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BRIEF REPORT

Oseltamivir-resistant influenza viruses isolated in South Korea from 2005 to 2010

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Abstract South Korean isolates of oseltamivir-resistant influenza viruses from 2005–2010 were investigated with a total 491 influenza viruses identified from 1702 specimens. Neuraminidase genes from 342 influenza viruses (71 A/H1N1, 74 pandemic A/H1N1 2009, 117 A/H3N2, and 80 B) were analyzed by RT-PCR with molecular markers for oseltamivir resistance. The H274Y mutation in the NA protein was identified in 100 % (n=40) of A/H1N1 viruses circulating in 2008–2009. Influenza A/H1N1 viruses harboring the H274Y substitution exhibited, on average, a 626-fold reduction in oseltamivir susceptibility and clustered with the A/Norway/1736/2007 strain. Close and timely monitoring for resistance to clinically available influenza antivirals should be consistently performed.

Keywords Influenza viruses · Neuraminidase · Oseltamivir resistance

Influenza viruses are leading etiological agents of acute respiratory-related diseases in humans and cause seasonal epidemics and occasional pandemics with substantial morbidity and mortality across the world [1]. Highly pathogenic avian H5N1 viruses in Asia were first detected in geese in China in 1996, and confirmed human cases of influenza A (H5N1) virus infections were reported in Hong Kong in 1997 [2]. The emergence and global spread of the pandemic A/H1N1 2009(A/H1N1pdm) viruses in 2009 have caused public concerns regarding the management of influenza virus infections [3]. Although annual vaccination remains the primary way to control influenza virus infections, antiviral drugs are a valuable alternative to prevent and treat pandemic influenza virus infections [4]. Amantadine has been used successfully against influenza A virus infections for over 30 years. A high prevalence of amantadine-resistant influenza viruses has been detected around the world since 2003 [5]. Two neuraminidase (NA) inhibitors, orally active oseltamivir and inhaled zanamivir, are the currently recommended drugs for treating influenza A and B virus infections [6]. Use of oseltamivir and/or natural genetic variation in the gene encoding NA can result in a reduction of oseltamivir susceptibility [7]. Oseltamivir-resistant seasonal A/H1N1 viruses emerged in Europe in 2007 [8]. The World Health Organization (WHO) reported that oseltamivir-resistant A/H1N1 viruses were isolated from more than 39 countries in 2008 [9–13]. Oseltamivir-resistant A/H5N1 viruses were isolated from infected patients in 2005 [14], and the recent identification of oseltamivir-resistant A/H1N1pdm viruses has raised public concerns worldwide [15–18]. The recent increasing

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resistance to oseltamivir among influenza viruses has necessitated a close surveillance system.

In South Korea, amantadine-resistant influenza A viruses have emerged: A/H1N1 in 2005–2006, A/H3N2 in 2003–2004, and A/H1N1pdm in 2008–2009 [19, 20]. Unlike amantadine resistance, only sporadic cases of oseltamivir-resistant A/H1N1pdm viruses have been reported recently [15], and systemic surveillance data for oseltamivir resistance has not been reported to date. In the present study, intensive surveillance of oseltamivir resistance was conducted using influenza viruses collected between 2005 and 2010 in Gyeonggi Province, a region located around Seoul, where approximately 12 million people (24 % of the population of South Korea) reside, and variations in the genotypic patterns of these isolates were analyzed.

A total of 1702 nasopharyngeal swabs were collected from outpatients with symptoms of influenza-like illness (ILI) from 10 general hospitals in Gyeonggi Province, South Korea, from December 1, 2005 to August 29, 2010. The influenza seasons examined represent 12-month periods from September to August (e.g., 2009–2010 includes isolates gathered from September 1, 2009 to August 31, 2010). Viruses were propagated in Madin-Darby canine kidney (MDCK) cells (ATCC No. CCL-34) until a cytopathic effect (CPE) was observed in over 80 % cells in a monolayer culture. Viral RNA was extracted from the supernatants of MDCK cell cultures using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). Virus types and subtypes were determined by reverse transcription polymerase chain reaction (RT-PCR) with specific primers targeting the hemagglutinin (HA) gene of A/H1N1 and A/H3N2 viruses and the nucleoprotein (NP) gene of B viruses (Supplementary Table 1) [20]. Quantitative real-time RT-PCR (qRT-PCR) was also used to detect the HA gene of A/H1N1pdm viruses (Supplementary Table 1) [20]. Monitoring resistance to oseltamivir was primarily based on sequence analysis of the NA gene. The NA genes of 262 influenza A and 80 influenza B viruses were amplified by RT-PCR using type- or subtype-specific primers (Supplementary Table 2). To analyze the NA genes (1422 bp of A/H1N1, 1455 bp of A/H3N2, 1413 bp of A/H1N1pdm, and 1487 bp of B), sequences were aligned using DNASTAR (DNASTAR Inc. Software, Madison, WI, USA). The influenza virus type- and subtype-specific molecular markers of oseltamivir resistance were used as reported previously [7, 21, 22]. Phylogenetic analysis was performed with 48 NA nucleotide sequences from influenza viruses, including 20 A/H1N1 viruses whose sequences are contained in the GenBank database (accession no. JX465434–JX465477 and KC690002–690005; Supplementary Table 3). They were compared with NA sequences of three vaccine and 22 reference strains from GenBank (accession no. CY033624, EU124136, CY030233, FJ445080, FJ403567, CY030866, HQ291902, CY043707, FJ403546, EU516112, FJ445025, FJ403552, EU551822, FJ403588, FJ403550, GQ476109, FJ743468, FJ743465, FJ687029, CY043555, GQ475954, FJ403585, HQ291904, KC475826 and EU566972). Phylogenetic trees were constructed by the neighbor-joining method using MEGA (version 5.0) with bootstrap analysis (n = 1000).

A total of 359 (21.1 %) influenza A and 132 (7.8 %) influenza B viruses were detected from 1702 specimens using RT-PCR or qRT-PCR during the study period (Supplementary Table 4). Of these, 262 influenza A (71 A/H1N1, 74 A/H1N1pdm, and 117 A/H3N2 strains) and 80 influenza B viruses were selected based on season, frequency, and type/subtype in order to determine the recent status and spread of oseltamivir-resistant influenza viruses. With assistance from molecular markers in the NA protein, all influenza viruses were screened by sequencing the NA gene (Table 1). All A/H1N1 viruses (n = 40) from the 2008–2009 season contained an H274Y mutation (N2 numbering here and throughout the text), the major cause of oseltamivir resistance in influenza viruses [7]. H274Y mutations were not detected in the A/H1N1pdm, A/H3N2, and B strains; and other substitutions in NA proteins, related to oseltamivir resistance were not detected in these viruses. These substitution mutations included A/H1N1 (D79G, H126N, Q136K, Y155H, S247G, G248R, and I266V), A/H1N1pdm (V116A, I117V/M, E119G/A/D, Q136K, K150N, D151A/V/N, D198G/E/N, I222M/R/V, and N294S), A/H3N2 (E41G, E119V/G/D, Q136K, D151A/V/N, R152K, V165I, I222R/Q, Q226H, G248R, K249E, D251G, H274N, R292K, and N294S), and B (E119V, R152K, D198E/N, I222T, S250G, T325I, R371K, and G402S).

NA inhibition (NAI) assays for influenza viruses was performed using oseltamivir carboxylate (Hoffman-La Roche, Switzerland) and a commercially available kit (NA-Star; Applied Biosystems, Foster City, CA, USA) [7]. Before the NAI assay, an NA activity test was performed with serially diluted supernatants of MDCK cell cultures to normalize the amount of the viruses and to reduce signal-to-noise ratio. NAI assays were conducted with 60 influenza A/H1N1 viruses and the oseltamivir-resistant A/Kitakyusyu/10/2006(H1N1) strain as a positive control. Luminescence was measured using a multiplate Victor3 reader (Perkin-Elmer, Shelton, CT, USA), and the 50 % inhibitory concentration (IC_{50}) was determined using Prism software (GraphPad Software, La Jolla, CA, USA).

To identify the biological phenotype, a fluorometric NAI assay was conducted on 35 A/H1N1 viruses from the 2008–2009 season carrying the H274Y substitution. A total of 25 A/H1N1 viruses harboring the H274 mutation, 8

Table 1 Frequency of oseltamivir resistance markers in the NA proteins of influenza A and B viruses isolated in Gyeonggi Province from 2005 to 2010

Season	H274Y mutation/influenza virus examined (%)			
	A/H1N1[a]	A/H1N1pdm[b]	A/H3N2[c]	B[d]
2005–2006	0/11 (0)	–	0/2 (0)	0/8 (0)
2006–2007	–	–	0/61 (0)	–
2007–2008	0/20 (0)	–	0/5 (0)	0/14 (0)
2008–2009	40/40 (100)	0/2 (0)	0/49 (0)	–
2009–2010	–	0/72 (0)	–	0/58 (0)
Total	40/71 (56)	0/74 (0)	0/117 (0)	0/80 (0)

[a] D79G, H126N, Q136K, Y155H, S247G, G248R, and I266V substitutions were not found [7]

[b] V116A, I117V/M, E119G/A/D, Q136K, K150N, D151A/V/N, D198G/E/N, I222M/R/V, and N294S substitutions were not found [21]

[c] E41G, E119V/G/D, Q136K, D151A/V/N, R152K, V165I, I222R/Q, Q226H, G248R, K249E, D251G, H274N, R292K, and N294S substitutions were not found [7]

[d] E119V, R152K, D198E/N, I222T, S250G, T326I, R371K, and G402S substitutions were not found [7, 22]

"–" indicates that no isolates were obtained

Table 2 The mean IC_{50} values in the fluorometric neuraminidase inhibition assay for influenza A/H1N1 viruses collected from 2005 to 2010

Season	Mean IC_{50} [nM] (range)[a]	Phenotype[b]	H274Y[c]
A/Kitakyusyu/10/2006[d]	71.39	R	Y
2005–2006 (n=8)	0.23 (0.14 – 0.29)[e]	S	H
2007–2008 (n=17)	0.16 (0.09 – 0.29)[e]	S	H
2008–2009 (n=35)	112.59 (50.74 – 176.41)[e]	R	Y

[a] The 50 % inhibitory concentration (IC_{50}) is the concentration of drug required to inhibit NA enzyme activity by 50 %

[b] S, oseltamivir susceptible; R, oseltamivir resistant

[c] H274Y indicates that a histidine (H) residue has been substituted for a tyrosine (Y) residue at position 274 of the NA protein

[d] Oseltamivir-resistant A/H1N1 positive control

[e] Mean IC_{50} values for each A/H1N1 virus are provided in Supplementary Table 5

strains from 2005–2006 and 17 strains from 2007–2008, were used as a panel of controls to confirm oseltamivir resistance (Table 2). The A/H1N1 viruses carrying the H274Y substitution exhibited elevated IC_{50} values for oseltamivir, ranging from 50.74 to 176.41 nM (mean IC_{50} value, 112.59 nM) (Table 2). In contrast, the mean IC_{50} value for the H274 A/H1N1 viruses from 2005–2006 was 0.23 nM, ranging from 0.14 to 0.29 nM, and for A/H1N1 viruses from 2007–2008, it was 0.16 nM, ranging from 0.09 to 0.29 nM (cumulative mean IC_{50} value, 0.18 nM) (Supplementary Table 5). The mean IC_{50} value for A/H1N1 viruses harboring the H274Y substitution in the 2008–2009 season was 626-fold higher than the cumulative mean IC_{50} of A/H1N1 viruses carrying the H274 from the 2005–2006 and 2007–2008 seasons.

To determine the genetic relationship of oseltamivir-resistant A/H1N1 viruses carrying the H274Y substitution, phylogenetic analysis of the NA nucleotide sequence was performed with four A/H1N1 viruses from 2005–2006, four A/H1N1 viruses from 2007–2008, and 12 A/H1N1 viruses from the 2008–2009 season. The phylogenetic results showed that 12 oseltamivir-resistant A/H1N1 viruses from the 2008–2009 season were clustered with the A/Norway/1736/2007(H1N1) strain (Fig. 1). They belonged to subclade 2B.II, whose members possess two amino acid substitutions in NA: H274Y and D357G. On the other hand, four oseltamivir-susceptible A/H1N1 viruses from the 2005–2006 season belonged to clade 2A, including the A/Solomon Island/3/2006(H1N1) strain, and four oseltamivir-susceptible A/H1N1 viruses collected from the 2007–2008 season belonged to clade 2C with A/Hong Kong/2652/2006(H1N1) strain.

Prior to the 2007–2008 influenza season, the frequency of resistance to oseltamivir was less than 0.5 % among field isolates worldwide [7, 23]. However, seasonal A/H1N1 viruses that were resistant to oseltamivir emerged in Europe in 2007 and spread globally in 2008 [7, 13]. In this study, we report the emergence of oseltamivir-resistant seasonal influenza A/H1N1 viruses carrying the H274Y substitution in South Korea during the 2008–2009 season. While markers for oseltamivir resistance were not detected in the NA protein of A/H1N1pdm, A/H3N2, and B viruses, the H274Y substitutions were detected exclusively in A/H1N1 viruses from the 2008–2009 season. In Japan, however, oseltamivir-resistant A/H1N1 viruses carrying the H274Y substitution were detected at a prevalence of 0.4 % and 100 % during the 2007–2008 and 2008–2009 season, respectively [10]. The H274Y substitution in the NA protein is known to lead to oseltamivir resistance in influenza A and B viruses [7]. Other substitutions in the NA proteins that were presumably associated with oseltamivir resistance were not found in every subtype of influenza viruses. Additionally, no substitutions related to zanamivir resistance in the NA protein were detected in the influenza A/H1N1, A/H1N1pdm, A/H3N2, and B viruses.

All A/H1N1 viruses from the 2008–2009 season harboring the H274Y substitution showed amantadine susceptibility based on M2 sequence analysis and virus yield reduction assays [5], while A/H1N1 viruses carrying H274 circulating during the 2007–2008 were all amantadine resistant and belonged to clade 2C, and all of them had the S31N substitution in the M2 protein [19, 20]. Phylogenetic

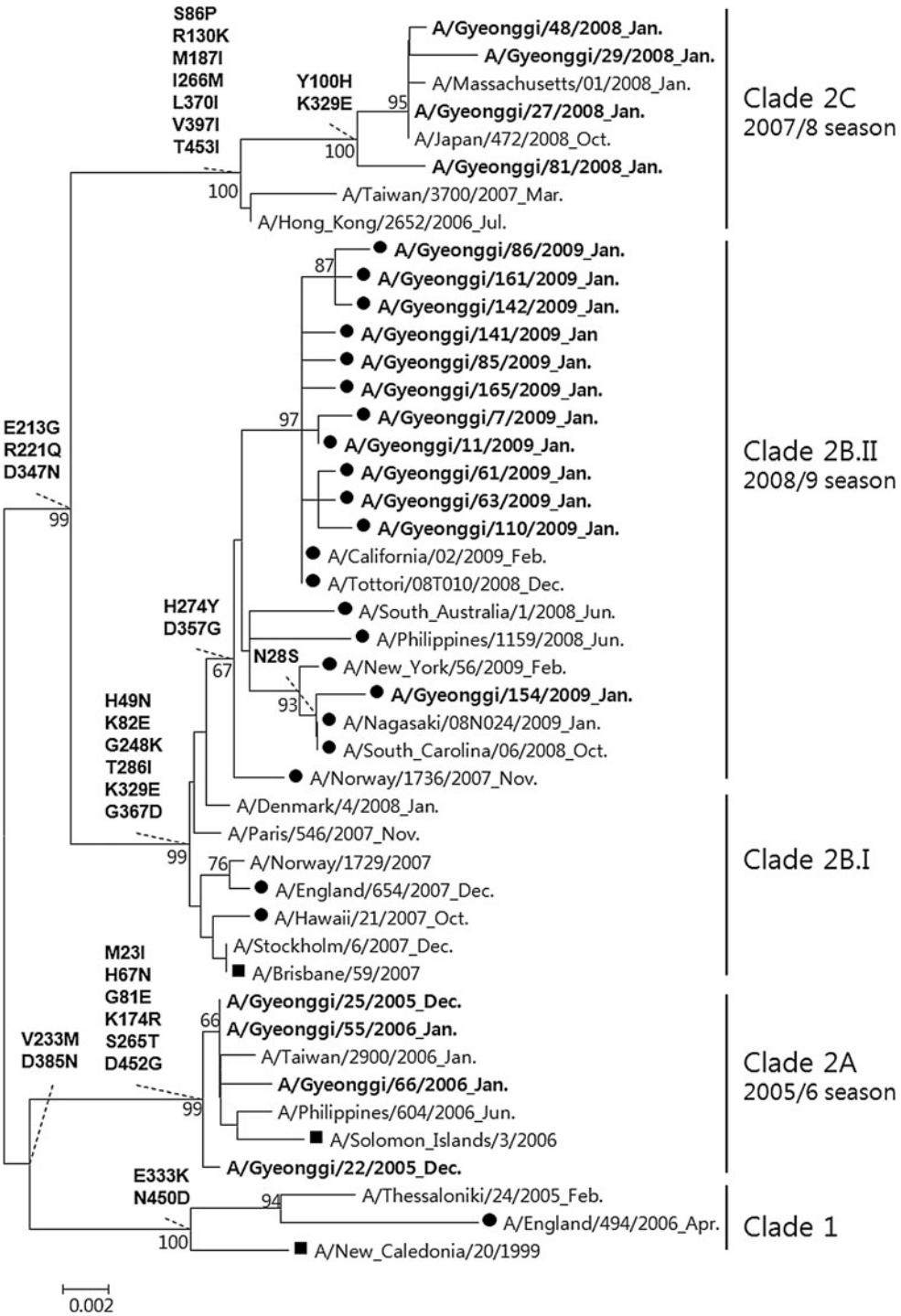

Fig. 1 Phylogenetic analysis of the neuraminidase (NA) genes of influenza A/H1N1 viruses during 2005–2010. Influenza A/H1N1 viruses isolated in this study are in boldface type. Filled squares (■) indicate vaccine strains, and filled circles (●) represent oseltamivir-resistant strains. Phylogenetic trees were constructed using a 1372-nucleotide region of the NA gene, by the neighbor-joining method, using MEGA (version 5.0), and bootstrap analysis (n = 1,000). Bootstrap values less than 60 % are omitted, and representative amino acid substitutions (N2 numbering) are shown at the branch points. The month of isolation is indicated at the end each strain designation

analysis of the NA proteins showed that oseltamivir-resistant A/H1N1 viruses from the 2008–2009 season clustered with the A/Norway/1736/2007 strain, belonging to subclade 2B.II, referred to as the "Northern European" lineage [8, 10, 13]. This lineage had common amino acid changes, H274Y and D357G in the NA protein when compared with the A/Brisbane/59/2007 strain (subclade 2B.I). Phylogenetic analysis of the HA gene revealed that oseltamivir-resistant A/H1N1 viruses from the 2008–2009 season had an A193T (H3 numbering) substitution in HA1 domain, a substitution that had been detected previously in the Northern European lineage [10]. This suggests that oseltamivir-resistant A/H1N1 influenza viruses originating in Northern Europe in 2007–2008 might have been extensively circulating in South Korea in 2008–2009. In South Korea, oseltamivir was licensed in November 2001

by Korea Food and Drug Administration (KFDA). The drug has been prescribed with caution by physicians for the treatment of influenza infections because of its severe side effects [24]. However, it was extensively used during the spread of influenza A/H1N1pdm viruses [25]. Based on the frequency of oseltamivir resistance and on the results of the phylogenetic study of A/H1N1 viruses, we assume that the extensive circulation of oseltamivir-resistant A/H1N1 viruses in 2008–2009 might have arisen due to influx of the virus from abroad rather than by use of the drug in South Korea.

Novel A/H1N1pdm (A/California/04/2009(H1N1)) viruses are reassortants that acquired M and NA gene segments from a Eurasian amantadine-resistant swine influenza virus [3]. The A/H1N1pdm viruses present during 2009–2010 were all amantadine resistant as well as being susceptible to oseltamivir in this study [20]. Although 10 sporadic cases of oseltamivir-resistant A/H1N1pdm viruses with the H274Y mutation in the NA protein were reported from patients receiving treatment with oseltamivir in South Korea [15], oseltamivir-resistant A/H1N1pdm viruses were not found during this study period. This might be a reason that oseltamivir-resistant A/H1N1pdm viruses have not been extensively disseminated into the local community. However, further surveillance is necessary to monitor the emergence of oseltamivir-resistant A/H1N1pdm influenza viruses, as these oseltamivir-resistant viruses are widespread in other places [26].

In the present study, oseltamivir-resistant A/H1N1 viruses were found to be highly dominant during the 2008–2009 season in Gyeonggi Province, a densely populated region surrounding Seoul in South Korea. Oseltamivir resistance during the 2008–2009 season was conferred through the H274Y mutation within the NA protein of A/H1N1 viruses. The rise in frequency of oseltamivir resistance needs to be seriously considered, because oseltamivir is the one of stockpiled antiviral agents in South Korea for the control of avian H5N1 and pandemic influenza infections. With the increased use of NA inhibitors to treat influenza infections, establishing active drug resistance monitoring programs is essential for better management of public health.

Conflict of interest The authors declare no conflicts of interest.

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