

Short Communication

Molecular Epidemiology of Adenovirus Type 3 Detected from 1994 to 2006 in Hyogo Prefecture, Japan

Tsuguto Fujimoto^{1,2*}, Itsuki Hamamoto¹, Kiyosu Taniguchi¹,
Masatsugu Chikahira² and Nobuhiko Okabe¹

¹National Institute of Infectious Diseases, Tokyo 162-8640, and

²Hyogo Prefectural Institute of Public Health and Environmental Sciences, Hyogo 652-0032, Japan

(Received November 14, 2007. Accepted December 28, 2007)

SUMMARY: The molecular epidemiology of 126 adenovirus type 3 (AdV3) isolates obtained in Hyogo Prefecture (population: 5.5 million) from 1994 to 2006 was studied. The hexon-coding region, including 7 hypervariable regions (HVRs) (1,419 bp), was sequenced. We found 5 nonsynonymous nucleotide substitutions in the HVRs. The results are strongly suggestive of positive Darwinian selection. We classified the AdV3 strains analyzed here into 3 genome types: AdV3x ($n = 44$), AdV3y ($n = 46$), and AdV3z ($n = 36$). AdV3x first appeared in 2001 in Hyogo Prefecture, and was detected predominantly during a large outbreak of AdV3 in 2003-2005. AdV3x was identical to a Korean strain responsible for a large outbreak of AdV3 in Korea in 1998-1999. We conclude that at least 3 genome types of AdV3 have circulated in Hyogo Prefecture, Japan, during the past 13 years (1994-2006). The findings also suggest that AdV3x was imported from Korea to Hyogo Prefecture in 2001.

Human adenoviruses (AdVs) are known to cause acute respiratory disease, pharyngoconjunctival fever, and epidemic keratoconjunctivitis (1-3). To date, a large family with 51 serotypes has been recognized. Among these serotypes, human adenovirus serotype 3 (AdV3) is the infectious agent most frequently isolated from patients with pharyngoconjunctival fever (PCF) (1).

National surveillance of PCF showed that the number of patients with PCF due to AdV3 increased in 2003 (4) and continued to increase until 2006. In Hyogo Prefecture (Fig. 1), which has a population of 5.5 million, an outbreak of PCF began in 2003 and continued until 2006 (5).

Recently, two new genome types, Ad3a16 and Ad3a18, were recognized during a large outbreak in 1998-1999 in Korea (6). Choi et al. have reported that new genome types of AdV3, isolated for the first time in 1998, are associated with 3 amino acid changes in the hexon-coding region, which potentially affects the antigenic characteristics of AdV3 (6). In this study, we report on the molecular epidemiology of AdV3 detected during the period extending from 1994 to 2006.

During 1994-2006, 3,711 clinical samples were tested as part of a surveillance program in Hyogo Prefecture. Among these clinical samples, 380 tested AdV3-positive by viral culture and polymerase chain reaction (PCR) analysis. From among these AdV3 samples, a total of 126 isolates (33%) were chosen for this study. At least 11% of the AdV3 samples per surveillance year were tested, except for in the year 1996, when no AdV3 was detected. The patients from whom these isolates were taken were diagnosed with PCF or another respiratory illness.

HeLa (HEp2), A549, RD, and Vero cells were used for the virus isolation procedure. Clinical samples were inoculated onto an 80% confluent cell monolayer in duplicate wells of a



Fig. 1. Location of Hyogo Prefecture in Japan.

24-well plate (Nippon Becton Dickinson, Tokyo, Japan). For cultivation, Dulbecco's modified Eagle's medium (Sigma, St. Louis, Mo., USA), supplemented with 2% heat-inactivated fetal calf serum and antibiotics, was used as the maintenance medium. The cells were passaged 3 to 8 times in order to allow time for the development of a cytopathic effect (CPE). When a CPE became evident, the isolates were tested by neutralizing antisera against AdVs purchased from Denkaseiken (Tokyo, Japan).

Viral DNAs were extracted using High Pure Viral Nucleic Acid Kits (Roche Applied Science, Basel, Switzerland). In the hexon-coding region, the hypervariable regions (HVRs) (7) were amplified and sequenced according to a modified version of the method reported by Takeuchi et al. (8): briefly, primers Hx5-NIID (5'-ATG GCT ACC CCT TCG ATG ATG CCC CAA T-3') and Hx3-NIID (5'-CTT ATG TGG TGG CGT TGC CGG CCG AGA ACG G-3') were used instead of HX5-1 and HX3-1. The underlined nucleotides were designed according to the AdV3 sequence in this study. Partial sequences of the hexon-coding region (1,419 bp), including the 7 HVRs

*Corresponding author: Mailing address: Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111, E-mail: fujimo-t@nih.go.jp

(7), have been determined.

The sequences were aligned using GENETIX, version 6.1.2 (Software Development Co., Tokyo, Japan). Phylogenetic and molecular evolutionary analyses were conducted using MEGA (version 4) developed by Tamura et al. (9). To construct a phylogeny tree, a neighbor-joining (NJ) method was used.

Among the 380 AdV3 isolates, 126 were sequenced and classified into 3 genome types. A comparison of the 1,419-bp hexon-coding regions revealed that there were 5 nucleotide replacements. All 5 nucleotide changes resulted in amino acid changes (Table 1). The 126 strains were analyzed and classified as follows: AdV3x ($n = 44$; 35%), AdV3y ($n = 46$;

Table 1. Sequence variation among adenovirus type 3 (AdV3) variants

Genome type	Genome position corresponding to AY599836 ¹⁾				
	18935	19028	19663	19699	19730
AdV3x	C (Thr) ²⁾	G (Gly)	C (His)	G (Ala)	A (Asp)
AdV3y	A (Asn)	T (Val)	A (Asn)	A (Thr)	C (Ala)
AdV3z	C (Thr)	T (Val)	A (Asn)	A (Thr)	C (Ala)
Region	HVR 2 ³⁾	HVR 3	HVR 7		

¹⁾: AY599836 is a deposit number of DNA from AdV3 in USA.

²⁾: Amino acids shown in parentheses.

³⁾: Hypervariable region.

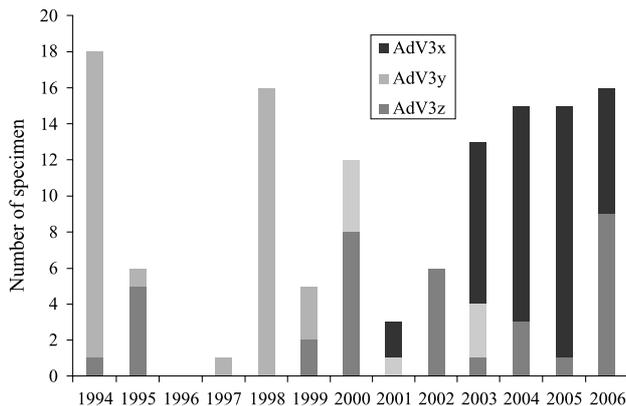


Fig. 2. Genetic classification of AdV3s detected in Hyogo Prefecture for 13 years (1994-2006).



Fig. 3. Phylogenetic tree of the hexon-coding region generated by neighbor-joining method using 14 AdV3 strains and 1 AdV7 strain. The scale bar represents substitutions per site. The numbers at the nodes of the tree are bootstrap values in percentages, which measure the robustness of the support for each particular node.

37%), and AdV3z ($n = 36$; 29%).

AdV3x first appeared in Hyogo Prefecture in 2001 (Fig. 2), and it was identical to the Korean strain (Gen Bank accession no. AY854178). AdV3x was constantly dominant during 2003-2005. AdV3y was dominant in 1994 and 1998. AdV3y has not yet been deposited in GenBank. Although AdV3y is highly homologous to AdV3z, there is a single base substitution, accompanied by an amino acid change. AdV3z is identical to AdV3, which was isolated in the United States (AY599836), and detection of this variant was relatively continuous throughout the surveillance period of this study.

Phylogenetic analysis of AdV3x, AdV3y, and AdV3z was undertaken using 11 AdV3s and 1 AdV7 sequences available from GenBank. AdV3x belongs to Clade 1, which consists of recent East Asian isolates, and AdV3y and AdV3z belong to Clade 2 (Fig. 3).

Several other molecular epidemiology AdV3 studies in Japan have been previously reported, i.e., Guo et al. in 1988 (2), Mizuta et al. in 1994 (10), and Shiao et al. in 1996 (11). However, there is limited information from recent molecular epidemiological studies of AdV3 in Japan. Thus, a total of 126 AdV3 strains detected during 1994-2006 were analyzed and divided into 3 genetic types, using the hexon-coding region that includes 7 HVRs (7).

According to Crawford and Schnurr (7), unique sequences of AdVs are limited to the 7 HVRs, and 1 or more of these regions contain the type-specific neutralization epitopes. That study demonstrated that AdV neutralization epitope(s) are complex as well as conformational. Pichla-Gollon et al. (12) reported that the specific sites recognized by neutralizing antibodies have not been identified for any AdVs. They studied the major neutralization site for chimpanzee adenovirus 68, and found that a single small surface loop defines a major neutralization site for the AdV hexon.

AdV3x, first detected in 2001 in Hyogo Prefecture, is identical to the strain that caused a large AdV3 outbreak in Korea in 1998-1999 (6). Choi et al. has suggested that the genetic heterogeneity of AdV3 could play a potential role in the appearance of new genome types and could also affect the antigenic characteristics of AdV3. They also suggested a relationship between the appearance of a new AdV3 genome type and the large outbreak in Korea (6).

From the data available in the present study, it appears possible that AdV3x was imported from Korea to Japan and caused a large outbreak of AdV3 in Japan. A total of 2,368,877 people from Korea entered Japan, and a total of 2,319,676 Japanese people visited Korea in 2006, according to the Japanese Ministry of Justice.

All genome substitutions among AdV3x, AdV3y, and AdV3z were nonsynonymous, which is strongly suggestive of positive Darwinian selection. Additional studies will be necessary to provide detailed antigenic characterizations of AdV3x, AdV3y, and AdV3z. We conclude that at least 3 genome types of AdV3 circulated in Hyogo Prefecture, Japan, during the past 13 years (1994-2006).

ACKNOWLEDGMENTS

We thank Dr. Teruo Okafuji and Dr. Takao Okafuji (Okafuji Pediatric Clinic), Dr. Shinsaku Yoshida (Toyooka Hospital), and Dr. Hiroshi Akutagawa (Tsukaguchi Hospital) for providing clinical samples.

This study was conducted with the support of a grant for Research Promotion of Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan. This work was supported in part by a grant from Daido Life Welfare Foundation, Osaka, Japan.

REFERENCES

1. Wold, W.S.M. and Marshall S.H. (2007): Adenoviruses. p. 2395-2436. In Knipe, P.M. and Howly, P.M. (ed.), *Fields Virology*. 5th ed. Lippincott Williams and Wilkins, a Wolters Kluwer Business, Philadelphia.
2. Guo, D.F., Shibata, R., Shinagawa, M., et al. (1988): Genomic comparison of adenovirus type 3 isolates from patients with acute conjunctivitis in Japan, Australia, and the Philippines. *Microbiol. Immunol.*, 32, 833-842.
3. Lyn, J., Michael, O.V., Roderick, C. J., et al. (2007): Outbreak of human adenovirus type 3 infection in a pediatric long term care facility-Illinois, 2005. *Clin. Infect. Dis.*, 45, 416-420.
4. National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare (2004): Adenovirus and pharyngoconjunctival fever, 2003. *Infect. Agents Surveillance Rep.*, 25, 94'-95'.
5. Fujimoto, T., Okafuji, T., Okafuji, T., et al. (2004): Evaluation of a bedside immunochromatographic test for detection of adenovirus in respiratory samples, by comparison to virus isolation, PCR, and real-time PCR. *J. Clin. Microbiol.*, 42, 5489-5492.
6. Choi, E.H., Kim, H.S., Park, K.H., et al. (2006): Genetic heterogeneity of the hexon gene of adenovirus type 3 over a 9-year period in Korea. *J. Med. Virol.*, 78, 379-383.
7. Crawford-M, L.K. and Schnurr, D.P. (1996): Adenovirus serotype evolution is driven by illegitimate recombination in the hypervariable regions of the hexon protein. *Virology*, 224, 357-367.
8. Takeuchi, S., Itoh, N., Uchio, E., et al. (1999): Serotyping of adenoviruses on conjunctival scrapings by PCR and sequence analysis. *J. Clin. Microbiol.*, 37, 1839-1845.
9. Tamura, K., Dudley, J., Nei, M., et al. (2007): MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evolution*, 24, 1596-1599.
10. Mizuta, K., Suzuki, H., Ina, Y., et al. (1994): Six-year longitudinal analysis of adenovirus type 3 genome types isolated in Yamagata, Japan. *J. Med. Virol.*, 42, 198-202.
11. Shiao, S., Aoki, K., Isobe, K., et al. (1996): Genome analysis of adenovirus type 3 isolated in Japan. *J. Clin. Microbiol.*, 34, 413-416.
12. Pichla-Gollon, S.L., Drinker, M., Zhou, X., et al. (2007): Structure-based identification of a major neutralizing site in an adenovirus hexon. *J. Virol.*, 81, 1680-1689.