Influenza A(H1N1)pdm09 virus exhibiting enhanced cross-resistance to oseltamivir and peramivir due to a dual H275Y/G147R substitution, Japan, March 2016

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An influenza A(H1N1)pdm09 virus carrying a G147R substitution in combination with an H275Y substitution in the neuraminidase protein, which confers cross-resistance to oseltamivir and peramivir, was detected from an immunocompromised inpatient in Japan, March 2016. This dual H275Y/G147R mutant virus exhibited enhanced cross-resistance to both drugs compared with the single H275Y mutant virus and reduced susceptibility to zanamivir, although it showed normal inhibition by laninamivir.

Detection of a dual H275Y/G147R mutant influenza A(H1N1)pdm09 virus

In the context of our nationwide monitoring for antiviral-resistant viruses, we previously reported that a large community cluster of influenza A(H1N1)pdm09 virus exhibiting cross-resistance to oseltamivir and peramivir had occurred in Hokkaido, Japan between November 2013 and February 2014 [1,2]. Of a total of 2,531 A(H1N1)pdm09 viruses investigated in the 2013/14 influenza season, 105 (4.1%) were shown to harbour the H275Y substitution in the neuraminidase (NA) protein. In the 2015/16 season (Figure 1), we screened 1,938 A(H1N1)pdm09 viruses by allelic discrimination [3] and detected 39 (2.0%) H275Y mutant viruses.

No epidemiological links were identified among the patients infected with the H275Y mutant viruses except for five nosocomial infections. Among 34 sporadic cases, 11 (32%) cases had no exposure to NA inhibitors before the specimen collection. This is consistent with our previous study that 19 (32%) of 59 sporadic cases had no exposure to NA inhibitors in the 2013/14 season [2]. One H275Y mutant virus with an additional G147R

substitution in the NA protein was detected from a sporadic case treated with peramivir in March 2016. Both substitutions were confirmed in the corresponding clinical specimen. Deep sequencing analysis of the specimen using MiSeq (Illumina, California, United States) revealed that the G147R substitution was detected in 21% of mixed sequence populations with 147G wild type, in contrast to H275Y, which was detected at a rate of 100%. The results suggested that the H275Y mutant virus had acquired the additional G147R substitution in the patient during peramivir administration. The NA H275Y mutation of the A(H1N1)pdm09 and the former seasonal A(H1N1) viruses confers cross-resistance to oseltamivir and peramivir [4] and the NA G147R is linked to slightly reduced susceptibility of the highly pathogenic avian A(H5N1) virus to oseltamivir and zanamivir [5]. Searching the EpiFlu Database of the Global Initiative on Sharing All Influenza Data (GISAID) yielded 18,172 A(H1N1)pdm09 viruses, of which nine were G147R mutant viruses (Table). No dual H275Y/G147R substitution appears to have been previously reported however.

Clinical course of the patient infected with the dual H275Y/G147R mutant virus

The patient, a woman in her early 50s with malignant lymphoma who was receiving chemotherapy, was hospitalised with myelosuppression in late February 2016. She received prophylaxis with laninamivir (40 mg) on the same day because her husband had been diagnosed as having influenza A virus infection. Three days later, she had onset of illness and tested positive for influenza A. At this time, peramivir was administered intravenously at a dosage of 600 mg daily for three intermittent periods of 5 days because of persistent influenza A virus infection. She developed left lower

FIGURE 1

Detection of influenza viruses, September 2015–April 2016 (week 36, 2015–18, 2016)^a, Japan (n = 5,778)

Weekly reports of influenza virus isolation/detection by the National Epidemiological Surveillance of Infectious Diseases.

^a Week 36 started on 31 August 2015.

lobe pneumonia 20 days post-disease onset and alveolar haemorrhage 12 days after, but no bacterial pathogens were isolated from sputum or blood samples. She died 5 days later. A nasal swab specimen of the patient was collected only at the end of the second intermittent administration of peramivir and an influenza A(H1N1)pdm09 strain A/Hiroshima/13/2016 was isolated in Madin-Darby canine kidney (MDCK) cells from the specimen (Table). We could not obtain a specimen from the husband.

Antiviral susceptibility of the dual H275Y/G147R mutant virus

After isolation in MDCK cells, A/Hiroshima/13/2016 possessed the H275Y and G147R substitutions in 100% population, respectively, although the G147R mutation was detected at a rate of 21% in the specimen. This result indicates that the virus carrying both substitutions had become predominant during MDCK cell culture.

We compared the susceptibilities of the dual H275Y/G147R mutant virus and single H275Y mutant viruses isolated during the same influenza season to four NA inhibitors approved in Japan: laninamivir, oseltamivir, peramivir and zanamivir (Figure 2).

Oseltamivir carboxylate, peramivir and zanamivir were purchased from Sequoia Research Products (Pangbourne, UK) and laninamivir was kindly provided by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). The susceptibilities of the viruses to NA inhibitors were determined by fluorescent NA inhibition assay with the NA-Fluor Influenza Neuraminidase Assay Kit (Applied Biosystems, California, United States). Results were expressed as the drug concentrations required to inhibit

NA activity by 50% (IC_{50}). To interpret the NA inhibitor susceptibility, we used the World Health Organization criteria, which are based on the fold change of IC_{50} values compared with the median IC_{50} values of the same subtype/lineage [6]. For influenza A viruses, normal (<10-fold increase of IC_{50} value), reduced (10–100-fold increase) or highly reduced (>100-fold increase) inhibition were defined.

The IC_{50} values of the viruses to laninamivir, oseltamivir, peramivir and zanamivir are shown in Figure 2. The median IC_{50} values of 19 single H275Y mutant viruses to oseltamivir and peramivir were 920- and 260-fold higher, respectively, than those of the 236 wild-type viruses. The dual H275Y/G147R mutant virus exhibited 2,600- and 1,400-fold higher IC_{50} values to oseltamivir and peramivir, respectively, compared with the wild-type viruses. These results indicate that the dual H275Y/G147R mutant virus showed highly reduced inhibition with high increases in oseltamivir and peramivir IC_{50} values compared with values for the single H275Y mutant viruses. Furthermore, the IC_{50} value of the dual mutant virus to zanamivir was ca fivefold higher than the median IC_{50} values of the wild type and the single H275Y mutant viruses, although the dual mutant virus showed normal inhibition by laninamivir.

Discussion

The first widespread community cluster of the H275Y mutant A(H1N1)pdm09 virus was detected in Newcastle, Australia in 2011 [7]. The H275Y substitution in the NA protein would destabilise the mutant virus. However, two additional V241I and N369K substitutions in the NA of H275Y mutant viruses were reported to increase their replication and transmission fitness, contributing to efficient transmission [8,9]. Almost all recently circulating A(H1N1)pdm09 viruses possess these permissive substitutions, suggesting an increased risk for H275Y mutant viruses to emerge and spread globally [10]. Indeed, during the 2013/14 influenza season, the H275Y mutant viruses from a large community cluster in Hokkaido, Japan carried these permissive substitutions. Following this finding, we subsequently increased nationwide monitoring for the H275Y mutant viruses in the 2015/16 season and detected an H275Y mutant with V241I and N369K and an additional G147R substitution in the NA protein from an immunocompromised inpatient. The IC_{50} fold changes of a number of NA inhibitors for the dual H275Y/G147R mutant virus compared with those for the single H275Y viruses showed clearly the synergistic effect of this dual substitution (Figure 2).

Hooper et al. reported that the G147R substitution in the NA protein has been detected in A(H1N1)pdm09, in the former seasonal A(H1N1) as well as in the A(H5N1) viruses where it conferred receptor-binding activity to the NA proteins of these viruses, similar to a D151G substitution in the NA of A(H3N2) viruses [11,12]. Phylogenetic analyses suggested that these G147R mutant viruses occurred sporadically in nature, while

FIGURE 2

Susceptibility to neuraminidase inhibitors of influenza A(H1N1)pdm09 viruses with H275Y and G147R substitutions detected in Japan, September 2015–April 2016 (n = 256)

IC₅₀: 50% inhibitory concentration.

The IC₅₀ values of the viruses to laninamivir, oseltamivir, peramivir and zanamivir were determined by fluorescent neuraminidase inhibition assay. Box-and-whisker plots of the IC₅₀ values (medians and interquartile ranges) are shown. The numbers at the bottom of each box-and-whisker plot indicate the fold change in IC₅₀ values compared with the median IC₅₀ values of 275H wild-type viruses.

the D151G substitution emerged during propagation of virus in MDCK cell culture [11,12].

Residue 147 is located in a 150-loop that includes residues 147 to 152 adjacent to the NA active site as shown in Figure 3 [13]. A previous study reported it as having an essential role in the conformation of the 150-loop [14]. Our structural analysis of the NA protein of the dual H275Y/G147R mutant virus using Molecular Operating Environment, MOE, (Chemical Computing Group Inc., Quebec, Canada) [15] suggests that the

G147R substitution may alter the stability of the 150-loop because the side chain of arginine is larger than that of glycine (Figure 3), negatively affecting the binding affinity to NA inhibitors.

The G147R substitution of N1 NA has been shown to slightly decrease enzymatic activity but not to affect the viral replication fitness [12]. These results, together with the findings of recent H275Y mutant viruses carrying permissive substitutions, V241I and N369K, suggest that the dual H275Y/G147R mutant virus had the

FIGURE 3

Three-dimensional structure of the neuraminidase protein of influenza A(H1N1)pdm09 virus with the H275Y and G147R substitutions

A. H275Y

B. H275Y/G147R

Structure models of the neuraminidase proteins of the single H275Y (A) and the dual H275Y/G147R (B) mutant viruses were constructed by homology modelling. The crystal structure of the A(H1N1)pdm09 neuraminidase protein (PDB ID: 4B7R) was used as the modelling template.

A 150-loop in the neuraminidase protein is shown in green.

potential to replicate efficiently. In this study, we found that the dual H275Y/G147R mutant virus grew well in cell culture. Furthermore, the patient infected with the dual H275Y/G147R mutant virus developed pneumonia without isolation of bacterial pathogens, suggesting viral pneumonia with this dual mutant virus.

Immunocompromised patients are at great risk for emergence of the antiviral resistant virus because of the selective pressure from prolonged exposure to antiviral drugs [16]. A high rate and prolonged shedding of the H275Y mutant A(H1N1)pdm09 virus in immunocompromised patients treated with oseltamivir and/or peramivir were reported previously [17]. As a specimen from the husband of the patient was unavailable, we cannot rule out that the patient was infected with a

virus readily carrying the G147R substitution. Results of this study nevertheless suggest that this substitution likely occurred in the patient during peramivir treatment. On the other hand, whether the H275Y mutation had already occurred or not before infection remains unclear. Other additional substitutions, I223R and S247N, in the NA protein of H275Y mutant A(H1N1)pdm09 viruses have been reported and showed a synergistic effect with the H275Y substitution on the reduction of NA inhibitor susceptibility [18,19]. Although the frequencies of these dual substitutions were low, the surveillance of antiviral-resistant viruses should be continued to protect public health and support clinical management, particularly for high risk populations.

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TABLE

Influenza A(H1N1)pdm09 viruses with G147R substitution submitted to the GISAID's EpiFlu Database

GISAID Isolate ID	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	NA substitution	
EPI_ISL_63723	Finland	2009-07-24	A/Finland/614/2009	Other Database Import		G147R	275H
EPI_ISL_108540	China	2011-01-08	A/Tianjinhedong/SWL44/2011	Other Database Import		G147R	275H
EPI_ISL_123113	Singapore	2011-02-09	A/Singapore/SGHo2/2011	Other Database Import		G147R	275H
EPI_ISL_98830	Argentina	2011-08-05	A/Argentina/656/2011	Instituto Nacional de Enfermedades Infecciosas	Centers for Disease Control and Prevention	G147R	275H
EPI_ISL_150164	India	2013-09-07	A/India/3743/2013	National Institute of Virology	Centers for Disease Control and Prevention	G147R	275H
EPI_ISL_212270	UK	2014-03-13	A/England/354/2014	Microbiology Services Colindale, Public Health England	Microbiology Services Colindale, Public Health England	G147R	275H
EPI_ISL_164370	India	2014-05-24	A/India/5964/2014	National Institute of Virology	Centers for Disease Control and Prevention	G147R	275H
EPI_ISL_207955	United States	2016-01-01	A/Wisconsin/02/2016	Wisconsin State Laboratory of Hygiene	Centers for Disease Control and Prevention	G147R	275H
EPI_ISL_209086	Russian Federation	2016-01-15	A/Orenburg/05/2016	Information not available	State Research Center of Virology and Biotechnology Vector	G147R	275H
EPI_ISL_220376	Japan	2016-03-11	A/Hiroshima/13/2016	Hiroshima Prefectural Technology Research Institute	National Institute of Infectious Diseases	G147R	H275Y

GISAID: Global Initiative on Sharing All Influenza Data; NA: neuraminidase.

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Conflict of interest

None declared.

Authors' contributions

Designed the analyses: ET, SF, SW, TO. Analysed and interpreted data: ET, SF, MS, KN, NK, TK, YS, TS, SW, TO. The members of the Influenza Virus Surveillance Group of Japan. Drafted the article: ET. Revised the article: SW, TO.

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