Increased virulence of neuraminidase inhibitor-resistant pandemic H1N1 virus in mice
Potential emergence of drug-resistant and virulent variants

Min-Suk Song1,2, Yun Hee Baek1, Eun-Ha Kim1, Su-Jin Park1, Semi Kim1, Gyo-Jin Lim1, Hyeok-il Kwon1, Philippe Noriel Q Pascua1, Arun G Decano1, Byeong-Jae Lee1, Young-Il Kim1, Richard J Webby2, and Young-Ki Choi1,*

1College of Medicine and Medical Research Institute; Chungbuk National University; Cheongju, Republic of Korea; 2Division of Virology; Department of Infectious Diseases; St. Jude Children’s Research Hospital; Memphis, TN USA

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Pandemic H1N1 2009 (A[H1N1]pdm09) variants associated with oseltamivir resistance have emerged with a histidine-to-tyrosine substitution in the neuraminidase (NA) at position 274 (H274Y). To determine whether the H274Y variant has increased virulence potential, A(H1N1)pdm09 virus, with or without the H274Y mutation, was adapted by serial lung-to-lung passages in mice. The mouse-adapted H274Y (maCA04H274Y) variants showed increased growth properties and virulence in vitro and in vivo while maintaining high NA inhibitor resistance. Interestingly, most maCA04H274Y and maCA04 viruses acquired common mutations in HA (S183P and D222G) and NP (D101G), while only maCA04H274Y viruses had consensus additional K153E mutation in the HA gene, suggesting a potential association with the H274Y substitution. Collectively, our findings highlight the potential emergence of A(H1N1)pdm09 drug-resistant variants with increased virulence and the need for rapid development of novel antiviral drugs.

The pandemic H1N1 2009 (A[H1N1]pdm09) virus has begun to act as a seasonal influenza since it caused an influenza outbreak in 2009. Before the pandemic, especially during the 2007–2008 season, most seasonal H1N1 viruses had a neuraminidase (NA) gene with a substitution of tyrosine for histidine at residue 274 (H274Y, N2 numbering) which confers resistance to oseltamivir (OS), the most widely used drug for clinical therapy of influenza-infected patients, albeit mostly with compromised viral fitness. Fortunately, the A(H1N1)pdm09 virus remained sensitive to neuraminidase inhibitors (NAIs) such as OS. However, the widespread use of NAIs to treat patients that are believed to be infected with A(H1N1)pdm09 in many countries led the virus to evolve into drug-resistant variants. Furthermore, unlike previous seasonal H1N1 drug-resistant variants of A(H1N1)pdm09 retained comparable viral fitness in pathogenesis and transmissibility to those of their OS-sensitive counterparts.

A(H1N1)pdm09 virus has exhibited remarkable transmissibility among humans and even reverse-zoonotic transmission, such as to pigs and turkeys, but the pathogenicity of the virus was milder than that of the pandemic virus in 1918. However, several studies have shown increased pathogenesis of A(H1N1)pdm09 in new host species through mouse adaptation and molecular-based substitutions related to enhanced virulence, although the tested viruses still remained NAI-sensitive. Of note, in two of these studies, the mouse-adapted variants also showed enhanced fitness in ferrets, suggesting mice are a relevant model for such studies.

The virulence potential of A(H1N1)pdm09 variants with drug-resistance mutations in the NA gene has been poorly understood. Increasing numbers of cases of oseltamivir (OS)-resistant variants, especially associated with the H274Y mutation, have been reported to the World Health Organization, implicating a potential threat of virulent and drug-resistant variants. Although several studies demonstrated that OS-resistant A(H1N1)pdm09 viruses are as virulent as their OS-sensitive virus counterparts, the viruses showed basically mild virulence in vivo. Here, the virulence potential of drug-resistant A(H1N1)pdm09 variants conferred by the H274Y mutation in the NA gene (CA04H274Y) was investigated, along with wild-type California/04/09 (CA04) virus in mice. Furthermore, to determine whether the CA04H274Y can become more virulent than its CA04 virus counterpart, parallel mouse adaptation of these OS-resistant and OS-sensitive parental strains was performed by serial lung-to-lung passage for five times. Both viruses were generated by reverse genetics and the H274Y mutation was given by site directed mutagenesis in CA04 virus. The research protocol for the use of mice in this study was in strict accordance and adherence with relevant policies regarding animal handling as mandated under the Guidelines for Animal Use and Care of the Korea Center for Disease Control.

Initially, 105 TCID50 in 30 µl were administered intranasally (i.n.) to groups of four BALB/c mice after light anesthesia with a mixture of Zoletil (Virbac) and Rompun (Bayer). Lungs from infected mice were collected 5 days (d) post-inoculation.
and the supernatant of the homogenate was administered to naïve mice for the subsequent four additional passages. Finally, to compare pathogenicity, $10^{3.0}$ TCID$_{50}$ of the mouse-adapted viruses and their parental strains (maCA04A-D and maCA04H274Y-A-D vs. CA04 and CA04H274Y, respectively), were administered in groups of five mice and were monitored for morbidity and mortality for 12 d p.i. If the infected mice lost more than 30% of their body weight, they were euthanized. Three of four independently passaged, mouse-adapted CA04 viruses (maCA04) were 100% lethal, but the other one had a 60% survival rate starting at 7 d p.i. (Fig. 1A and B). All 4 independently passaged, mouse-adapted CA04 (H274Y) viruses (maCA04H274Y) killed all infected mice within 7 d p.i. (Fig. 1C and D). These findings show that the OS-resistant variants can cause mortality and morbidity equivalent to that of the mouse-adapted wild-type A(H1N1)pdm09 virus after serial lung-to-lung passages.

To confirm the genetic stability of the conferred H274Y mutation in the NA gene and examine whether any additional mutations occurred in the viral genome during adaptation, the whole genome sequences of 8 mouse-adapted viruses were analyzed and compared with their parental viruses. We found 10 amino acid substitutions in 6 genes of the 8 mouse-adapted viruses during adaptation (Table 1); one synonymous silent mutation (G1066C) was additionally noted within the corresponding H1 HA2 protein region of the maCA04 viruses (data not shown). The conferred H274Y mutation in the NA gene of the CA04H274Y virus was retained after mouse adaptation in all 4 independent parallel passages, showing that the OS-resistance-inducing mutation did not create genetic instability or need other compensatory mutations in the NA gene to increase virulence. Three mutations (S183P and D222G in HA [H1 numbering] and D101G in NP) were almost synonymously found in our mouse-adapted CA04 (maCA04A-C and CA04H274Y [maCA04H274Y-A-D] viruses (Table 1), and were also correspondingly noted in previous mouse-adaptation studies. The maCA04 D virus did not kill all mice and retained the wild-type sequence of 222D in the HA gene (Fig. 1B and Table 1). The D222G mutation in HA gene has been associated with severe-to-fatal cases of human A(H1N1) pdm09 infections and lethal swine H1N2 virus infection in ferrets. Interestingly, all maCA04H274Y, but not maCA04 viruses, acquired a synonymous K153E mutation in the HA gene, suggesting a potential association with the H274Y mutation. On the other hand, maCA04C virus had further mutations in PB1 (N105T and R721K) and HA (K119E) while maCA04H274Y also incurred a S714R substitution in PB2. The E158G and T97I mutations found in PB2 and PA, respectively, of several mouse-adapted viruses in our study have been reported to be associated with increased polymerase activity or virulence. For further characterization of the virulent phenotype of OS-resistant variants, maCA04H274Y-C and maCA04A viruses, which only differ in HA$_{196}$ (E or K, respectively) were plaque-purified and selected as described elsewhere, and tested for morbidity, mortality, and viral lung titer. Groups of 5 mice were inoculated i.n. with $10^{3.0}$ TCID$_{50}$ of the parental and plaque-purified mouse adapted viruses (CA04, CA04H274Y, maCA04, and maCA04H274Y) and monitored for survival and body weight loss daily for 12 d. Similar to the results of infection with the passaged viruses in lungs, mice infected with the plaque-purified mouse-adapted viruses (maCA04 and maCA04H274Y) had dramatic weight loss and succumbed to infection within 9 d p.i. (Fig. 1E and F) although maCA04H274Y killed all inoculated mice at 7 dpi. Viral replication kinetics in mouse lungs (n = 12) revealed that the mouse-adapted viruses yielded titers more than 10-fold higher than those of parental viruses at 1 and 3 d p.i., and no difference was observed between maCA04 and maCA04H274Y at any time, which implies that the adaptation of the OS-resistant H274Y variant in mice increased growth properties to as high as that of the wild-type virus in vivo (Fig. 1G). The increased yields of mouse-adapted viruses in mouse lungs was also observed in MDCK cells and eggs, which yielded significantly higher titers (>$10^{3}$-fold, $P < 0.05$) than their parental strains (Table 2). To determine the 50% mouse lethal dose (MLD$_{50}$) of the viruses, we inoculated groups of 5 mice i.n. with 10-fold serial dilutions containing $10^{1}$ to $10^{6}$ TCID$_{50}$ of the viruses. The maCA04 and maCA04H274Y viruses showed more than $10^{3.5}$-fold higher MLD$_{50}$ values, comparable replicative ability and virulence in mice which corresponded to previous studies. Here, we show that parental CA04 and CA04H274Y viruses had comparable replicative ability and virulence in mice which corresponded to previous studies. However, we also show for the first time that mammalian adaptation of the CA04H274Y virus through serial passage in mice resulted to increased growth properties and virulence in vitro and/or in vivo with no apparent alteration of
Figure 1. Comparison and characterization of virulence between mouse-adapted and parental viruses in BALB/c mice. Five independent serial lung-to-lung passages for each virus (CA04 and CA04\_H274Y) were performed. After adaptation, 10^{10} TCID\_50 of the passaged viruses and their parental strains were administered intranasally (i.n.). Body weight loss and survival rate of the parental CA04 and CA04\_H274Y with their corresponding mouse-adapted variants (maCA04\_A, maCA04\_B, maCA04\_C, maCA04\_D, and maCA04\_H274Y\_A, maCA04\_H274Y\_B, maCA04\_H274Y\_C, and maCA04\_H274Y\_D, respectively) were monitored in groups of 5 mice for 12 d p.i. (A–D). The number of passages is represented by ‘p’ followed by the name of the individually passaged viruses on the figure. For further characterization of lethal mouse-adapted oseltamivir-resistant variants, mouse-adapted viruses were plaque-purified and selected. Next, 10^{3} TCID\_50 of plaque-purified mouse-adapted CA04, mouse-adapted CA04\_H274Y, and their parental viruses were administered to mice i.n. Body weight loss (E) and survival rate (F) were monitored daily for 12 d p.i. Groups of 12 mice were also given 10^{3} TCID\_50, and lung samples were collected 1, 3, 5, and 7 d p.i. to determine lung viral titers (3 heads/day) (G). Standard hematoxylin and eosin staining of lung tissues infected by CA04 (H), CA04\_H274Y (I), mouse-adapted CA04 (J), and mouse-adapted CA04\_H274Y (K) was examined 5 d p.i. (magnification × 100). *P < 0.05 (unpaired t test, two-tailed) of viral lung titers between wild-type CA04 and CA04\_H274Y viruses and mouse-adapted CA04 and CA04\_H274Y viruses. Error bar shown in (C, E, and G) represents standard error mean (SEM).
Table 1. Amino acid substitutions identified after mouse adaptation of pandemic H1N1 2009 and oseltamivir-resistant variants

| Virus strain       | PB2     | PB1     | PA   | HA   | NP   | NA |
|--------------------|---------|---------|------|------|------|----|
|                    | 158     | 714     | 105  | 721  | 97   | 119|
| CA/04/09           | E       | S       | N    | R    | T    | K  |
| maCA04_A           | E       | S       | N    | R    | T    | K  |
| maCA04_B           | E       | S       | N    | R    | I    | K  |
| maCA04_C           | E       | S       | T    | K    | T    | E  |
| maCA04_D           | E       | S       | N    | R    | T    | K  |
| CA/04/09_H274Y     | E       | S       | N    | R    | T    | K  |
| maCA04_H274Y_A     | G       | S       | N    | R    | T    | K  |
| maCA04_H274Y_B     | E       | R       | N    | R    | T    | K  |
| maCA04_H274Y_C     | E       | S       | N    | R    | T    | K  |
| maCA04_H274Y_D     | G       | S       | N    | R    | T    | K  |

\*The number of the amino acid residue by H1 numbering. \*The number of the amino acid residue by N2 numbering. \*The boldface represents substitution of parental amino acid.

Table 2. Characteristics of growth efficiency and virulence, and neuraminidase-inhibitor susceptibility of wild-type and mouse-adapted pandemic H1N1 influenza viruses and their oseltamivir-resistant counterpart

| Virus strain       | MDCK cells (Log_{10} TCID_{50}/ml ± SD) | Eggs (Log_{10} EID_{50}/ml ± SD) | MLD_{50} (dpi) | MST | Neuraminidase inhibitors IC_{50} ± SD, nM (Ratio) |
|--------------------|----------------------------------------|----------------------------------|----------------|-----|-----------------------------------------------|
| CA/04/09           | 6.1 ± 0.3                              | 6.7 ± 0.5                        | >5.5           | >12 | 0.86 ± 0.03 (1)                               |
| maCA04             | 7.8 ± 0.2*                             | 8.9 ± 0.4*                       | 2              | 8   | 2.53 ± 0.04 (2.9)                            |
| CA04_H274Y         | 6.1 ± 0.3                              | 7.1 ± 0.1                        | >5.5           | >12 | 1071 ± 0.07 (124.5)                          |
| maCA04_H274Y       | 7.7 ± 0.1*                             | 8.4 ± 0.3*                       | 1.5            | 6   | 128.1 ± 0.04 (149)                           |

\*Compared with that of the wt CA/04/09 virus. \*P < 0.05 compared with the value for the respective parental viruses (unpaired t test, two-tailed). SD, standard deviation; MLD, mouse lethal dose; MST, median survival time; wt, wild type; ma, mouse adapted.

NAI resistance implicating undiminished viral fitness conferred by the H274Y mutation. Increased in pathogenicity of CA04 and CA04_H274Y viruses appeared to be associated with previously described specific mutations, particularly HA-D222G, which also showed viral fitness comparable to that of the wild type.24,25 Altogether, our findings emphasize the potential emergence of drug-resistant variants associated with high virulence as well as the need for the development of novel antiviral agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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