



ORIGINAL ARTICLE

Generation and Characterization of Recombinant Influenza A(H1N1) Viruses Resistant to Neuraminidase Inhibitors

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Abstract

Objectives: To examine the effect of neuraminidase (NA) mutations on the NA inhibitor (NAI) resistance phenotype, the recombinant influenza A/Chungbuk/4448/2008(H1N1) virus isolated in South Korea during the 2008–2009 season was generated by reverse genetics.

Methods: Site-directed mutagenesis was introduced on the NA gene of A/Chungbuk/4448/2008(H1N1) virus, and a total of 23 single, double, and triple mutants were generated. Resistance phenotype of these recombinant viruses was determined by NA-inhibition (NAI) assays based on a fluorometric method using two NAIs (oseltamivir and zanamivir).

Results: NA-inhibition assays showed that all the single and double mutants containing the Y275 except the single Y275-E119V mutant conferred important levels of resistance to oseltamivir, whereas all the single, double, and triple mutants containing the E119V mutation were associated with the resistance to zanamivir.

Conclusion: Considering the effect of mutations in NA gene on the resistance to NAIs, it is important to monitor the possible emergence and dissemination of multidrug-resistant variants in the human population due to amino acid changes at NA gene as well as to develop novel NAIs.

1. Introduction

Influenza infects approximately 20% of the world's population, and more than half a million individuals die every year of influenza-associated complications [1]. In 2009, the pandemic influenza A(H1N1) virus spread

quickly among humans worldwide to cause the first influenza pandemic of the 21st century [2].

Vaccination and antiviral treatment are essential for the prevention and control of influenza infection. With the use of antiviral drugs for the clinical management of influenza, neuraminidase (NA) inhibitors (NAIs) have

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been proved to be very effective against influenza A and B viruses [3]. However, NAI-resistant strains have been reported frequently [4,5], in case of the emergence during the treatment of drug or dissemination of drug-resistant variants.

Several subtype-specific mutations in framework or catalytic residues of NA that confer resistance to NAIs have been reported [1,6–8]. The dominant change conferring oseltamivir resistance in the current seasonal influenza viruses is a mutation in the NA gene, H275Y (N1 numbering). The frequency of isolates with the H275Y mutation has increased with each flu season, including in countries where oseltamivir is not prescribed regularly. Particularly, most isolated seasonal influenza A(H1N1) viruses during the 2008–2009 season were found to encode the H275Y substitution in the NA gene, conferring resistance to oseltamivir [9]. Other NA mutations (N2 numbering: E119G, H274Y, R292K, and N295S) that have been reported to confer resistance to NAIs were each introduced into recombinant A/Vietnam/1203/04(H5N1) influenza virus [10].

Here we selected A/Chungbuk/4448/2008(H1N1) virus isolated in South Korea during the 2008–2009 season, which was one of the viruses isolated from throughout the country by the Korean Influenza Surveillance Scheme [11]. To investigate the effect of different NA mutations on the NAI resistance phenotype of A/Chungbuk/4448/2008(H1N1) virus, we generated recombinant A/Chungbuk/4448/2008(H1N1) virus using a reverse genetics system, introduced the different NA mutations into the background of the recombinant A/Chungbuk/4448/2008(H1N1) virus, and compared their NAI resistance phenotypes.

2. Materials and methods

2.1. Cells and viruses

Madin Darby canine kidney (MDCK) cells and human embryonic kidney cells transformed with large T antigen (293T cells) were obtained from American Type Culture Collection (Manassas, VA, USA). A/Chungbuk/4448/2008(H1N1) virus isolated in South Korea during the 2008–2009 season was used for the generation of recombinant viruses.

2.2. Antiviral compounds

Tartrate salt of oseltamivir carboxylate (active form of Tamiflu) was provided generously by F. Hoffmann-La Roche, Inc. (Basel, Switzerland); it was dissolved in distilled water such that the final oseltamivir carboxylate concentration was 10 mM. Zanamivir (Relenza), which was provided by GlaxoSmithKline (Stevenage, UK), was dissolved in distilled water to a final zanamivir concentration of 10 mM. Aliquots were stored at -20°C until use.

2.3. Generation of recombinant viruses and site-directed mutagenesis

Recombinant viruses were generated using the eight-plasmid reverse genetics system with pHW2000 (the vector was provided kindly by Robert Webster, St. Jude Children's Research Hospital, Memphis, TN, USA) [12]. The eight genes from the cultured A/Chungbuk/4448/2008(H1N1) virus were amplified by reverse transcription polymerase chain reaction (RT-PCR) and incorporated into the pHW2000 plasmid. All eight plasmids were transfected into a coculture of 293T and MDCK cells for 3 days, and then further prepared in 10-day-old embryonated chicken eggs. The collected supernatant from the transfected cells was injected into the allantoic cavities of the eggs and incubated at 37°C for 3 days to amplify the rescued viruses. The viruses generated in eggs were stored at -80°C until use.

To investigate mutations at NA key residues (N1 numbering: I117V, I117M, E119V, I223V, Y275H, R293K, N295S, and S334N), site-directed mutagenesis was conducted on the NA gene cloned plasmid using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA). All recombinant plasmids were sequenced to ensure the absence of undesired mutations. The eight plasmids were then cotransfected to 293T mixed with MDCK cells. Supernatants were collected at 3 days post-transfection and used to inoculate specific pathogen-free (SPF) eggs. Introduced mutations of the recombinant wild type virus as well as the NA mutants were confirmed by sequencing.

2.4. NA activity assay and NA inhibition assay

The NA activity of each virus sample was determined by a modified fluorometric assay that measured 4-methylumbelliferone released from the fluorogenic substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA; Sigma-Aldrich, St. Louis, MO, USA) by the enzymatic activity of the influenza virus NA [13,14].

The drug resistance phenotype was determined by NA inhibition assays using the MUNANA (Sigma) substrate, with minor modifications [13–15]. The viruses were tested for susceptibility to oseltamivir and zanamivir. To determine the drug concentration required to inhibit 50% of the NA activity [50% inhibitory concentration (IC_{50})], 50 μL of virus, diluted according to the NA activity assay, was mixed with various concentrations of inhibitor in microtiter plates (FluoroNunc plates; Nalge Nunc International, Penfield, NY, USA). The final reaction mixture concentration of the NAIs ranged from 0.01 nM to 10,000 nM. The virus–inhibitor mixture was incubated at room temperature for 45 minutes prior to the addition of 50 μL of the MUNANA substrate (0.3 mM) and then incubated at 37°C for 60 minutes. The reaction was terminated by the addition of 100 μL of the stop solution. The IC_{50} for each drug was calculated from the dose–response curve using GraphPad Prism software, version 5 (San Diego, CA, USA). The sensitive viruses

belonged to an IC_{50} range of 0.001–15 nM for zanamivir and 0.001–25 nM for oseltamivir carboxylate. The resistant viruses belonged to both inhibitors in the IC_{50} range from 43 nM to as high as 8020 nM [15].

3. Results

3.1. Generation of recombinant wild type and NA mutant influenza viruses by reverse genetics system

The recombinant wild-type [A/Chungbuk/4448/2008(H1N1)] virus was generated by reverse genetics. That is, the eight genes of A/Chungbuk/4448/2008(H1N1) virus (*PB2*, *PB1*, *PA*, *HA*, *NP*, *NA*, *M*, and *NS*) were amplified by RT-PCR (data not shown). The PCR products were then inserted into pHW2000 vector. After the cell transfection with these eight plasmids, supernatants were collected at 3 days post-transfection and used to inoculate SPF eggs. The generation of recombinant wild-type virus was confirmed by sequencing.

We used mutagenesis and plasmid-based reverse genetics to generate recombinant A/Chungbuk/4448/2008(H1N1) viruses containing either the wild-type NA (Y275) or a single amino acid change at NA residue 117 (I→V), 117 (I→M), 119 (E→V), 223 (I→V), 275 (Y→H), 293 (R→K), 295 (N→S), or 334 (S→N). For example, the recombinant Chungbuk-Y275-I117V virus contained an I→V amino acid change at residue 117 of NA in the wild-type virus [A/Chungbuk/4448/2008(H1N1)] and the recombinant Chungbuk-Y275H-I117V virus contained I→V and Y→H amino acid changes at residues 117 and 275 of NA in the wild-type virus, respectively (numbering is based on the N1 NA protein throughout). Totally, eight single mutants (Y275-I117V, Y275-I117M, Y275-E119V, Y275-I223V, Y275H, Y275-R293K, Y275-N295S, and Y275-S334N), 10 double mutants (Y275H-I117V, Y275H-I117M, Y275H-E119V, Y275H-I223V, Y275H-R293K, Y275H-N295S, Y275H-S334N, Y275-N295S-I117V, Y275-N295S-I117M, and Y275-N295S-I223V), and five triple mutants (Y275H-E119V-I117V, Y275H-E119V-I117M, Y275H-N295S-I117V, Y275H-N295S-I117M, and Y275H-N295S-I223V) were generated (Table 1). Introduced mutations of the recombinant NA mutants were confirmed by sequencing.

3.2. Susceptibility of recombinant wild-type and NA mutant influenza viruses to NAIs in vitro

An enzymatic NA inhibition assay was used to characterize the susceptibilities of recombinant A/Chungbuk/4448/2008(H1N1) virus and each of the 23 mutants to oseltamivir and zanamivir (Table 2). NA-inhibition assays showed that E119V mutation of the wild-type virus containing tyrosine (Y) at NA residue 275 (Chungbuk-Y275-E119V) and E119V mutation of

Table 1. Generation of the recombinant influenza A(H1N1) viruses.

NA mutation ^a	Chungbuk-Y275 ^b		Chungbuk-Y275H ^c	
	Single mutant	Double mutant	Single mutant	Double mutant
I117V	Chungbuk-Y275-I117V		Chungbuk-Y275H-I117V	
I117M	Chungbuk-Y275-I117M		Chungbuk-Y275H-I117M	
E119V	Chungbuk-Y275-E119V		Chungbuk-Y275H-E119V	Chungbuk-Y275H-E119V-I117V Chungbuk-Y275H-E119V-I117M
I223V	Chungbuk-Y275-I223V		Chungbuk-Y275H-I223V	
Y275H				
R293K	Chungbuk-Y275-R293K		Chungbuk-Y275H-R293K	
N295S	Chungbuk-Y275-N295S	Chungbuk-Y275-N295S-I117V Chungbuk-Y275-N295S-I117M Chungbuk-Y275-N295S-I223V	Chungbuk-Y275H-N295S-I117V Chungbuk-Y275H-N295S	Chungbuk-Y275H-N295S-I117V Chungbuk-Y275H-N295S-I117M Chungbuk-Y275H-N295S-I223V
S334N	Chungbuk-Y275-S334N		Chungbuk-Y275H-S334N	

^aNAI numbering; ^bWild-type virus; ^cNA mutant virus at NA residue 275 (Y→H). NA = neuraminidase.

Table 2. Susceptibility of the recombinant influenza A(H1N1) viruses to NAIs.

Recombinant virus	Neuraminidase inhibition assay			
	Oseltamivir		Zanamivir	
	Mean IC ₅₀ ± SD (nM) ^a	Fold change ^b	Mean IC ₅₀ ± SD (nM) ^a	Fold change ^b
Chungbuk-Y275H	1.1 ± 0.5	1.0	2.0 ± 0.6	1.0
Chungbuk-Y275H-I117V	2.0 ± 0.4	1.8	1.1 ± 0.4	0.5
Chungbuk-Y275H-I117M	0.4 ± 0.1	0.4	0.4 ± 0.1	0.2
Chungbuk-Y275H-E119V	16.9 ± 2.0	15.4	271.5 ± 17.7	135.7
Chungbuk-Y275H-I223V	1.9 ± 0.2	1.7	0.6 ± 0.4	0.3
Chungbuk-Y275H-R293K	0.6 ± 0.1	0.5	35.7 ± 6.3	17.8
Chungbuk-Y275H-N295S	44.3 ± 7.0	40.2	2.2 ± 1.0	1.1
Chungbuk-Y275H-S334N	0.7 ± 0.1	0.6	0.4 ± 0.1	0.2
Chungbuk-Y275H-E119V-I117V	7.4 ± 0.4	6.7	781.3 ± 39.5	390.6
Chungbuk-Y275H-E119V-I117M	21.5 ± 2.2	19.5	362.0 ± 22.0	181.0
Chungbuk-Y275H-N295S-I117V	66.1 ± 13.5	60.0	4.0 ± 1.0	2.0
Chungbuk-Y275H-N295S-I117M	57.7 ± 17.2	52.4	7.5 ± 2.2	3.7
Chungbuk-Y275H-N295S-I223V	107.2 ± 8.3	97.4	3.1 ± 0.4	1.5
Chungbuk-Y275	552.4 ± 67.6	1.0	1.3 ± 0.9	1.0
Chungbuk-Y275-I117V	751.8 ± 51.1	1.3	1.0 ± 0.4	0.7
Chungbuk-Y275-I117M	434.8 ± 12.6	0.7	0.8 ± 0.2	0.6
Chungbuk-Y275-E119V	23.4 ± 2.4	0.04	263.3 ± 47.3	202.5
Chungbuk-Y275-I223V	642.8 ± 24.0	1.1	0.7 ± 0.2	0.5
Chungbuk-Y275-R293K	557.5 ± 12.8	1.0	2.2 ± 0.4	1.6
Chungbuk-Y275-N295S	118.9 ± 20.0	0.2	2.4 ± 0.4	1.8
Chungbuk-Y275-S334N	456.7 ± 38.2	0.8	0.6 ± 0.2	0.4
Chungbuk-Y275-N295S-I117V	369.6 ± 40.5	0.6	34.8 ± 10.8	26.7
Chungbuk-Y275-N295S-I117M	133.2 ± 12.5	0.2	21.3 ± 5.0	16.3
Chungbuk-Y275-N295S-I223V	330.4 ± 55.1	0.5	26.3 ± 7.7	20.2

^aAverage of three independent assays (fluorometric NA inhibition assay); ^bFold difference in IC₅₀ values between mutants and wild-type (Chungbuk-Y275) or mutants and Chungbuk-Y275H, by drug. IC₅₀ = 50% inhibitory concentration; NA = neuraminidase; NAI = neuraminidase inhibitor; SD = standard deviation of IC₅₀ values.

the mutant virus containing histidine (H) at NA residue 275 (Chungbuk-Y275H-E119V) conferred important levels of resistance to zanamivir, with 202.5-fold and 135.7-fold increases in IC₅₀ values compared with that of the wild-type virus (Chungbuk-Y275) and Chungbuk-Y275H mutant virus, respectively, whereas these mutants were susceptible to oseltamivir. Compared with the single Chungbuk-Y274H mutant, triple Chungbuk-Y275H-E119V-I117V and Y275H-E119V-I117M mutants had increased IC₅₀ values for zanamivir (390.7- and 181.0-fold), whereas these mutants were susceptible to oseltamivir. All the single, double, and triple mutants containing the E119V mutation were associated with the resistance to zanamivir.

All the single and double mutants containing tyrosine (Y) at NA residue 275 of the wild-type virus except the single mutant (Chungbuk-Y275-E119V) conferred important levels of resistance to oseltamivir, whereas the wild-type virus and these mutants were associated with the susceptibility to zanamivir.

The N295S mutation in the wild-type virus (Chungbuk-Y275-N295S) was associated with a reduction in oseltamivir resistance (0.2-fold decrease in IC₅₀ compared with that of the wild-type virus), whereas N295S mutation of the mutant virus containing histidine

(H) at NA residue 275 (Chungbuk-Y275H-N295S) was associated with an increase in oseltamivir resistance (40.3-fold increase in IC₅₀ compared with that of Chungbuk-Y275H mutant virus). Compared with the single Chungbuk-Y274-N295S mutant, double Chungbuk-Y275-N295S-I117V, Y275-N295S-I117M, and Y275-N295S-I223V mutants had slightly increased IC₅₀ values for oseltamivir (3.1-fold, 1.1-fold, and 2.8-fold), whereas these mutants were susceptible to zanamivir. Compared with the double Chungbuk-Y274H-N295S mutant, triple Chungbuk-Y275H-N295S-I117V, Y275H-N295S-I117M, and Y275H-N295S-I223V mutants also had slightly increased IC₅₀ values for oseltamivir (1.5-fold, 1.3-fold, and 2.4-fold), whereas these mutants were susceptible to zanamivir.

The single I117V, I117M, I223V, R293K, N295S, or S334N mutation did not seem to contribute significantly to a phenotype resistant to oseltamivir as well as to zanamivir.

4. Discussion

Although many subtype-specific NA mutations conferring resistance to NAIs in influenza A(H1N1),

A(H3N2), and A(H5N1) viruses have been reported previously [1,4–8,10,16–18], differences in viral backgrounds can account for a differential effect of these mutations on antiviral resistance or viral fitness.

Hence, we generated a recombinant wild-type A(H1N1) virus and its 23 mutants using a reverse genetics system to examine the effect of NA mutations on the NAI resistance phenotype. We used A/Chungbuk/4448/2008(H1N1) virus, which was isolated in South Korea during the 2008–2009 season as a wild-type virus.

NA-inhibition assays showed that all the single and double mutants containing the Y275, except the single Y275-E119V mutant, conferred important levels of resistance to oseltamivir, whereas all the single, double, and triple mutants containing the E119V mutation were associated with the resistance to zanamivir (Table 2).

In combination with Y275, the I117V or I223V mutation has a synergistic effect on oseltamivir resistance, increasing IC₅₀ values of oseltamivir by 1.3-fold and 1.1-fold, respectively, compared to the values caused by the Y275 mutation alone. Addition of the N295S mutation to the Y275H mutation has a synergistic effect on oseltamivir resistance, increasing the IC₅₀ value of oseltamivir to 40.3-fold of that of the Y275H mutation alone, whereas the addition of the N295S mutation to the Y275 has an antagonistic effect on oseltamivir resistance, decreasing the IC₅₀ value of oseltamivir to 0.2-fold of that of the Y275 alone.

The I117M mutation was detected in a pandemic (H1N1) 2009 virus from South Korea that was isolated from a patient during oseltamivir treatment [19]. The I117V mutation was detected in influenza A(H5N1) virus [20]. A previous study has reported that I117V has a synergistic effect of increasing resistance with H275Y, whereas the I117M mutation does not alter oseltamivir sensitivity in the recombinant viruses with NA from the pandemic (H1N1) 2009 virus and the remaining genes from A/PR/8/34 [21]. In this study, we had similar results in case of the recombinant A/Chungbuk/4448/2008(H1N1) virus.

In summary, I117V or I223V mutation has increased the resistance to oseltamivir in combination with Y275 virus, whereas N295S mutation has decreased resistance to oseltamivir in combination with Y275 virus. Different amino acid substitutions at key NA residues can cause considerably different effects on NAI susceptibility.

Here, we showed the NA activities of recombinant wild-type and NA mutant influenza viruses to NAIs *in vitro*. Further *in vivo* and clinical studies are required to investigate the effect of these drug-resistant mutants.

Considering the effect of mutations in NA gene on the resistance to NAIs, it is important to monitor the possible emergence and dissemination of multidrug-resistant variants in the human population due to amino acid changes at NA gene as well as the to develop novel NAIs.

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