INTRODUCTION

Influenza A viruses are classified under the Orthomyxoviridae family and consist of several subtypes, based on their hemagglutinin (HA) and neuraminidase (NA) composition. In some cases, reassortment of the 8 viral RNA segments may occur if different influenza viruses infect the same host, thereby generating new viral strains (antigenic shift). Mutations in the genes of influenza viruses are responsible for antigenic drift, which can lead to the emergence of new influenza strains that are not well recognized by the host’s immune system, thereby causing an outbreak of influenza.
A viruses are enhanced by the absence of RNA proofreading enzymes; in particular, substitutions in the HA protein alter the viral antigenic epitopes sufficiently to avoid the host immune response (antigenic drift). These antigenic changes can trigger the generation of new strains and subgroups, leading to pandemics or new epidemics worldwide.1-3

Influenza A virus subtypes H3N2 (A/H3N2) and H1N1 (A/H1N1), and influenza B virus have been circulating in the human population every winter, and account for 3 to 5 million cases of severe illness and 250,000 to 500,000 deaths annually, mostly caused by secondary bacterial pneumonia in young children and the elderly.4-7 To reduce the public health burden of influenza, the World Health Organization (WHO) recommends annual influenza vaccination and recommends the four strains to be included in vaccine composition. Seasonal vaccine strains are recommended twice a year because of the differences in the duration of winter in the Southern Hemisphere and Northern Hemisphere. Vaccine strain recommendations for effective influenza vaccination are based on antigenic analysis and viral genome data worldwide in addition to global surveillance systems of the circulating viruses. In addition, genome sequencing and analysis of circulating influenza viruses have provided comprehensive understanding of evolutionary models as well as epidemiologic insight based on analysis of antigenic determinants, drug resistance, and a variety of sequence-based bioinformatics methods.2,8,9

Here, we analyzed the HA and NA genes of influenza A viruses prevalent in Korea during the 2011-2016 seasons. These viruses were identified through the Hospital-based Influenza Morbidity & Mortality (HIMM) surveillance system.10 In addition, we investigated the effect of mutations in the 8 segmented genes of influenza A viruses on risk of mortality for infected patients.

2 | MATERIALS AND METHODS

2.1 | Sample collection and viral isolation

Through the HIMM system, clinical and virological surveillance was conducted for patients visiting the emergency departments or being hospitalized due to influenza-like illness at 10 university hospitals in Korea from 2011 to 2016 (n = 13,620). We obtained nasopharyngeal swab samples during the 2011-2016 season and identified influenza viruses using RT-PCR. Influenza A virus-positive samples were identified as either influenza A/H3N2 or 2009 pandemic A/H1N1 (A(H1N1) pdm09) and subjected to sequencing for the determination of HA and NA genes. A total of 254 sequences were used in the phylogenetic study. Using influenza A virus-positive samples, Madin-Darby canine kidney (MDCK) cells were inoculated with severe acute respiratory infection (SARI) samples and cultured for 48-72 h. The hemagglutination assay was performed to detect the influenza virus growth.11 Unfortunately, samples were not obtained for the 2011-2013 seasons. Therefore, analysis of fatal cases was performed only for the 2013-2016 seasons.

| TABLE 1 | Influenza types in Korea during the 2011-2016 seasons based on RT-PCR |
| Seasons | Number of cases enrolled in HIMM | Number of samples analyzed | FluA | FluB | Co-infection | A/H1N1 | A/H3N2 |
|---------|----------------------------------|---------------------------|------|------|-------------|--------|--------|
| 2011-2012 | 2252 | 927 (41.1%) | 1243 (55.2%) | 82 (3.6%) | 731 (78.9%) | 182 (19.6%) | 2 |
| 2012-2013 | 1667 | 681 (40.9%) | 882 (52.9%) | 104 (6.2%) | 676 (99.3%) | 4 (0.6%) | 93 |
| 2013-2014 | 3082 | 1295 (42.0%) | 1328 (43.1%) | 459 (14.9%) | 978 (75.5%) | 307 (23.7%) | 22 |
| 2014-2015 | 3901 | 1298 (33.3%) | 2002 (51.3%) | 601 (15.4%) | 962 (74.1%) | 273 (21.0%) | 70 |
| 2015-2016 | 2718 | 524 (19.3%) | 1543 (56.8%) | 651 (24.0%) | 349 (66.6%) | 651 (24.0%) | 20 |
| Total | 13,620 | 4725 (34.7%) | 6798 (51.4%) | 2718 (19.3%) | 1897 (13.9%) | 3696 (78.2%) | 211 |
Phylogenetic tree of HA and NA genes of influenza A virus in Korea during the 2011-2016 seasons. The (A) HA and (B) NA sequences of A/H3N2 isolate in Korea showed clustering in the phylogenetic tree. The sequences of clade 3C.2a in HA tree are shown in red font (B) NA of A/H3N2. The phylogenetic tree of A/H1N1 isolate (C) HA and (D) NA sequences reveals distinct groups. The phylogenetic tree was inferred from the recommended H1N1 vaccine strains (A/California/07/2009), H3N2 vaccine (A/Perth/16/2009, A/Victoria/361/2011, A/Texas/50/2012, A/Switzerland/9715293/2013), and clinical isolates identified in Korea (2011-2016). The font colors represent the vaccine strain (blue).
### 2.2 Viral RNA extraction and sequencing

Viral RNA was extracted using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) and then reverse-transcribed using influenza A virus universal primers (Uni 12, 5′-AGC AAAAGCAGGG-3′) with the PromegaScript 1st strand cDNA synthesis kit (Takara, Shiga, Japan). PCR amplification was carried out using primers specific for the viral RNA segments coding for HA (forward, 5′-AGCAAAAGCAGGGG-3′; reverse, 5′-AGTAGA AAC AAGGGTGTTTT-3′), NA (forward, 5′-AGCRAAAGCAGGGTATTTTT-3′; reverse, 5′-AGTAGAAACAAGGGTATTTTT-3′), nucleoprotein (NP; forward, 5′-AGCRAAAGCAGGGTATTTTT-3′; reverse, 5′-AGTAGAAACAAGGGTATTTTT-3′), non-structural protein (NS; forward, 5′-AGCRAAAGCAGGGTATTTTT-3′; reverse, 5′-AGTAGAAACAAGGGTATTTTT-3′), matrix protein (M; forward, 5′-AGCRAAAGCAGGGTATTTTT-3′; reverse, 5′-AGTAGAAACAAGGGTATTTTT-3′), RNA polymerase subunits PA (forward, 5′-AGCRAAAGCAGGGTATTTTT-3′; reverse, 5′-AGTAGAAACAAGGGTATTTTT-3′), and RNA polymerase subunits PB1 (forward, 5′-AGCRAAAGCAGGGTATTTTT-3′; reverse, 5′-AGTAGAAACAAGGGTATTTTT-3′). After amplification, the sequence readouts of the PCR products were analyzed. The sequences obtained from this study were deposited in GenBank under accession no. KY063619 through KY063705, KY509553-KY509793.

| Reference viruses | Post-infection sheep antisera | A/Victoria/361/2011 | A/Texas/50/2012 | A/Switzerland/9715293/2013 | Genetic clade |
|-------------------|-------------------------------|---------------------|----------------|--------------------------|---------------|
| A/H3N2 vaccine strains | >1280 | >1280 | >1280 | | |
| Test viruses |
| 2013-2014 season isolates | | | | | |
| A/Ansang/D765/2014 | 640 | 320 | 1280 | 3C.3a |
| A/Cheongju/G1532/2014 | 320 | 160 | 640 | 3C.3a |
| A/Cheongju/G1629/2014 | 640 | 320 | 1280 | 3C.3a |
| A/Ansang/D799/2014 | 320 | 160 | 1280 | 3C.3a |
| A/Cheongju/G1739/2014 | 640 | 320 | 1280 | 3C.3a |
| 2014-2015 season isolates | | | | | |
| A/Cheongju/G2156/2015 | >1280 | 1280 | >1280 | 3C.3a |
| A/Seoul/K98/2015 | 160 | 80 | 1280 | 3C.3a |
| A/Ansang/D858/2015 | 320 | 160 | 1280 | 3C.3a |
| A/Ansang/D906/2015 | 320 | 160 | 1280 | 3C.3a |
| A/Cheongju/G1971/2015 | 160 | 80 | >1280 | 3C.3a |
| A/Cheongju/G2103/2015 | 640 | 320 | 1280 | 3C.3a |
| A/Seoul/A1284/2015 | 1280 | 1280 | 1280 | 3C.3a |
| A/Seoul/A1251/2015 | 1280 | 1280 | 1280 | 3C.3a |
| A/Seoul/A1342/2015 | 80 | 40 | 80 | 3C.2a |
| A/Ansang/D830/2015 | 160 | 80 | 160 | 3C.2a |
| A/Ansang/D909/2015 | 160 | 40 | 80 | 3C.2a |
| A/Seoul/A1299/2015 | 160 | 40 | 80 | 3C.2a |
| A/Cheongju/G2477/2015 | 80 | 40 | 160 | 3C.2a |

### 2.3 Sequence analysis

For the phylogenetic study, the sequences were compared with the NCBI-registered full-length nucleotide sequences of influenza A/H3N2 and A(H1N1)pdm09 viruses and vaccine strains (H1N1: A/California/07/2009; H3N2: A/Perth/16/2009, A/Victoria/361/2011, A/Texas/50/2012, and A/Switzerland/9715293/2013). The sequences were aligned using the BioEdit program. MEGA 6.06 was used to build the phylogenetic tree, using the maximum-likelihood method by obtaining the initial tree for the heuristic search based on the neighbor-joining method through a matrix of pairwise distances evaluated by the maximum composite likelihood approach. The bootstrap scores were set to 1000 (bootstrap values over 50 are shown above the tree branches).

### 2.4 Hemagglutination inhibition (HAI) assay

HAI titers were determined using standard procedures. In brief, antisera were pre-treated overnight with receptor-destroying enzyme (RDE) at 37°C and heat inactivated at 56°C for 30 min. Twofold serial dilutions of RDE-treated antisera (50 μL) starting at a 1:10 dilution were incubated with 4 HA unit/25 μL of each virus and incubated at RT for 1 h. Next, 50 μL of 0.75% guinea pig red blood cells (gRBCs) was added, followed by 1-h incubation at RT. We confirmed the co-agulation of gRBCs and interpreted HAI results.
| Season       | Isolated virus                  | Age/sex | BMI  | Body temperature at presentation (°C) | Pre-existing condition                                      | Cause of death                  | Microbiologic test                      | Pneumococcal urinary antigen test | Antiviral drug treatment (days from symptom onset to administration of antiviral) |
|--------------|---------------------------------|---------|------|---------------------------------------|------------------------------------------------------------|-------------------------------|-----------------------------------------|-----------------------------------|----------------------------------------------------------------------------------|
| 2011-2012    | A/Ansan/D16/2012 (H3N2)         | 78/M    | 27.5 | None                                  | Diabetes, hypertension, chronic cerebrovascular disease    | Pneumonia                     | Streptococcus pneumoniae              | Not done                         | Positive                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Ansan/D342/2013 (H3N2)        | 72/M    | 20.7 | 38.6                                  | Chronic cardiovascular disease, AML                         | Pneumonia                     | Pseudomonas aeruginosa                 | Pseudomonas aeruginosa             | Negative                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Cheongju/G792/2013 (H1N1)     | 74/F    | 15.2 | 38                                    | Cancer                                                     | Pneumonia                     | Normal flora                           | Not done                         | Positive                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Cheongju/G856/2013 (H3N2)     | 93/F    | 20   | Non                                   | Chronic cerebrovascular disease                            | Pneumonia                     | No growth                              | Not done                         | Negative                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
| 2012-2013    | A/Seoul/A468/2013 (H3N2)        | 89/M    | 23.7 | 37.6                                  | None                                                       | Multi-organ failure            | Streptococcus pneumoniae              | Not done                         | Positive                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Ansan/D342/2013 (H3N2)        | 72/M    | 20.7 | 38.6                                  | Chronic cardiovascular disease, AML                         | Pneumonia                     | Pseudomonas aeruginosa                 | Pseudomonas aeruginosa             | Negative                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Cheongju/G1532/2014 (H3N2)    | 86/F    | 17.8 | 36.8                                  | Hypertension                                               | Pneumonia                     | Normal flora                           | Staphylococcus aureus              | Negative                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Cheongju/G1629/2014 (H3N2)    | 86/M    | 16.5 | 37.5                                  | COPD                                                       | Pneumonia                     | MRSA                                    | Not done                         | Negative                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
| 2013-2014    | A/Ansan/D765/2014 (H3N2)        | 80/M    | 23.1 | 38.6                                  | Chronic respiratory diseases, COPD                         | Pneumonia, sepsis             | Normal flora                           | Not done                         | Olseltamivir 75 mg bid (1)           |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Cheongju/G1532/2014 (H3N2)    | 86/F    | 17.8 | 36.8                                  | Hypertension                                               | Pneumonia, sepsis             | Normal flora                           | Staphylococcus aureus              | Negative                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Cheongju/G1629/2014 (H3N2)    | 86/M    | 16.5 | 37.5                                  | COPD                                                       | Pneumonia                     | MRSA                                    | Not done                         | Negative                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
| 2014-2015    | A/Seoul/A1284/2015 (H3N2)       | 97/F    | 20.2 | 37.8                                  | Diabetes, chronic respiratory diseases, anemia, dementia, osteoporosis | Pneumonia                     | Pseudomonas fluorescens                | Not done                         | Positive                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Seoul/A1251/2015 (H3N2)       | 92/M    | 17.9 | 38.2                                  | Diabetes, asthma, chronic respiratory diseases, TB, BPH   | Pneumonia, sepsis             | MRSA, Escherichia coli                 | Not done                         | Negative                          |
|              |                                 |         |      |                                       |                                                            |                               |                                        |                                    |                                    |
|              | A/Cheongju/G2156/2015 (H3N2)    | 83/F    | 17.3 | None                                  | Hypertension, chronic respiratory diseases                | Pneumonia                     | Not done                                | Not done                         | Negative                          |

BMI, body mass index; BPH, benign prostatic hyperplasia; COPD, chronic obstructive pulmonary disease; TB, tuberculosis; AML, acute myeloid leukemia; MRSA, methicillin-resistant Staphylococcus aureus.
2.5 Neuraminidase inhibitor (NAI) assay

The NA-Fluor™ assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manufacturer’s protocol. Neuraminidase inhibitor was prepared in 10-fold serial dilutions at 4× the final concentration in assay buffer (16.65 mM MES, 2 mM CaCl₂, pH 6.5), and 25 μL was added to wells of a black flat-bottom 96-well microplate. Viral samples were diluted in assay buffer, and 25 μL was added to the NAI serial dilutions, and the samples were then incubated for 30 min at 37°C. NA-Fluor Substrate (50 μL) was added to create a final assay concentration of 100 μM 4-Methylumbelliferone sodium salt, and the samples incubated at 37°C for 1 h. No-virus controls were included on each assay plate. Assay was terminated by addition of 100 μL of Stop Solution, and plates were read on a Victor 3 plate reader using Ex 355 nm/Em 460 nm settings. For data analysis, the relative fluorescence unit
values of the no-virus control wells were subtracted from the virus-containing well values and data were processed using Graphpad® Prism software.

### 2.6 | Statistics analysis

To elucidate the factors associated with the death of influenza cases, fatal and non-fatal cases were selected (1:3 ratio) on the collection date, sex, and age in the 2013-2016 seasonal isolates. The A(H1N1)pdm09 fatal case sample was excluded because one fatal case was not appropriate for statistical analysis. The 2011-2013 fatal case samples were excluded due to lack of appropriate non-fatal case samples. Therefore, we analyzed the data pertaining to a total of 24 samples including 6 fatal and 18 non-fatal cases in the 2013-2016 season using Fisher’s exact test. A P-value <.05 was considered statistically significant.

| NP | NS1 | NS2 | PA | PB1 | PB2 | Genetic clade (HA based) |
|----|-----|-----|----|-----|-----|--------------------------|
| S359P, M374I, M440V | D209N, K229E | - | E351D, R356K, M407I | D581N | V606A | 3C.1 |
| V197A, M374I | V22F, D209N, K229E | K39R | E351D, M407I | - | M66T, R389K | 3C.1 |
| K470M | E26K, K78R, D209N, K229E | - | M407I | R361K | Y55F, R194Q, L384I | 3C.2 |
| K357R | E26K, I182V, D209N, K229E | - | G240V, M407I, V668I, N675K | - | - | 3C.2 |
| S217G | E26K, M124I, D209N, K229E | - | Q256K, I308V, I554V, K605R, V669I | - | - | 3C.3a |
| S217G, S482N | E26K, M124I, D209N, K229E | - | Q256K, I308V, I554V, K605R, V669I | - | - | 3C.3a |
| S217G | E26K, M124I, D209N, K229E | - | Q256K, I308V, I554V, K605R, V669I | R52K, L424M | V338I, S709N | 3C.3a |
| S217G | E26K, M124I, A202T, D209N, K229E | - | Q256K, I308V, I554V, K605R, V669I | T400K | A559V, S643T | 3C.3a |
| I336L, S217G | E26K, M124I, D209N, K229E | - | Q256K, I308V, D396E, I554V, K605R, V669I | - | R390K, M476L | 3C.3a |
| M481I | E26K, D209N, K229E | - | N272S, D396E, M407I, V668I, N675K | - | V63I, I589T | 3C.2a |
| M481I | E26K, D209N, K229E | - | N272S, D396E, M407I, V668I, N675K | W580C | V63I, I589T | 3C.2a |
| V100I, L108I | L90I, I111T, V117M, I123V, N205S | T48A | I30T, Y161F, P224S, E252V, N321K, A343T, V407I, R673S | G154D, I397M, I435T, H456Y | D195N, R293K, V344M, I354L, V731I | 7 |
TABLE 5 Neuraminidase inhibition of fatal case isolates

| Sample | 2013-2014 season | 2014-2015 season | 2015-2016 season |
|--------|------------------|------------------|------------------|
|        | D765             | G1532            | G1629            | G2156             | A1284             | A1251             | A/Switzerland/9715293/2013 |
| IC50 (μM) | 0.275           | 0.283            | 0.279            | 0.245             | 0.155             | 0.210             | 0.218             |
| 95% CI  | 0.259-0.292      | 0.266-0.300      | 0.267-0.292      | 0.224-0.269       | 0.147-0.163       | 0.192-0.230       | 0.204-0.232       |

3 | RESULTS

3.1 | HA and NA diversity of seasonal influenza A virus in Korea, 2011-2016

A total of 13,620 patients were enrolled through the HIMM surveillance system during the 2011-2016 seasons. These include 4,725 patients positive for influenza A or B viruses with RT-PCR, and 3,696 (78.2%) of them were confirmed with influenza A virus, including subtype A(H1N1)pdm09 and H3N2 (Table 1). During this period, A/H3N2 was the predominant epidemic strain with the exception of 2015-2016 season during which A/H1N1 co-circulated. Because of this dominant pattern, we collected 43 sequences of A(H1N1)pdm09 in the 2012-2014 and 2015-2016 seasons, and 211 sequences of A/H3N2 in the 2011-2015 seasons. A phylogenetic tree was constructed to confirm the substitution patterns of HA and NA genes in the influenza A virus.

The HA phylogenetic tree was developed using 43 HA sequences of A(H1N1)pdm09, 211 HA sequences of A/H3N2 and the recommended vaccine strains (A/Perth/16/2009, A/Victoria/361/2011, A/Texas/50/2012, A/Switzerland/9715293/2013, and A/California/07/2009). In Figure 1, most of the A/H1N1 sequences clustered in clade 6B with the exception of A/Cheongju/G1629/2013. The clade 6B was differentiated into two groups by the amino acid substitutions V152T and V173I in group 1 and S84N, S162N, and S203T in group 2. These groups were generally congruent in the A(H1N1)pdm09 HA and NA phylogenetic tree. In the A/H3N2 analysis, we confirmed variations for the cluster differences (3C.2a and 3C.3a) in the HA and NA genes. In addition, the NA sequence of the 2012-2013 season (n = 2) carried an I222V substitution, an NA inhibitor resistance mutation.13-16 The NA sequence of several samples carried substitutions: I20T in A/Ansan/D765/2013, A/Cheongju/G856/2013(H3N2).

In the analysis of internal genes, the M sequence of all the samples investigated harbored the S31N genetic marker for adamantane resistance in M2. By comparing the sequences from each season with the A/H3N2 vaccine strain (A/Victoria/361/2011), we found several amino acid substitutions (Table 4). Interestingly, the PA gene of A/Ansan/D765/2013 (H1N1) carried L40V, N44S, S52N, N200S, V241I, G262E, H273N, and I321V substitutions were located in the HA1 region, the E47K, S124N, I183V, and V193A substitutions were located in the HA2 region (Table 4).

3.2 | Genetic analysis of 8 segmented genes in the fatal influenza A virus isolates

Viral virulence can be increased by mutating non-structural proteins (Table 5). Therefore, internal gene sequences from influenza A viruses from fatal cases were used in the genetic analysis. Genetic analysis of 8 segments (HA, NA, M, NP, NS, PA, PB1, and PB2) was performed using sequences of fatal isolates [A(H1N1)pdm09; n = 1, A/H3N2; n = 11] and vaccine strains. Most of the fatal cases had underlying conditions, such as diabetes, chronic respiratory diseases, and chronic medical illnesses (Table 3).

Compared with vaccine strain, amino acid mutations were found in the A/Cheongju/G792/2013(H1N1) HA gene: the P83S, S84G, S143G, K163I, G170R, S185T, A197T, S203T, A261T, G262E, H273N, and I321V substitutions. In addition, several substitutions were identified in the other 6 segments (Table 4).

In the A/H3N2 analysis, we confirmed variations for the cluster differences (3C.2a and 3C.3a) in the HA and NA genes. In addition, the NA sequence of the 2011-2012 season (n = 2) carried an I222V substitution, an NA inhibitor resistance mutation.13-16 The NA sequence of several samples carried substitutions: I20T in A/Ansan/D342/2013, D339N in A/Cheongju/G1629/2014, Y67F in A/Ansan/D765/2014, and D151N, W383C in A/Seoul/A1251/2015. The NAi assay was conducted using vaccine strains and viruses isolated in the 2013-2016 seasons to determine whether NA gene mutations affected virulence due to antiviral resistance. The NAi assay demonstrated the absence of resistant viruses in the fatal influenza cases (Table 5).

In the analysis of internal genes, the M sequence of all the isolates investigated harbored the S31N genetic marker for adamantane resistance in M2. By comparing the sequences from each season with the A/H3N2 vaccine strain (A/Victoria/361/2011), we found several amino acid substitutions (Table 4). Interestingly, the PA gene of A/Ansan/D765/2014, A/Cheongju/G1532/2014, A/Cheongju/G1629/2014, A/Cheongju/G2156/2015, and A/Switzerland/9715293/2013 carried 5 amino acid mutations: Q256K, I308V, I554V, K605R, and V669I. These PA substitutions...
were consistent with clade 3C.2a in the HA phylogenetic tree. As shown in Table 4, we confirmed that the substitutions of NA, NS1, and PA coincided with HA-based genetic clade. In addition, the PB1 and PB2 genes revealed multiple non-synonymous substitutions (Table S2).

Statistical analysis was performed to study the association between amino acid substitution and death in fatal cases (Table 6). Although some substitutions (I39M of M2 and M481I of NP) showed a P value of .054, they were not significant in the correlation analysis of genetic substitution and mortality. As shown in Table 7, our correlation analysis revealed that chronic lung disease was more frequently associated with fatal than non-fatal cases.

In conclusion, statistical analysis showed no significant association between the viral genetic differences and mortality; however, the mortality was increased by host factors such as chronic lung disease.

### Table 6 Amino acid substitutions associated with mortality based on Fisher’s exact test

| Substitutions | Fatal cases (n = 6) | Non-Fatal cases (n = 18) | Fisher’s P-value |
|---------------|--------------------|--------------------------|------------------|
| M2_I39M       | 2                  | 0                        | .054             |
| NP_I136L      | 1                  | 4                        | 1.000            |
| NP_S217G      | 4                  | 15                       | .568             |
| NP_M481I      | 2                  | 0                        | .054             |
| NP_S482N      | 1                  | 0                        | .250             |
| NS1_M124I     | 4                  | 15                       | .568             |
| NS1_A202T     | 1                  | 0                        | .250             |
| PA_Q256K      | 4                  | 15                       | .568             |
| PA_N272S      | 2                  | 3                        | .568             |
| PA_I308V      | 4                  | 15                       | .568             |
| PA_M407I      | 2                  | 3                        | .568             |
| PA_A455G      | 1                  | 0                        | .250             |
| PA_I554V      | 4                  | 15                       | .568             |
| PA_K605R      | 4                  | 15                       | .568             |
| PA_V668I      | 2                  | 3                        | .568             |
| PA_V669I      | 4                  | 15                       | .568             |
| PA_N675K      | 2                  | 3                        | .568             |
| PB1_R52K      | 1                  | 0                        | .250             |
| PB1_T400K     | 1                  | 0                        | .250             |
| PB1_L424M     | 1                  | 0                        | .250             |
| PB1_W580C     | 1                  | 0                        | .250             |
| PB2_V63I      | 2                  | 4                        | .618             |
| PB2_V338I     | 1                  | 1                        | .446             |
| PB2_R390K     | 1                  | 4                        | 1.000            |
| PB2_M476L     | 1                  | 4                        | 1.000            |
| PB2_A560V     | 1                  | 0                        | .250             |
| PB2_I589T     | 2                  | 3                        | .568             |
| PB2_S644T     | 1                  | 0                        | .250             |
| PB2_S710N     | 1                  | 0                        | .250             |

### Table 7 Demographic characteristics of patients with influenza

|                        | Fatal cases (n = 6) | Non-fatal cases (n = 18) | P value* |
|------------------------|--------------------|--------------------------|----------|
| Age (year), mean ± SD  | 87.3 ± 6.2         | 82.0 ± 7.1               | .12      |
| Sex (male), n (%)      | 3 (50.0)           | 9 (50.0)                 | 1        |
| Comorbidity            | 4 (66.7)           | 14 (77.8)                | .62      |
| Diabetes mellitus      | 2 (33.3)           | 5 (27.8)                 | 1        |
| Cardiovascular disease | -                  | 5 (27.8)                 | .28      |
| Cerebrovascular disease| -                  | 6 (33.3)                 | .28      |
| Neuromuscular disease  | -                  | 4 (22.2)                 | .54      |
| Chronic lung disease   | 4 (66.7)           | 2 (11.1)                 | .02      |
| Asthma                 | 1 (16.7)           | 2 (11.1)                 | 1        |
| Chronic kidney disease | -                  | 1 (5.6)                  | 1        |
| Chronic liver disease  | -                  | 3 (16.7)                 | .55      |
| Malignancy             | -                  | 1 (5.6)                  | 1        |

*Fisher’s exact test.
a twofold HAI titer of the A/Switzerland/ virus. In addition, the isolates in the 3C.2a clade differ from the vaccine strain in antigenicity due to the 8- to 32-fold differences from the vaccine strain in the HAI titer.

This difference seems to be due to structural variation in HA from genetic substitution. Our study demonstrated the variation patterns of epidemic influenza viruses in Korea using genetic as well as antigenic analyses. These data can be used to select vaccine strains and to analyze virus substitution patterns over time.

H3N2 seasons become increasingly severe, with higher numbers of hospitalizations and deaths. We studied the molecular genetics of influenza A viruses and factors associated with patient death following infection with influenza A/H3N2 during the 2011-2016 seasons in Korea. First, we identified genetic substitutions in viruses from fatal cases. The I222V substitution in the NA protein was found in only the two A/H3N2 viruses from the 2011-2012 season. However, other antidrug mutations, H274Y (N2 numbering) and I191V, were not confirmed. Previous studies have shown that the single I222V/M substitution in the NA protein is associated with marginal levels of resistance to oseltamivir, while synergistically increased drug resistance was associated with E119V and H274Y substitutions.15,16,19,22-26 The S31N substitution in the M2 protein was frequently detected in the more recent viral sequences and reference sequences.27 In addition, V51I and I39M substitutions were identified in the 2011-2012 and 2014-2015 fatal case sequences. Among these two substitutions, V51I may enhance the fitness of M2 protein to increase the frequency of adamantane resistance associated with S31N mutation and the substitution of V51-affected viral replication.28 The I39 of M2 was located in the transmembrane region, and substitution of the transmembrane region could affect M2 function, aiding in resistance to M2 inhibitors and transport to the cell surface. In the NP sequences, M374I and M482I were identified only in fatal case sequences. These substitutions have been reported to be involved in a T-cell epitope presented by MHC molecules.29,30 In addition, it was previously reported that other substitutions were identified in the A/Seoul/A1284/2015, A/Seoul/A1251/2015. These internal proteins are involved in a variety of host responses and associated with the infectivity and replication of viruses; therefore, substitutions in these proteins can have a variety of effects.31,32 A significant association between underlying diseases in patients and mortality revealed a significant correlation with chronic lung disease, confirming a well-known relationship.

In conclusion, we observed clade changes in influenza A viruses in Korea from 2011 to 2016. Prediction of clade change using bioinformatics analysis of these data, along with antigenic analyses, can help select effective vaccine strains. We confirmed that the severity of influenza A virus infection was related to underlying patient diseases, such as chronic lung disease, and further studies are needed to confirm associations between mortality and genetic substitutions in the viruses.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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