Original Article

A Novel Complex Recombinant Form of Type 48-Related Human Adenovirus Species D Isolated in Japan

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SUMMARY: Recently, new genotypes of human adenoviruses (HAdVs) have been reported and many of them have been found to be recombinant forms of different known types of HAdV species D (HAdV-D). The objective of this study was to document the evolutionary features of a novel isolate (HAdV_Chiba_E086/2012) obtained from the eye swab of a patient with conjunctivitis in Japan. Viral DNA was extracted from the isolate to sequence the whole genome by the Sanger method and aligned with available genome sequences of HAdV-Ds. The phylogenetic trees of the nucleotide sequences of the penton base, hexon, and fiber genes and the E3 region showed that HAdV_Chiba_E086/2012 is closest to HAdV genotype 65 (HAdV-GT65), HAdV-48, HAdV-GT60 and HAdV-22 at 98%, 99%, 95% and 98% identity, respectively, suggesting that this isolate is a novel recombinant form to be designated as P65H48F60. Further phylogenetic and recombination analyses of the genome alignment of the new isolate implied that nested recombination events involving HAdV-GT59, GT65, 48, GT60, 22, and some ancestral lineages or their close relatives have shaped its genome. These results showed that HAdV_Chiba_E086/2012 is the first HAdV-48-related HAdV found in Japan, which has the most complicated evolutionary history among the known HAdVs so far.

INTRODUCTION

Human adenoviruses (HAdVs) are common causative agents of several diseases, including respiratory infections, acute gastroenteritis and epidemic conjunctivitis. HAdV infections also associated with the development of a serious adenovirus syndrome in immunocompromised patients who have undergone stem cell transplantation. HAdVs are divided into seven species (HAdV-A to G) based on molecular phylogenetic analyses of their genome sequences (1).

HAdV-D is the only adenoviral species that is specific to human and is known to be the most diverged species (1). A lot of new genotypes of HAdV-D, which were defined in this report as the HAdV genotypes without serological determinations, that is those from type number 53 onward, have been recently reported and characterized in terms of evolution and neutralization assays, e.g., HAdV genotype 53 (HAdV-GT53) (2) and HAdV-GT64 (3) (abbreviated as HAdV-GT53 and so on for such genotypes in this report). Except for a few types,

such as HAdV-GT54, most of the new genotypes have been suggested to be originated from recombination between the types which were designated by serological typing criteria, i.e., HAdV-1 to 51, particularly in the penton base, hexon and fiber genes (2–4). It has also been speculated that recombinant viruses exhibiting similar phenotypes to their original viruses (e.g., in tropism and pathogenesis) would have been recognized as known types (3–6). HAdV-GT53 is one of the most complex recombinant forms at least 4 original viruses were involved in the origin of the HAdV GT53 genome.

HAdV-48, a type of HAdV D was first isolated from the feces of an AIDS patient (7). According to a sero-epidemiological analysis conducted in 1989, the seroprevalence of HAdV-48 is significantly higher in HIV-seropositive subjects; however, infection is not limited to immunocompromised patients. Sexual preference is also not a significant determinant of the seroprevalence of HAdV-48 in HIV-seronegative individuals (8). According to the National Epidemiological Surveillance of Infectious Diseases (NESID) (9) as of August 2013, no cases of HAdV-48 were reported in Japan between 1981 and 2012. However, a novel HAdV strain (hereafter referred to as HAdV_Chiba_E086/2012) was isolated in April, 2012 at the Chiba Prefectural Institute of Public Health from the eye swab of a patient with conjunctivitis, and an initial survey of its hexon gene sequence as a usual typing procedure of

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HAdV (10) indicated its close relationship to HAdV-48. In order to elucidate the evolutionary origin(s) of HAdV_Chiba_E086/2012, we analyzed the whole genome of this isolate. Here we report the complex structure of the genome in terms of the evolutionary origins.

MATERIALS AND METHODS

Sample source and clinical findings: A 63-year-old woman first noticed a foreign body sensation with ocular discharge in her left eye on April 24, 2012, defined as day 1 of her illness. On day 2, when she noted increased mucus production and significant swelling in her left eyelid, the woman presented to an eye clinic, and a sample for culture was collected from left conjunctiva. She was treated with antibiotic eye drops and dexamethasone sodium phosphate. By day 8, she had recovered from her symptoms. The ocular sign was unilateral moderate follicular conjunctivitis without multiple subepithelial corneal infiltrates. This study was conducted as part of NESID, carried out under Japanese law. In that case, no ethical committee's approval is required. Informed consent was obtained from the patient.

Virus isolation and nucleic acid extraction: HAdV was isolated from the patient using a Caco-2 cell line and propagated in an A549 cell line maintained in Eagle's minimal-essential medium supplemented with 2% fetal bovine serum. The cultures were observed for 2 weeks for cytopathic effects (CPEs) using both cell lines. After a CPE (100%) was observed, the infected cells in the medium were frozen and thawed 3 times to release virus from the cells. The medium including HAdV particles were centrifuged at $1,500 \times g$ for 20 minutes, and the resulting supernatant was used for viral nucleic acid extraction using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany). The extracted DNA was quantified using real-time PCR (11).

Genome sequencing: The entire genome of this isolate was sequenced using a PCR-based Sanger method with primers that were designed based on the consensus sequence of the complete genomes of 21 HAdV-Ds available in the International Nucleotide Sequence Database (INSD). We first amplified 23 segments that cover the whole genome with overlaps. We then sequenced each PCR product with sequence primers. PCR was performed by AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, CA, USA), and the PCR products were purified with Exonuclease I (Takara, Shiga, Japan) and Alkaline Phosphatase (Takara), followed by sequencing reaction with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and sequencing using an AB3130xl Genetic Analyzer (Applied Biosystems). We examined the chromatogram of each sequence read and confirmed that none contained polymorphisms that would suggest mixture of different genomes. Both termini of the genome were sequenced using the 5' RACE System, Version 2.0 (Invitrogen, Carlsbad, CA, USA). All the chromatograms were base-called and assembled with phredPhrap (12,13). All the sites of the single assembled contig were confirmed to have a phred Q value of ≥ 20 .

Phylogenetic and sequence analyses: We annotated

the coding-exons in the HAdV_Chiba_E086/2012 genome based on the sequence comparison of the protein products of the open reading frames identified in the genome with those of HAdV-8 (accession number AB448767) and submitted the annotated genome data to INSD through the DNA Data Bank of Japan (DDBJ). The entire genome sequence thus determined was aligned with 45 HAdV-D genomes obtained from INSD database (see below), which were selected from each serotype and genotype of HAdV-D, using the Iterative Refinement Method algorithm (FFT-NS-I) of MAFFT version 6 (14) to produce a multiple genome alignment (MGA).

Applying the Recombination Detection Program (RDP) (15) to the MGA, reliable recombination events were identified by selecting only those supported by >3 of the following 7 algorithms: RDP (16), Chimaera (17), BootScan (18), GENECONV (19), MaxChi (20), SiScan (21) and 3Seq (22) with p -values of <0.001 . The default values were used for all parameters except "Reference sequence selection" which was set to "Internal and External Reference" in this study.

For the purpose of typing HAdV_Chiba_E086/2012 based on the sequence identities of its penton base, hexon and fiber genes in comparison to those of the known HAdV types (4,23), the alignment regions of these genes were extracted from the MGA to construct neighbor-joining trees (as shown below) in order to reveal the sister groups of HAdV_Chiba_E086/2012 in these genes. Molecular phylogenetic trees of recombination regions were separately constructed with the neighbor-joining method (24) after calculating the evolutionary distances estimated under the Tamura and Nei model (25) using MEGA5.1 (www.megasoftware.net).

Nucleotide and accession numbers: The genome sequence of HAdV_Chiba_E086/2012 was deposited in the INSD under accession number AB765926. The accession numbers of the HAdV genomes used in this study are as follows: HAdV-8, AB448767; HAdV-22/H8 (Hiroshima 1989), AB605242; HAdV-22/H37 (Hiroshima 1987), AB605240; HAdV-9, AJ854486; HAdV-10, AB724351; HAdV-13, JN226747; HAdV-15, AB562586; HAdV-17, HQ910407; HAdV-19, AB448771; HAdV-20, JN226749; HAdV-22, FJ619037; HAdV-23, JN226750; HAdV-24, JN226751; HAdV-25, JN226752; HAdV-26, EF153474; HAdV-27, JN226753; HAdV-28, FJ824826; HAdV-29, AB562587; HAdV-30, JN226755; HAdV-32, JN226756; HAdV-33, JN226758; HAdV-36, GQ384080; HAdV-37, AB448776; HAdV-38, JN226759; HAdV-39, JN226760; HAdV-42, JN226761; HAdV-43, JN226762; HAdV-44, JN226763; HAdV-45, JN226764; HAdV-46, AY875648; HAdV-47, JN226757; HAdV-48, EF153473; HAdV-49, DQ393829; HAdV-51, JN226765; HAdV-GT53, AB605243; HAdV-GT54, AB448770; HAdV-GT56, HM770721; HAdV-GT58, HQ883276; HAdV-GT59, JF799911; HAdV-GT60, HQ007053; HAdV-GT62, JN162671; HAdV-GT63, JN935766; HAdV-GT64, EF121005; HAdV-GT65, AP012285; and HAdV-GT67, AP012302.

RESULTS

HAdV_Chiba_E086/2012 was successfully isolated

and propagated efficiently using the A549 cell lines. The isolate was confirmed to be a single clone by previously described method (26). We extracted DNA from this isolate, amplified it with PCR with HAdV-D-specific primers, and sequenced it using AB3130xl. We confirmed that none of the chromatograms obtained contained polymorphic sites, supporting that the isolate originated from a single clone. We then base-called and assembled the reads into a single contig of 35,198 bp using phredPhrap (12,13). The phred Q values of all sites of this contig were ≥ 20 . The base composition of this genome is A (22.5%), T (20.3%), G (28.6%) and C (28.6%).

The phylogenetic trees of the nucleotide sequences of the penton base, hexon, and fiber genes showed that HAdV_Chiba_E086/2012 is the closest to HAdV-GT65, HAdV-48 and HAdV-GT60 at 98%, 99% and 95% identity, respectively, and vice versa. Loops 1 and 2 of hexon and the fiber knob region showed the same relationships with HAdV-48 and HAdV-GT60, respectively. These results suggest that HAdV_Chiba_E086/2012 is a new recombinant form that may be represented as P65H48F60.

To characterize HAdV_Chiba_E086/2012 from the viewpoint of its molecular phylogeny, we first con-

structed a MGA of the HAdV_Chiba_E086/2012 genome with 45 available HAdV-D genomes using MAFFT (14). We then conducted a recombination analysis using the RDP program (15) to split recombined genomic regions before their molecular phylogenetic analyses in order to avoid incorrect inferences about the molecular phylogeny of HAdV_Chiba_E086/2012 resulting from mixing genomic regions of different evolutionary histories. Figure 1 depicts the regions identified as recombined segments by >3 algorithms in the RDP program.

The RDP analysis detected 8 regions in the genome that seem to have been recombined with HAdV-48, HAdV-GT65, HAdV-29, HAdV-22 and HAdV-GT60 or their close relatives (for simplicity, the phrase “HAdV-XX or its close relatives” will be represented by “HAdV-XX-like viruses” below) and 3 unknown or ancestral lineages (Fig. 1). The HAdV-48-like region (Region 1) contains the HAdV-GT65-like region. Similarly, the HAdV-22-like region contains the HAdV-29-like and GT60-like regions, as well as 2 unknown regions. The short HAdV-GT65-like region corresponds to the RGD loop region of penton base.

To clarify the overall molecular phylogeny among all genomes analyzed for each putative recombined region,

Region	Counterpart type	Breakpoint					Algorithm			
		Left	Right	RDP	GENECONV	BootScan	MaxChi	Chimaera	SiScan	3Seq
0	GT59	1	11,851	—	—	—	—	—	—	—
		23,977	24,137	—	—	—	—	—	—	—
		26,312	26,487	—	—	—	—	—	—	—
		34,696	35,198	—	—	—	—	—	—	—
1	48	11,852	23,976	2.36E-52	6.50E-72	NS	3.97E-11	1.14E-17	7.51E-42	9.23E-141
2	GT65	14,278	14,781	9.47E-45	2.57E-42	9.85E-16	1.34E-14	1.20E-14	2.29E-13	6.83E-31
3	Unknown	24,138	26,311	2.47E-05	5.56E-05	5.09E-07	NS	NS	1.08E-05	1.71E-23
4	29	26,488	27,269	7.56E-24	7.33E-23	NS	5.41E-18	1.14E-14	NS	6.83E-81
5	22	26,488	34,695	4.23E-15	NS	2.01E-05	NS	NS	2.48E-43	8.62E-277
6	Unknown	31,192	31,468	2.70E-14	1.44E-17	5.03E-12	1.16E-11	8.06E-08	NS	1.07E-04
7	GT60	31,216	33,568	2.87E-36	6.10E-44	5.81E-41	5.13E-24	3.68E-18	1.28E-42	1.53E-221
8	Unknown	32,536	33,556	1.22E-14	3.55E-15	NS	3.21E-09	6.55E-08	5.77E-14	NS

Fig. 1. The recombination signals that were supported by >3 different algorithms in the RDP analysis. The putative recombined regions identified by the MGA (see the Materials and Methods) are shown in the table and schematically represented beneath the table. Regions 1 to 8, corresponding to the 8 bars in the schematic representation, are the putative recombined regions that were supported by >3 algorithms in the RDP analysis. Region 0 is a set of 4 divided regions that were not included in any of the putative recombined regions. The “Counterpart type” is the HAdV-D type which was identified by the RDP analysis as the recombination counterpart. Breakpoint positions are shown in the base position in the HAdV_Chiba_E086/2012 genome. The p -values calculated by 7 different algorithms are shown for each region. NS means not significant ($p \geq 0.001$). The region numbers are shown with ‘#’ in the schematic representation followed by the counterpart type in parentheses. The coding exon regions and protein product names are shown under the scale bar that represents the physical position in base pair in the HAdV_Chiba_E086/2012 genome. The gray lines represent the intron regions between coding exons. The positions and the transcription orientations of the genes in the genome are shown in blue.

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Fig. 2. Neighbor-joining trees of Regions 0 to 8. (A–I) Partial neighbor-joining trees of Regions 0 to 8 (see Fig. 1). A HAdV_Chiba_E086/2012-containing subtree was extracted from the entire neighbor-joining tree of each region. The percent bootstrap value of 1,000 replicates is shown along each branch. The accession numbers of the source genome sequences are shown in parentheses. Details of the methods of constructing these trees are shown in the Materials and Methods.

Table 1. Putative recombination regions that were identified by the RDP analysis

Region	Coordinate				No. of gap-free sites	Sister group	% Identity	Bootstrap support ¹⁾
	In HAdV_Chiba_E086/2012		In MGA					
	Left	Right	Left	Right				
0	1	11,851	1	11,927	12,415	GT59	99.2	100
	23,977	24,137	24,159	24,319				
	26,312	26,487	26,519	26,694				
	34,696	35,198	35,443	36,015				
1	11,852	23,976	11,928	24,158	11,663	48	98.4	100
2	14,278	14,781	14,369	14,872	411	GT65	99.6	99
3	24,138	26,311	24,320	26,518	2,098	—	—	NS
4	26,488	27,269	26,695	27,476	728	—	—	NS
5	26,488	34,695	26,695	35,442	7,468	22	95.3	100
6	31,192	31,468	31,866	32,176	242	GT60	93.8	99
7	31,216	33,568	31,890	34,314	2,236	GT60	96.0	100
8	32,536	33,556	33,278	34,298	1,021	GT59	99.7	100

¹⁾ The bootstrap value of the branch that supports the clade of HAdV_Chiba_E086/2012 and the sister group indicated in Fig.

2. NS means the bootstrap value of the clade of HAdV_Chiba_E086/2012 and its sister group in Fig. 2 is < 75.5.

we constructed a neighbor-joining tree of each region separately (Fig. 2, Table 1). This analysis confirmed that the counterpart types identified by the RDP analysis were mostly the sister groups to HAdV_Chiba_E086/2012 in the trees in Fig. 2 for putative recombined regions. In the RDP analysis, the counterpart types of Regions 3 and 4 were unknown and HAdV-29, respectively. However, Figs. 2D and 2E show that HAdV_Chiba_E086/2012 is the sister group to the clade of

HAdV-30 and HAdV-13 and the clade of HAdV-29 and HAdV-GT63, respectively, although the bootstrap probabilities of the branches supporting these relationships were not significant. Although Region 6 was recognized as a region different from Region 7 in the RDP analysis, the sister groups of Regions 6 and 7 of HAdV_Chiba_E086/2012 were the same HAdV-GT60 in the phylogenetic analysis. Indeed, Region 7 overlaps all but the 24-bp tip region of Region 6. In addition, the

sister group of Region 8 of HAdV_Chiba_E086/2012 was clearly HAdV-GT59. Therefore, Region 7 can be divided into 2 halves, with the left half (31,216–32,535) being HAdV-GT60's sister group and the right half (32,536–33,568) being HAdV-GT59's sister group. The left half of Region 7 corresponds to the region of the fiber gene.

DISCUSSION

This is the first report of a HAdV-48-related isolate in Japan. Using the typing system based on the hexon structure (27), this virus is typed as 48, and using the system based on the gene trees of the penton base, hexon and fiber of the MGA (data not shown) (4,23), it is designated as P65H48F60, thus indicating that this virus is a novel recombinant form. By analyzing MGA using the RDP program and phylogenetic analyses to identify recombined regions in the genome precisely, we found that the HAdV_Chiba_E086/2012 genome has the most complex evolutionary history among genome the known HAdV genomes in terms of genomic recombination (Figs. 1 and 2). It should be noted that the close relationship between the penton base gene of HAdV_Chiba_E086/2012 and that of HAdV-GT65 does not mean that this gene was entirely copied from a HAdV-GT65-like virus. In reality, the HAdV-GT65-like region in the HAdV_Chiba_E086/2012 genome is embedded within the penton base gene, and the remaining region of this gene, which is much larger than the HAdV-GT65-like region, shows a close relationship with HAdV-48. The large difference from HAdV-48 in the HAdV-GT65-like region masked this evolutionary information.

The phylogenetic analysis of the putative recombined regions indicated that the terminal regions of the HAdV_Chiba_E086/2012 genome are most closely related to those of HAdV-GT59. This implies that HAdV_Chiba_E086/2012 originated from a HAdV-GT59-like virus, because it is less likely that these segments were independently copied from HAdV-GT59 simultaneously so as to satisfy the complementarity constraints on these terminal regions (28,29). The results of our analysis also indicate that HAdV_Chiba_E086/2012 has experienced several recombination events with HAdV-48, HAdV-GT65, HAdV-22, HAdV-29, and HAdV-GT60 or their recombinant viruses to form the current HAdV_Chiba_E086/2012 genome as shown in Fig. 1.

Among these putative parents of HAdV_Chiba_E086/2012, only HAdV-22 has been previously clinically reported in ocular infections (30) and it has also been suggested to be a putative recombination parent of HAdV-GT53 (31) and its variants (32,33), which were all isolated from epidemic keratoconjunctivitis patients. Although originally isolated from a respiratory infection, an experimental study has suggested that HAdV-GT60 may have a potential corneal tropism (34). In addition, the fiber knob has been reported to mediate the attachment of the virus to the receptor on the host cell (35,36), thus implying that the fiber is a determinant of tissue tropism of the virus. Therefore, we speculate that the conjunctivitis causativity of HAdV_Chiba_E086/2012 may have been acquired from HAdV-GT60 and the HAdV-22-like region may also be involved in

tissue tropism and/or pathogenicity.

The results of the RDP analysis showed several interesting recombination patterns. First, at least 2 nested recombination events were identified: recombination with a HAdV-GT65-like virus within the HAdV-48-like region; and recombination with a HAdV-GT60-like virus and probably with a HAdV-29-like virus within the HAdV-22-like region. Second, 3 regions were identified as those recombined with some unknown or ancestral viruses of several different HAdVs. Based on these observations, we may infer a possible recombination process that shaped the genome of HAdV_Chiba_E086/2012. For example, HAdV-GT59 (HAdV-48 (HAdV-GT65), Anc (HAdV-30, HAdV-13), HAdV-22 (Anc (HAdV-29, HAdV-GT63), HAdV-GT60, HAdV-GT59)), where $X(Y_1, Y_2)$ means that region X includes recombined regions Y_1 and Y_2 in this physical order and Anc (Z) means an ancestor of Z. The nested structures would have been generated via nested recombination events or by recombination with recombinant forms.

Third, we noticed that many of the recombined segments were mapped on certain functional/structural blocks of genes or other genomic regions. For example, the HAdV-GT65-like segment sharply corresponds to the RGD loop of the penton base gene. The fiber gene exhibits an exclusive and close relationship with HAdV-GT60, as if this gene had been selectively copied from HAdV-GT60. Region 4 corresponds to CR1- α . Such strong correlations between recombined segments and the genomic structure/function imply that HAdV_Chiba_E086/2012 was selected from among a large variety of recombinant forms of different types of HAdV due to its high fitness or by other mechanisms, rather than by chance. Indeed, the genes of the major epitope-containing capsid proteins are all involved in the recombination events. Switching of the hexon variable regions to a low seroprevalence epitope of HAdV-48 (37) and producing the novel combination of the epitope-containing capsid proteins, represented as P65H48F60, may have increased the success rate of host infection by HAdV_Chiba_E086/2012 since the low seroprevalence indicates that only a small number of hosts have experienced HAdV-48-like viruses. The change to the HAdV-GT60-like fiber may have altered the tropism of this virus from bronchoalveolar (as in HAdV-GT59) (6) to ocular (34). If this virus is an adaptive form, it would have the potential to cause epidemic ocular infections. To clarify the virulence and infectious capacity of HAdV_Chiba_E086/2012, continuous surveillance of ocular viruses should be implemented.

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Conflict of interest None to declare.

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