



Early findings of oseltamivir-resistant pandemic (H1N1) 2009 influenza A viruses in Taiwan

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ABSTRACT

In this study, we investigated the frequency of oseltamivir resistance in pandemic (H1N1) 2009 influenza A viruses in Taiwan and characterized the resistant viruses. From May 2009 to January 2010, 1187 pandemic H1N1 virus-positive cases in Taiwan were tested for the H275Y substitution in the neuraminidase (NA) gene that confers resistance to oseltamivir. Among them, eight hospitalized cases were found to be infected with virus encoding the H275Y substitution in their original specimens collected after oseltamivir treatment. The epidemiologic investigation indicated that each of the cases occurred sporadically and there was no evidence of further transmission. We monitored the variation of amino acid residues at position 275 of the NA gene in a series of specimens taken at various time-points and observed that viruses encoding the H275Y substitution differ in their fitness in vivo and in MDCK cells. Phylogenetic analysis indicated that the hemagglutinin (HA) sequences of oseltamivir-resistant pandemic H1N1 viruses exhibited greater diversity than the NA sequences and progressive changes of the HA genes from clade A1 into A2 and from there into clade A3 were observed. The resistant viruses seemed to occur in combination with diverse HA genes and a dominant NA gene. Enzymatic analysis of the viruses revealed that the ratio of NA/HA activities in oseltamivir-resistant viruses was reduced considerably compared to those in wild-type ones.

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1. Introduction

Influenza viruses cause annual epidemics in many countries and occasional worldwide pandemics. It was estimated that 250,000–500,000 deaths are associated with influenza epidemics every year (WHO, 2009a). Oseltamivir, one of the marketed influenza virus neuraminidase inhibitors, has been used for the treatment and prophylaxis of influenza and was stockpiled for pandemic influenza. The incidence of oseltamivir-resistant virus varied from 0.3 to 18% during the clinical trials (Ward et al., 2005; Whitley et al., 2001) and the oseltamivir-resistant influenza viruses were detected rarely among circulating viruses before 2007 (WHO, 2007). From the season of 2007–2008, oseltamivir-resistant seasonal influenza A (H1N1) viruses were detected in Europe and spread globally (Hauge et al., 2009; Hurt et al., 2009a; Meijer et

al., 2009). By the season of 2008–2009, most isolated seasonal influenza H1N1 strains were found to encode the H275Y substitution in the NA gene, conferring resistance to oseltamivir (WHO, 2010a). Emergence and spread of oseltamivir-resistant viruses pose a concern regarding the strategies for treating and preventing influenza.

An influenza outbreak caused by swine-origin influenza A (H1N1) viruses was detected initially in Mexico and USA in March–April 2009 (CDC, 2009a). The viruses spread rapidly and caused the first influenza pandemic of the 21st century. This pandemic (H1N1) 2009 influenza virus was sensitive to oseltamivir but resistant to the M2 inhibitors amantadine and rimantadine (CDC, 2009b). Since the first oseltamivir-resistant virus was reported in June 2009, an additional 225 cases out of more than 20,000 specimens were reported and confirmed worldwide as of February 2010 (WHO, 2010b). Sixteen cases of these oseltamivir-resistant viruses had no known association with oseltamivir treatment and seven were identified as a cluster with epidemiological linkage (Bai et al., 2009; Mai et al., 2009; WHO, 2010b). This finding raises a concern that oseltamivir-resistant pandemic (H1N1) 2009 influenza virus might spread and become predominant.

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In order to investigate the frequency of oseltamivir-resistant pandemic (H1N1) 2009 influenza A viruses in Taiwan, we collected the pandemic H1N1 virus-positive original specimens as well as the respective isolates after culture and analyzed the sequences of their NA genes. In addition, to understand the developmental process of oseltamivir-resistant pandemic H1N1 viruses, we analyzed the clinical specimens taken at different time-points, including those collected before or after oseltamivir treatment as well as their respective cultured isolates. The findings in the present study highlight the need for intensive surveillance of resistant viruses and the importance of direct gene analysis from clinical specimens for drug resistance surveillance.

2. Materials and methods

2.1. Collection of specimens and viral isolates

This study was conducted within the frame of the national influenza surveillance in Taiwan. Influenza isolates or original specimens from hospitalized patients, outpatients in communities, and clustering patients were collected and submitted to Taiwan CDC. In general, throat and nasal swabs were collected from patients who had influenza-like illness with or without severe complications and transported to the laboratories of the influenza surveillance network in Taiwan for analysis. From May 2009 to January 2010, of the 1187 pandemic H1N1 virus-positive cases, 604 were from hospitalized patients, 402 were from outpatients in communities, and 171 were from clusters. Among them, eight cases from hospitalized patients were found to encode the H275Y substitution in the NA gene in their original specimens. Amino acid sequences were predicted from the viral NA nucleotide sequences spanning position 275 following conventional RT-PCR and eight cases were characterized as oseltamivir-resistant by the presence of a tyrosine codon (TAC) at position 275 (N1 numbering). When the case was positive for the H275Y substitution, an epidemiological investigation was undertaken to determine the source of resistant viruses and whether further transmission had occurred. All of the H275Y-positive specimens were collected from patients who had been treated with oseltamivir. To express each of the original specimens and the respective cultured isolates from the eight cases more clearly and easily, we also used representative codes, comprised by case number, the nature of the preparation (original specimens or cultured isolates, denoted as O or C, respectively), the day of collection, and the amino acid sequence at position 275 in the NA gene. In case 1, for example, the oseltamivir-sensitive virus, A/Taiwan/6662/2009, isolated from day 1 could be represented as case 1-C1H and the resistant one, A/Taiwan/6663/2009, isolated from day 4 as case 1-C4Y. Similarly, in case 7, the virus in the original specimen collected at day 6, and the respective cultured isolate, A/Taiwan/7949/2009, harbored the mixture of the 275H and 275Y in NA gene, were denoted as case 7-O6H/Y and case 7-C6H/Y, respectively.

2.2. RNA extraction and identification of pandemic H1N1 viruses

Viral RNA was extracted from 140 µl of the supernatant of original swab specimens and cultured viral isolates using QIAamp Viral RNA Mini Kits, according to the Manufacturer's instructions (Qiagen, Santa Clara, CA). Automated extraction also was conducted using the MagNa Pure LC extraction system (Roche). Extracted RNA was first analyzed to determine the presence of pandemic H1N1 viruses by one-step real-time RT-PCR (Yang et al., 2009). Oseltamivir resistance was determined by conventional RT-PCR using QIAGEN OneStep RT-PCR Kit (Qiagen) and primers published by WHO spanning position 275 (N1 num-

bering, 274 in N2 numbering) of the neuraminidase (NA) gene (NA-536F 5'-GGTCAGCAAGCGCWGTCATGA-3' and NA-1326R 5'-GCTGCTYCCRCTAGTCCAGAT-3') (WHO, 2009b). Nucleotide sequences of the amplified PCR products were determined and used for further analysis.

2.3. Genetic analysis of the pandemic H1N1 virus

The full-length sequences of HA and NA genes of the virus isolates encoding the H275Y substitution also were determined using the primers and protocol published by WHO (WHO, 2009b). To provide a more detailed comparison of the genetic characteristics and relationship of the oseltamivir-resistant viruses in our study, the full-length sequences of the HA and NA genes were analyzed together with two sets of the selected reference strains. The first set comprised the dominant oseltamivir-sensitive pandemic H1N1 isolates defined by the number of virus isolates with more than 20 (for HA) or 10 (for NA) identical amino acid sequences of HA and NA genes submitted to GenBank before March 25, 2010. Selected based on the HA genes were A/New York/31/2009 (*n*=469), A/Mexico/4115/2009 (*n*=305), A/Canada-QC/RV1595/2009 (*n*=53), A/California/VRDL61/2009 (*n*=52), A/Australia/1/2009 (*n*=27), A/California/05/2009 (*n*=23) and A/Wisconsin/629-S1384/2009 (*n*=22). Selected for the NA genes were A/Mexico/4482/2009 (*n*=1007), A/California/04/2009 (*n*=212), A/California/05/2009 (*n*=52), A/Louisiana/03/2009 (*n*=24), A/Pennsylvania/10/2009 (*n*=13), A/Michigan/02/2009 (*n*=11) and A/Toronto/3184/2009 (*n*=10). The second set comprised all of the oseltamivir-resistant viruses with the full-length HA and NA genes available in GenBank, including A/Denmark/528/2009 (*n*=9), A/Osaka/180/2009, A/Washington/29/2009, A/Washington/28/2009, A/Texas/47/2009 and A/Nagasaki/HA-58/2009. The vaccine strain A/California/07/2009 also was included. Multiple sequence alignments, protein translation and phylogenetic analysis were performed on the basis of nucleotide sequences using the software MEGA4 (Tamura et al., 2007) and BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). A phylogenetic tree was constructed by the neighbor-joining method and 1000 bootstrap replications were performed to evaluate the reliabilities.

2.4. NA inhibition assay of the cultured pandemic H1N1 virus isolates

The 50% inhibitory concentration (IC₅₀) analysis of oseltamivir and zanamivir for the pandemic H1N1 isolates was determined using the NA-Star Influenza Neuraminidase Inhibitor resistance Detection Kit (Applied Biosystems) according to the Manufacturer's recommendations. Briefly, 25 µl of half-log dilutions of neuraminidase inhibitor (NI) from 0.03 to 1000 nM were mixed with 25 µl of virus dilution with a hemagglutination titer greater than or equal to 16 in each well of a white 96-well microplate and incubated 10–20 min at 37 °C. Two wells of the mixture destined to be negative controls contained only assay buffers, instead of NI, and culture medium, instead of virus, also were included. Then, 10 µl of diluted substrate was added to each well and incubated for 10–30 min at room temperature, followed by the addition of 60 µl of accelerator, the chemiluminescent signal being measured immediately. The software GraphPad Prism version 4.00 was used to determine the IC₅₀ values. For NA enzyme activity determination, 25 µl of virus dilution was mixed with 25 µl of assay buffers, instead of NI. One well of negative control contained assay buffers and culture medium also was included. All of the experiments of IC₅₀ and NA enzyme activity determination were done with three replicates.

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