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Original Article

Spread of predominant neuraminidase and hemagglutinin co-mutations in the influenza A/H3N2 virus genome☆

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ABSTRACT

Genetic variation of influenza neuraminidase (NA), unlike for hemagglutinin (HA), has not been fully characterized. Therefore, we determined the relation between mutations in the NA and HA genome segments of 205 influenza A/H3N2 viruses isolated from patients in Japan during the five seasons from 2010 to 2015. The amino acid (AA) sequences of the NA and HA proteins in these isolates were then determined. In the 2011–2012 season, there was the emergence of isolates with NA and HA sequences containing AA93G (NA93G) and AA278K (HA278K), respectively (24/48 isolates, 50.0%). This was in contrast to NA93D-HA278N being detected exclusively in the previous 2010–2011 season (24/24 isolates, 100.0%). The isolates with the NA93G-HA278K substitutions became predominant in the following 2012–2013 season (95.8%, 46/48 isolates). The NA and HA phylogenetic trees of the 2011–2012 and 2012–2013 seasons were segregated by clades with NA93D-HA278N or NA93G-HA278K. In the subsequent 2013–2014 and 2014–2015 seasons, the strong relationship between NA93D-HA278N and NA93G-HA278K observed in the previous seasons, was no longer present and NA93G-HA278N (33/52 isolates, 63.5% in the 2014–2015 season) became predominant. In addition, the clades within the NA and HA trees could no longer be segregated based on NA AA93 and HA AA278. These findings suggest that the co-mutation of NA and HA AA sequences is present and may contribute to the formation of an epidemic lineage.

1. Introduction

Epidemics of influenza viruses occur annually, and result in high morbidity and mortality in humans. A/H3N2 virus is the most common and virulent of the influenza subtypes. Antigenic drift, changes in antigenicity through the accumulation of mutations in the hemagglutinin (HA) gene, a major surface protein, is chiefly responsible for the continuing circulation of these viruses [1]. As a result, influenza vaccines must be frequently updated based on analyses of new variants; however, protection by influenza vaccines has typically been suboptimal [2,3]. Neuraminidase (NA) is also a mutable surface protein, and its significance as an antigen has been recognized for several decades [4]. However, unlike the HA gene, mutations in the NA gene, despite possibly undergoing antigenic drift, are not routinely examined and have yet to be extensively analyzed.

We have developed a network of clinics throughout Japan, where influenza virus samples are routinely collected from patients who have given informed consent and tested positive for influenza infection using a rapid influenza antigen kit [5–8]. In the present study, 205 influenza A/H3N2 viruses were isolated for genetic analysis at these clinics during the five seasons between 2010 and 2015. The amino acid (AA) sequences of the NA and HA proteins in the A/H3N2 viruses were determined by next-generation sequencing. Interestingly, AA mutations in NA were directly correlated with mutations in HA, and viruses with NA and HA co-mutations formed a distinct phylogenetic clade and a lineage that caused an epidemic in the following season. To the best of our

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knowledge, this study is the first to identify NA and HA co-mutation that is associated with a change of influenza epidemics.

2. Materials and Methods

2.1. Sample collection

Nasopharyngeal swabs for influenza virus isolation were collected from patients who had a positive result on a rapid influenza antigen test, given at one of the member clinics of our nationwide study network of general practitioners [5–8]. Informed consent was obtained from all patients, all of whom were outpatients. No patients were included who had a severe chronic respiratory disease, renal disease, liver disease, or heart failure. All viral samples were collected before the initiation of neuraminidase inhibitors (NAIs). In this study, the isolates of 205 samples (24 from the 2010-11 season, 48 from the 2011-12 season, 48 from the 2012-13 season, 33 from the 2013-14 season and 52 from the 2014-15 season) were genetically analyzed.

2.2. Viral RNA extraction and RT-PCR

Nasopharyngeal swabs from patients were soaked in virus transport medium and 75 μL of the medium was cultured using Madin-Darby canine kidney (MDCK) cells. Viral RNA was extracted from infected MDCK cell culture supernatants using the Maxwell 16 LEV simply RNA Cells Kit (Promega, Madison, WI). The A/H3N2 subtype was determined by PCR [9]. MDCK-passaged viruses were used for the sequencing. RT-PCR for the preparation of sequencing, which was different from that for the determination of subtype, was performed using the H3N2 RNA samples. PCR primers, synthesized based on the 3′ and 5′ terminal nucleotides that are common to all human influenza A virus segments [10] were as follows: forward primer (Uni-12), 5′-ACGCGTGATCAGCAAAAG-CAGG-3′ and reverse primer (Uni-13), 5′-ACGCGTGATCAGTA-GAAACAAGG-3′. The PCR consisted of 31 cycles of a denaturing step at 94 °C for 30s, an annealing step at 57 °C for 30s, and an extension step at 72 °C for 2 min.

2.3. Next generation sequencing

A DNA library for Illumina sequencing was prepared using the Nextera XT DNA Sample Prep kit (Illumina, San Diego, CA). Sequencing was conducted via a paired-end, 2 × 250 bp cycle run, using the Illumina MiSeq sequencing system and MiSeq Reagent Kit version 2 (300 Cycle) (Illumina) [11].

2.4. Bioinformatic analysis

Data processing was performed using the pipeline prepared by Amelieff Co [11]. The reference sequence was A/New York/396/2005 (H3N2) (GenBank accession numbers for the eight gene segments: CY002079, CY121123, CY121122, CY121117, CY121120, CY121119, CY121118, and CY121121). The HA amino acid (AA) sequence was deduced from the obtained nucleotide sequence.

2.5. Nucleotide sequence accession number

The sequence data from this study were deposited into the DDBJ/EMBL/GenBank nucleotide sequence databases under the following accession numbers: LC090941-LC091036, LC111574-LC111606, and LC155845-LC155896.

3. Result

In the 2010–2011 season, only the isolates with NA sequences containing 93D (NA93D) and HA sequences containing 278N (HA278N) were detected (Table 1). Next, based on sequence analysis of isolates from the 2011–2012 season, a direct relationship was identified between NA residue 93 and HA residue 278. Thus, the isolates with NA93D also possessed HA278N; in contrast, NA93G corresponded to HA278K in this season. This indicates that the NA93G-HA278K isolates had newly emerged in the 2011–2012 season (24/48 isolates, 50.0%). There were no isolates found with NA93G-HA278N or NA93D-HA278K in the 2011–2012 season. In the 2012–2013 season, no isolates with NA93D-HA278N were detected (0.0%, 0/48 isolates). Rather, most isolates from this season shared the NA93G-HA278K substitutions (95.8%, 46/48 isolates) (Table 1). Fig. 1 presents a phylogenetic tree constructed based on NA and HA AA alignments of the A/H3N2 strains isolated in Japan during the 2011–2012 and 2012–2013 seasons. The isolates with NA93D from the 2011–2012 season formed a distinct clade, while all of the remaining sequences contained NA93G. Based on the HA sequence, the isolates from the 2011–2012 season could be segregated into two distinct clades, defined by the presence of either HA278N or HA278K. Interestingly, the isolates with NA93G-HA278K that newly emerged in the 2011–2012 season were genetically more similar to those from the 2012–2013 season than those with NA93D-HA278N, as observed in both the NA and HA trees (Fig. 1).

In the following 2013–2014 season, the isolates with NA93G-HA278N increased in frequency, unlike the combinations observed in the two previous seasons (14/33 isolates, 42.4%). The NA93D-HA278N isolates reemerged in the 2014–2015 season. However, the strong relationship of NA93D-HA278N and NA93G-HA278K, seen in the 2011–2012 season, was no longer present due to the predominance of NA93G-HA278N (33/52 isolates, 63.5%). Fig. 2 presents the NA and HA phylogenetic trees of isolates from the 2013–2014 and 2014–2015 seasons. Unlike the NA tree constructed for isolates from the 2011–2012 season, the clades of the NA tree could no longer be segregated based on 93D or G. Similarly, in contrast to the clades in the HA tree in the 2011–2012 season, the clades in the trees for the 2013–2014 and 2014–2015 seasons could no longer be segregated based on 278N or K. Finally, isolates with the NA93D-HA278K combination were rarely detected throughout the five seasons studied.

Table 1
Amino acid change at sites 93 in neuraminidase and 278 in hemagglutinin in influenza A/H3N2 viruses isolated in Japan over the five seasons from 2010 to 2015.

Amino acid pattern	2010–2011 season	2011–2012 season	2012–2013 season	2013–2014 season	2014–2015 season
	No. of isolates (%)	No. of isolates (%)	No. of isolates (%)	No. of isolates (%)	No. of isolates (%)
NA93D-HA278N	24 (100.0)	24 (50.0)	0 (0.0)	0 (0.0)	15 (28.8)
NA93G-HA278N	0 (0.0)	0 (0.0)	2 (4.2)	14 (42.4)	33 (63.5)
NA93G-HA278K	0 (0.0)	24 (50.0)	46 (95.8)	17 (51.5)	4 (7.7)
NA93D-HA278K	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.1)	0 (0.0)
Total	24 (100.0)	48 (100.0)	48 (100.0)	33 (100.0)	52 (100.0)

NA, neuraminidase; HA, hemagglutinin.

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