been prevalent since 2006 in Osaka. Furthermore, we report-

ed the isolation of a novel recombinant HAdV-D strain related to HAdV-37 and HAdV-56 in 2011 (17). Taken together, the downtrend of original HAdV-D types in Japan may be related to the spread of novel types. Moreover, several recent studies have reported the detection of HAdV-8 and -37 in Japan (22,23). This suggests a regional variation in HAdV-D endemic.

HAdV-53 is an intertypic recombinant of HAdV-8, -22, and -37 and reactive to HAdV-8 specific antiserum (7,8). Therefore, in our historical surveillance based on neutralization tests using specific antisera against HAdV-8, -19, and -37, HAdV-53 strains have been misclassified as HAdV-8. Furthermore, neutralization tests showed that HAdV-54 is weakly reactive to antisera against both HAdV-8 and -9 (9). In fact, an HAdV-54 strain isolated in Osaka in 2003 was misclassified as HAdV-8 using neutralization tests available at that time. Based on these results, we propose that strains serologically classified as HAdV-8 in previous surveillance programs may include HAdV-8, -53, and/or -54.

In conclusion, we demonstrated that genetic analysis is beneficial for understanding HAdV epidemiology. In the present study, molecular analyses elucidated the serological misclassification of EKC-associated HAdV-D in Osaka, Japan. We argue that retyping is necessary to accurately classify HAdV isolates, notably serologically classified HAdV-D strains, because novel types have become widespread.

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Conflict of interest None to declare.

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