Screening and mechanistic study of key sites of the hemagglutinin-neuraminidase protein related to the virulence of Newcastle disease virus

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ABSTRACT Newcastle disease is a kind of avian infectious disease caused by Newcastle disease virus (**NDV**). The virulence of NDV is dependent mainly on the fusion (**F**) protein and hemagglutinin-neuraminidase (**HN**) protein. The genomes of 2 viruses, NDV-Blackbird and NDV-Dove, are 99.9% similar, while NDV-Blackbird is a velogenic virus, and NDV-Dove is a lentogenic virus. Further analysis revealed that the F proteins of the 2 strains were identical, and only 5 amino acid sites on the HN proteins were inconsistent. Five different HN mutant plasmids were constructed and analyzed in this study. The results showed that the mutation F110L caused a significant increase in fusion-promotion activity caused by an increase in neuraminidase activity. Because of the increase in receptor-binding activity caused by G116R, there was a significant increase in fusion-promotion activity. The mutation G54S resulted in a slight decrease in the fusion-promotion activity caused by a slight decrease in receptor-binding activity. The slight increase in the fusion-promotion activity caused by A469V was associated with a significant increase in neuraminidase activity. Therefore, the amino acids L110 and R116 played a key role in determining the virulence difference between NDV-Blackbird and NDV-Dove, which could lay a foundation for illuminating the virulence differences of NDV strains, as well as the development of attenuated vaccines.

Key words: Newcastle disease virus (NDV), hemagglutinin-neuraminidase (HN) protein, virulence, cell fusion promotion

INTRODUCTION

Newcastle disease (**ND**) is one of the most important poultry diseases that significantly harm the poultry industry worldwide (Gao et al., 2019). Newcastle disease virus (**NDV**) is the etiological pathogen of ND, also called avian paramyxovirus serotype 1 (Wang et al., 2017). NDV are classified into 3 major pathotypes: velogenic, mesogenic, and lentogenic (Jin et al., 2016). NDV is a single-stranded negative-strand RNA virus with a genomic structure of 3′-Leader-NP-P-M-HN-F-L-Trailer-5′ (Millar and Emmerson, 1988), and the homologous interaction between the fusion (**F**) protein and hemagglutinin-neuraminidase (**HN**) protein is a prerequisite for NDV replication, syncytia formation, and pathogenicity (Huang et al., 2004; Kim et al., 2011; Paldurai et al., 2014).

The HN protein is a type II membrane glycoprotein that has 3 main functions: receptor-binding activity, cell fusion–promotion activity, and neuraminidase (**NA**) activity (Morrison, 2001). Hemadsorption (**HAd**) activity can be used to determine the receptor-binding activity. The HN protein consists of 4 parts: the intracellular zone (**CT**), the transmembrane zone (**TM**), the stalk, and the globular head. The globular head contains

2 receptor-binding sites: Site I is involved in both receptor-binding and NA activities, and site II is involved in receptor-binding activity without NA activity (Porotto et al., 2012; Jin et al., 2016). The receptor-binding sites can bind the sialic acid receptor on the cell surface when the virus infects cells. NA can hydrolyze the sialic acid receptor to promote the spread of viruses and prevent the aggregation of new viruses (Iorio et al., 2001; Ganar et al., 2014). The stalk consists of 4 parallel helix bundles (4-helix bundles), which are formed by 11 amino acids (83-114 **aa**) (Yuan et al., 2011). The stalk can interact with the F protein and play a major role in fusion-promotion activity (Melanson and Iorio, 2004; de Leeuw et al., 2005; Kim et al., 2011). In summary, the HN protein plays an important role in the virulence, replication, and tissue tropism of NDV (Huang et al., 2004; de Leeuw et al., 2005).

The amino acid sequence at the cleavage site of the F protein has been identified as the key to the cleavage ability of the F protein, which is considered the basis for the virulence of NDV. In general, the cleavage site of mesogenic and velogenic viruses is ^{112}R/K-R-Q-R/K-R$\downarrow$F^{117}, and the cleavage site of lentogenic viruses is ^{112}G/E-K/R-Q-G/E-R$\downarrow$L^{117} (Dortmans et al., 2011). Under the activation of the stalk of the HN protein, the F protein can be cleaved from F_0 into F_1 and F_2, and the phosphor peptide of F_1 inserts into the cell surface to form a fusion pore to initiate membrane fusion (Mahon et al., 2011).

Two viruses (NDV-Blackbird and NDV-Dove) are isolated from wild birds in the early infection stage of 2008 (Liu et al., 2013), and we find that both strains belonged to genotype IX and that their genomes were 99.9% similar. However, the intracerebral pathogenicity index of the 2 viruses is 1.638 and 0.425, and the mean death time of the 2 viruses is 37.2 h and 64.8 h, respectively. NDV-Blackbird is a velogenic virus, but NDV-Dove is a lentogenic virus. Whole-genome sequence analysis revealed that there were only 11 different aa in the encoded structural protein and 4 different aa in the nonstructural protein. The site differences were distributed in the P protein (77 aa and 105 aa), M protein (16 aa), HN protein (54 aa, 110 aa, 116 aa, 469 aa, and 522 aa), and L protein (1,076 aa, 1,728 aa, and 1,876 aa). In addition, the nonstructural proteins V and W both have 2 different aa. The aa sites of the F protein were identical, with the cleavage site of ^{112}R-R-Q-R-R$\downarrow$F^{117}, which is the cleavage site of velogenic and mesogenic viruses. In summary, the 2 viruses had significant differences in virulence, indicating that, in addition to the F protein, other factors could be involved in regulating virulence.

Mutations in the transmembrane region of the HN protein affect the structure of the protein. Mutations such as L44A decreased HAd, NA, and fusion-promotion activities (McGinnes et al., 1993). Y526 and E547 are located between site I and site II, and their mutations resulted in the loss of NA and HAd activities (Zaitsev et al., 2004). The mutations E258D, E258S, and Y299A eliminated only NA activity (Connaris et al., 2002). The mutations T216A, F553A, and R557A prevented the fusion-promotion activity of the HN protein and resulted in different degrees of decrease in HAd and NA activities (Takimoto et al., 2002). The mutation R516 A in the region of site II reduced HAd activity and decreased syncytia formation, while NA activity was unaffected (Porotto et al., 2012). The mutation L110A decreased the fusion-promotion activity and NA activity of the HN protein (Stone-Hulslander and Morrison, 1999). The mutation G116P increased

Figure 1. Comparison of syncytia sizes and growth characteristics between NDV-Blackbird and NDV-Dove. (A) Baby hamster kidney (BHK21) cells were inoculated with 2 viruses of 0.01 multiplicity of infection (MOI) for 24 h after infection (hpi), respectively. The syncytia were photographed on an inverted microscope with 400-fold magnification, Bar = 20 μm. (B) The diameters of 40 syncytia were separately measured by the software ImageJ as the fusion index of 2 viruses. (C) Chicken embryo fibroblasts (DF-1 cells) were inoculated with 2 viruses at an MOI of 0.01. Growth characteristics were evaluated until infection of 72 hpi. *$P < 0.05$, significant; **$P < 0.01$, very significant; ***$P < 0.001$, extremely significant.

Figure 2. Analysis of HAd and NA activities between NDV-Blackbird and NDV-Dove. BHK21 cells were inoculated with 2 viruses at an MOI of 2 for 8 hpi and then determined for hemadsorption (HAd) (A) using HAd assay and neuraminidase (NA) activity (B) using neuraminidase assay kit, respectively. ***$P < 0.001$, extremely significant.

the fusion-promotion activity but had no effect on receptor-binding and NA activities (Adu-Gyamfi et al., 2016). The mutation V469A significantly decreased the fusion-promotion activity of the HN protein, and the receptor-binding and NA activities were decreased to different extents (Sun et al., 2015). In summary, mutations in various regions of the HN protein may result in changes in one or more of the HAd, NA, and fusion-promotion activities, and the virulence of NDV is related to the HN protein. Therefore, under the premise that the aa sequence of the F protein was identified, we suspected that 5 aa sites may lead to changes in the function of the HN protein, which ultimately caused the difference in virulence between the 2 viruses.

At present, there are many studies on the effects of HN protein aa mutations on HAd, NA, and fusion-promotion activities. However, 2 NDV strains with extremely high similarity but obvious differences in pathogenicity and virulence have not been found. Therefore, this study was based on these 2 viruses to identify the effects of the HN protein on the fusion ability, proliferation ability, and pathogenicity of NDV, which could help identify key sites in the HN protein causing virulence differences. Then, we can use reverse genetic technology to replace the sites of the NDV vaccine with the identified key sites to improve the effectiveness of the modified vaccine. Finally, our study will provide a theoretical basis for the prevention and treatment of NDV and vaccine development.

MATERIALS AND METHODS

Viruses and Cells

Two viruses, NDV-Blackbird and NDV-Dove, were isolated from wild birds and stored by our laboratory. Chicken embryo fibroblasts (**DF-1** cells) and baby hamster kidney (**BHK21**) cells were obtained from the American Type Culture Collection, preserved in our laboratory, and grown in Dulbecco's Modified Eagle Medium (**DMEM**; Gibco, Grand Island, NY) with 10% fetal bovine serum (**FBS**; Gibco, Grand Island, NY). The cells were also maintained in DMEM containing 1% FBS at 37°C under a 5% CO_2 atmosphere.

Sequence Analysis of Five aa Sites

The positions of 5 different aa sites were shown in the three-dimensional structure of the HN protein using PyMOL software (Schrodinger LLC, Cambridge, UK) (Li et al., 2004; Yuan et al., 2011). A total of 763 natural NDV strains (Supplementary Table 1) were collected from the GenBank database for complete HN protein sequences, and the fusion (**F**) protein cleavage sequences of these strains were ^{112}R/K-R-Q-R/K-R↓F^{117} (the cleavage site of velogenic and mesogenic viruses). Then, sequence logos of the 5 aa sites were analyzed to display the position-specific features of multiple sequence alignments using Lasergene software (DNA-Star, Madison, WI), and the results were shown using Weblogo (University of California, Berkeley, CA) (Wang et al., 2017).

Construction of HN and F Protein Expression Plasmids

First, viral RNA from NDV-Blackbird and NDV-Dove was extracted using a StarSpin Animal RNA Mini Kit (Genstar, Beijing, China) according to the manufacturer's protocol, and cDNA was synthesized from the viral RNA using a reverse transcription PCR kit (Genstar, Beijing, China). Then, the specific primers (TSINGKE, Beijing, China) HN-F and HN-R (Supplementary Table 2) were used to amplify the HN gene using high-fidelity enzymes (TaKaRa, Beijing, China). These amplified HN genes were inserted into the eukaryotic expression vector plasmid pCAGGS containing XhoI and BglII restriction sites. When designing the primers, a HA tag was added to the C-terminus of the HN protein for detection. Furthermore, these

obtained plasmids were named pBHN and pDHN. In this way, the primers F-F and F-R were designed to amplify the F genes and inserted into the vector pCAGGS containing EcoRI and BglII restriction sites, with a Flag tag added at the C-terminus of the F protein for detection, which was named pB/DF. Then, using the HN gene of NDV-Dove as a backbone, 5 aa sites on the NDV-Dove HN gene were mutated to match the sites on NDV-Blackbird using overlapping PCR. Then, 5 different HN mutant plasmids were constructed and named pDHN-G54S, pDHN-F110L, pDHN-G116R, pDHN-A469V, and pDHN-T522I. Taking pDHN-G54S as an example, the method for constructing HN mutant plasmids was the following. First, the primers G54S-F and G54S-R were designed, which mutated glycine (G) to serine (S) at the 54 aa sites. Then, using pDHN as a template, the first fragment was amplified with the primers HN-F and G54S-R, and the second fragment was amplified with G54S-F and HN-R. Then, the 2 fragments were used as templates for overlapping PCR, and then the PCR products were inserted into the vector pCAGGS. Finally, a successful HN mutant plasmid was constructed.

Plaque Assay

The plaque technique was used to determine virus titers, as previously described (Chu et al., 2018). BHK21 cells were cultured in 24-well plates, and when the cell density reached 90% or higher, the viruses were serially diluted 10-fold ($10^{-1} \sim 10^{-6}$) with serum-free DMEM. Each duplicate well contained 200 µL of diluted virus solution, followed by adsorption at 37°C under 5% CO_2 for 1 h after inoculation (**hpi**). The cells were washed, 500 µL of DMEM containing 1% methylcellulose was added, and then the plate was further cultured for 3 D after inoculation (**dpi**). Subsequently, the medium was removed, 500 µL of PBS containing 4% paraformaldehyde was added to fix the cells for 30 min, 500 µL of 0.1% crystal violet solution was added to stain the cells for 30 min, the staining solution was aspirated, and the cells were washed again. Each plaque originated as a virion, and thus, the virus titers were counted.

Viral Growth Characteristics Assay

The proliferation of the 2 viruses in DF-1 cells was evaluated. First, 1×10^6 cells were cultured in each well in a 6-well culture plate. After 12 h, the cells were inoculated with one of the 2 viruses at a multiplicity of infection (**MOI**) of 0.01. After adsorption at 37°C under 5% CO_2 for 1 hpi, the cells were washed, and 500 µL of DMEM containing 1% FBS was added. Then, 100 µL of supernatant was aspirated every 6 hpi, and 100 µL of DMEM containing 1% FBS was added until infection for 72 hpi. The supernatants collected at different time points were assayed for virus titers by the plaque technique.

Fusion Index Assay

The sizes of the syncytia formed by 2 viruses in BHK21 cells were evaluated. First, BHK21 cells were cultured in a 6-well culture plate, and when the cell density reached 90% or higher, the cells were inoculated with 2 viruses at an MOI of 0.01. After adsorption at 37°C under 5% CO_2 for 1 hpi, cells were washed, and 2 mL of DMEM containing 1% methylcellulose was added. At 24 hpi, 40 syncytia were randomly photographed using an inverted microscope (IX73; OLYMPUS, Tokyo, Japan) at 400-fold magnification, and the diameters of the syncytia were counted using the ImageJ software (National Institutes of Health, Bethesda, MD) to compare the membrane fusion indexes of the 2 viruses.

Fusion Promotion Activity Assay

One microgram of HN mutant plasmid and 1 µg of F plasmid were cotransfected into BHK21 cells. After 36 h, 40 fusion regions were randomly photographed using an inverted microscope, and the areas of the fusion regions were counted using the ImageJ software to compare the fusion-promotion activity of those HN mutant proteins.

HAd Activity Assay

In this study, we determined the receptor-binding activity of the HN protein by a HAd activity assay. BHK21 cells were incubated with 2 viruses at an MOI of 2 for 8 hpi at 37°C. As described previously, with some modifications (Li et al., 2004), the medium was removed, and the cells were washed with precooled PBS. Then, 1 mL of a 2% chicken red blood cell (**CRBC**) suspension was added to each well. After incubation on ice for 30 min, 100 µL of 50-mmol NH_4Cl solution was added to lyse the CRBC bound to cells infected by the virus, and the mixture was collected into a 1.5-mL centrifuge tube and placed for 5 min. After centrifugation (12,000 rpm/min, 4°C, 5 min), 100 µL of each supernatant was aspirated to a 96-well plate. The absorbance was measured at a wavelength of 540 nm using an ELISA reader (Epoch; BioTek, Winooski, VT). The HAd activity of the 2 viruses was determined.

Then, 1.8 µg of HN mutant plasmids were individually transfected into BHK21 cells in a 12-well plate, and after 24 h, the HAd activity at the protein level was determined by the methods described previously.

NA Activity Assay

We determined the NA activity of the HN protein by an NA activity assay. BHK21 cells were incubated with 2 viruses at an MOI of 2 for 8 hpi at 37°C, the medium was removed, and the cells were washed. Then, 400 µL of EDTA-PBS solution was added at 4°C for 30 min, collected into a 1.5-mL centrifuge tube, and centrifuged to remove the supernatant. Then, 20 µL of

radioimmunoprecipitation assay buffer (Solarbio, Beijing, China) was added, and the samples were freeze-thawed 3 times at $-80°C$. After centrifugation (12,000 rpm/min, 4°C, 5 min), the supernatant was transferred to a 96-well plate, and the NA activity of the 2 viruses was detected using a NA assay kit (Beyotime, Shanghai, China) with a microplate reader (Spark; TECAN Group AG, Männedorf, Switzerland).

Transfection was performed as previously described, and the NA activity at the protein level was determined by the methods described previously.

Immunofluorescence Assay

An immunofluorescence assay (**IFA**) was used to qualitatively analyze the expression of HN mutant proteins. As described previously, 1 μg of HN mutant plasmid was individually transfected into BHK21 monolayer cells and cultured for 24 h. Then, the cells were washed, and 200 μL of PBS containing 4% paraformaldehyde was added to fix the cells for 15 min. Then, the cells were washed 3 times, 500 μL of PBS containing 1% bovine serum albumin (**BSA**) was added, and the cells were incubated for 30 min at room temperature. Furthermore, a HA label-specific mouse primary antibody (Invitrogen, Carlsbad, CA) and fluorescein isothiocyanate–conjugated secondary antibody (goat antimouse IgG; Abcam, Cambridge, UK) were diluted with 1% BSA (1:500). Then, 200 μL of diluted primary antibody was added and incubated for 2 h, and the cells were washed 5 times. Then, 200 μL of diluted secondary antibody was added and incubated for 2 h in the dark. Finally, the cells were observed and photographed using a fluorescence microscope (IX73; OLYMPUS, Tokyo, Japan).

Flow Cytometry Assay

A flow cytometry (**FCM**) assay was used to quantitatively analyze the expression efficiency of HN mutant proteins on the cell membrane. As described previously, 3.5 μg of HN mutant plasmid was individually transfected into BHK21 cells and cultured for 24 h. Then, the supernatant was removed, and the cells were digested with 100 μL of 0.25% trypsin-EDTA (Gibco, Grand Island, NE) for 2 min. Then, 500 μL of PBS containing 10% FBS was added to terminate the digestion, and the cells were collected in a 1.5-mL centrifuge tube. Then, the cells were centrifuged, and 200 μL of 4% BSA was added to fix the cells for 30 min at room temperature. The primary antibody and the secondary antibody were the same as those in the IFA assay and diluted with 4% BSA (1:500). After washing the cells, 100 μL of diluted primary antibody was added and incubated for 2 h, with the bottom of the tube flicked every 10 min. Then, the cells were washed 2 times, and 100 μL of diluted secondary antibody was added for 2 h in the dark, with the bottom of the tube flicked every 10 min. Then, 500 μL of PBS containing 1% paraformaldehyde was added to fix the cells for 10 min. Finally, the cells

were filtered through the nitrocellulose filter membrane into a flow tube, and the cell surface fluorescence intensity was detected by a flow cytometer (BD Biosciences, San Jose, CA).

Western Blotting

Western blotting was used to detect the cleavage promotion of HN mutant proteins. As described previously, 1 μg of HN mutant plasmid and 1 μg of F plasmid were cotransfected into BHK21 cells. After adsorption at 37°C under 5% CO_2 for 36 h, the total protein was extracted from cells with radioimmunoprecipitation assay buffer (Solarbio, Beijing, China), separated by SDS-PAGE electrophoresis, and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The polyvinylidene fluoride membrane was blocked with 10% skim milk for 2 h at room temperature, and then a HA or Flag label-specific mouse primary antibody (1:3000; Invitrogen, Carlsbad, CA) was diluted with PBS and incubated with the membrane overnight at 4°C. The membrane was washed 5 times with PBS containing Tween-20 for 8 min intervals. The membrane was incubated with a secondary antibody (HRP-conjugated goat antimouse IgG, 1:3000; Invitrogen, Carlsbad, CA) for 2 h, and then the membrane was washed 5 times with PBS containing Tween-20 for 8 min each time. Finally, we used an ECL super sensitive kit (DiNing, Beijing, China) to expose the membrane by a chemiluminescence imager (MiniChemi610; Sagecreation, Beijing, China). The protein load was normalized to GAPDH signal (1:3000; Sungene Biotech, Tianjin Binhai New Area, China).

Statistical Analysis

Measurement results of other samples were displayed as a percentage of NDV-Dove (or pDHN), and the measurement results of NDV-Dove (or pDHN) were set to 100%. All data represent the mean and standard deviation of 3 independent experiments obtained from the GraphPad Prism 5.0 software (GraphPad Software Inc, San Diego, CA). Statistical analyses were performed with Student t tests (95% confidence levels) using this software: ns, nonsignificant; $*P < 0.05$, significant; $**P < 0.01$, very significant; $***P < 0.001$, extremely significant.

RESULTS

Biological Characteristics of the Two Viruses

The time of syncytium generation for NDV-Blackbird was earlier than that for NDV-Dove, and the size of the syncytium was also larger than that for NDV-Dove (Figures 1A and 1B; Table 1). The proliferation kinetics of NDV-Blackbird were significantly higher than those of NDV-Dove before 36 h after infection (**hpi**) and reached the highest virus titer, with 5.89 log10 at 36 hpi. However, after 36 hpi, the virus titers of NDV-

Figure 3. Shown of differences in amino acid (aa) sites of the hemagglutinin-neuraminidase (HN) protein between NDV-Blackbird and NDV-Dove. (A) There were 11 different aa in the encoded structural protein of 2 viruses. (B) The three-dimensional structure of the HN protein showing the positions of 3 different aa sites. The monomer of HN protein was shown in ribbon mode, globular head in magenta, and stalk in cyan. The residues were shown in spheres model, 110 aa was shown in yellow, 469 aa was shown in green, and 522 aa was shown in blue. However, the sites of 54 and 116 were not shown. (C) Diversity of residues of 5 aa sites was analyzed using WebLogo. (D) Statistical results of the numbers and proportions of aa species in 5 aa positions.

Dove were higher than those of NDV-Blackbird and reached a maximum of approximately 6.12 log10 at 42 hpi (Figure 1C). These decreases in virus titers of NDV-Blackbird may be caused by decreases in the number of surviving cells. The HAd and NA activities of NDV-Blackbird were significantly higher than those of NDV-Dove (Figures 2A and 2B; Table 1).

Sequence Analysis of Five aa Sites

In our study, we showed 11 different aa in the encoded structural protein of 2 viruses and then showed 3 different aa sites in the three-dimensional structure of HN protein between the 2 viruses (Figures 3A and 3B). However, the sites at residues 54 and 116 are not shown.

To determine whether the mutations of the 5 aa of the 2 viruses are major aa, we analyzed 763 complete HN protein sequences from GenBank. These results showed that there were 10 kinds of aa at 54 aa, with R (248) and H (371) residues at frequencies of 32.5% and

48.6% and G (39) and S (65) residue frequencies of 5.1% and 8.5%, respectively. There were 3 kinds of aa at 110 aa, whose frequencies of L (759) and F (3) residues were 99.5% and 0.4%, respectively. There were 4 kinds of aa at 116 aa, whose frequencies of G (750) and R (9) were 98.3% and 1.2%, respectively. At the 469 aa position, there were 4 kinds of aa with frequencies of 99.5% and 0.3% for V (759) and A (2) residues, respectively. At

Table 1. Biological activities between NDV-Blackbird and NDV-Dove.

Virus	Fusion[1]	HAd activity	NA activity
NDV-Dove	100%	100%	100%
NDV-Blackbird	299.6%***	116.2%***	942.8%***

***$P < 0.001$, extremely significant.

Abbreviations: HAd, hemadsorption; NA, neuraminidase; NDV, Newcastle disease virus.

[1]Those results of the average of cell fusion, HAd , and NA activities of 2 viruses (% of NDV-Dove), respectively.

the 522 aa position, there were 4 kinds of aa, whose frequencies of T (744) and I (16) residues were 97.5% and 2.1%, respectively (Figures 3C and 3D).

These data indicated that in the natural strains of NDV with a fusion (**F**) protein cleavage sequence of ^{112}R/K-R-Q-R/K-R↓F^{117}, the major aa at position 54 aa in the HN protein sequence may be R or H residues, 110 aa was L, 116 aa was G residue, 469 aa was V, and 522 aa was T. These results indicated that the mutations in the HN protein of the 2 NDV strains were major aa sites and indicated that the mutations occurred between the major sites.

Analysis of Fusion-Promotion Activity of HN Mutant Proteins

To determine whether the 5 aa mutants modulate the fusion-promotion activity of the HN protein, 40 syncytia were photographed by inverted microscopy after cotransfection of the HN mutant plasmid and F plasmid. The fusion regions of 40 syncytia were counted as the index of fusion-promotion activity of HN protein. Among them, the syncytia formed by pBHN (699.5%) were significantly larger than those formed by pDHN (as a percentage). Compared with those for pDHN, the syncytia formed by G54S were slightly smaller (78.1%), and the syncytia formed by A469V, G116R, and F110L gradually increased, at 168.6, 395.7, and 564.7%, respectively, but those formed by T522I (149.0%) were not significantly different (Figures 4A and 4B; Table 2). These results

indicated that the L110 and R116 aa sites were key sites for fusion-promotion activity.

Analysis of Western Blotting

After cotransfection of the HN mutant plasmid and F plasmid, Western blotting was used to detect the cleavage promotion of HN mutant proteins, and then the Western blots were quantified by densitometry using the ImageJ software. The F protein of all cotransfected samples was cleaved into F_1, but there was no significant difference in the F_1/HN ratios between different HN mutant proteins (Figures 4C and 4D; Table 2). These results indicated that there were no differences in cleavage promotion among the HN mutant proteins.

Analysis of HAd Activity of Different HN Mutant Proteins

Then, a HAd assay was used to determine the receptor-binding activity of the HN protein. After adsorption of CRBC by different HN mutant proteins, the HAd activity was quantified by measuring the absorbance at 540 nm. These results showed that the HAd activities of pBHN (222.1%), G116R (133.8%), A469V (223.2%), and T522I (198.9%) were higher than that of pDHN (as a percentage), that of G54S (77.8%) was slightly lower than that of pDHN, and that of F110L (53.4%) was significantly lower than that of pDHN (Figure 5A; Table 2). These results indicated that the sites R116, V469, and I522 could increase receptor-

Figure 4. Analysis of fusion-promotion and cleavage-promoting activities of the HN mutant proteins. (A) HN mutant plasmid and F plasmid were cotransfected into BHK21 cells for 36 h, and then the fusion regions were photographed on an inverted microscope with 400-fold amplification, Bar = 20 μm. (B) The areas of 40 fusion regions were separately measured by using the software ImageJ as the fusion index of these HN mutant proteins. (C) After 36 h of cotransfection, the amount of each HN or F (F_0 and F_1) protein was determined by Western blot analysis using the HA or Flag label-specific mouse primary antibody and corresponding secondary antibody, and then determined the fusion-promotion activity of HN mutant proteins. (D) Those results of Western blot were quantified by densitometry using the ImageJ software. *$P < 0.05$, significant; ***$P < 0.001$, extremely significant.

Table 2. Biological activities of HN mutant proteins.

HN	Fusion[1]	Cleavage	HAd	NA	Expression
pDHN	100%	100%	100%	100%	100%
pBHN	699.5%***	88.1%	222.1%***	2991.4%**	91.0%
pDHN-G54S	78.1%*	85.3%	77.8%*	123.5%	120.4%
pDHN-F110L	564.7%***	76.5%	53.4%***	252.1%***	114.1%
pDHN-G116R	395.7%***	86.0%	133.8%***	64.0%***	96.7%
pDHN-A469V	168.6%*	108.2%	223.2%***	1911.7%**	82.9%
pDHN-T522I	149.0%	78.7%	198.9%***	279.4%***	99.5%

*$P < 0.05$, significant; **$P < 0.01$, very significant; ***$P < 0.001$, extremely significant.

Abbreviations: HAd, hemadsorption; HN, hemagglutinin-neuraminidase; NA, neuraminidase.

[1]Those results of the average of the fusion-promotion, cleavage-promoting, HAd, NA, and cell surface expression activities of HN mutant proteins (% of pDHN), respectively.

binding activity and that S54 and L110 could decrease receptor-binding activity.

Analysis of NA Activity of Different HN Mutant Proteins

After transfection of HN mutant plasmids, a NA assay kit was used to quantitatively detect the NA activity of different HN mutant proteins, and the NA activities of HN mutant proteins had different activities than pDHN (as a percentage). The NA activity of pBHN (2,991.4%) was significantly higher than that of pDHN, and that of G54S (123.5%) was not significantly different ($P = 0.25$). The mutation A469V activity (1,911.7%) was significantly higher than pDHN activity, and F110L (252.1%) and T522I activities (279.4%) were higher than pDHN activity; however, G116R activity (64.0%) was significantly lower than pDHN activity (Figure 5B; Table 2). These results showed that the L110, V469, and I522 sites could increase NA activity and that R116 could decrease NA activity.

Analysis of the Cell Surface Expression Efficiency of HN Mutant Proteins

To determine whether the change in the protein expression efficiency caused the changes in biological activities of the five HN mutant proteins, IFA and FCM assay were used to measure the cell surface expression efficiency of HN mutant proteins. IFA assays showed yellow-green fluorescence for each HN protein, indicating that both HN proteins were successfully expressed on the cell surface. The FCM assay further detected the fluorescence intensity on the cell surface, and the results showed that the cell surface expression efficiency of the HN mutant protein was not significantly different from that of pDHN (Figures 6A and 6B; Table 2), which proved that the biological activities of HN mutant proteins were not caused by changes in the cell surface expression efficiency.

DISCUSSION

Although the cleavage site of the fusion (**F**) protein has an important influence on the degree of membrane fusion and pathogenicity, it is not the only determinant factor (Kim et al., 2011). It has been reported that the virulence or cell fusion activities of NDV strains with identical cleavage sites also show a large difference (Shengqing et al., 2002; de Leeuw et al., 2003). Using the LaSota lentogenic virus as a backbone and replacing the HN protein of a lentogenic virus by the HN protein of a velogenic virus increased the tissue infection of the recombinant virus (Oldoni et al., 2005; Wakamatsu et al., 2006). These studies proved that

Figure 5. Analysis of HAd and NA activities of HN mutant proteins. HN mutant plasmid was transfected into BHK21 cells for 24 h, and then determined for HAd activity (A) using HAd assay, and NA activity (B) using neuraminidase assay kit, respectively. *$P < 0.05$, significant; **$P < 0.01$, very significant; ***$P < 0.001$, extremely significant.

Figure 6. Analysis of cell surface expression efficiency of the HN mutant proteins. (A) The expression of the HN mutant proteins was detected by immunofluorescence assay (IFA). The HN mutant plasmid was transfected into BHK21 cells for 24 h, then the cells were incubated with primary antibody of anti-HA and secondary antibody of anti-Fluorescein Isothiocyanate (FITC), and then cells were photographed on a fluorescent inverted microscope with 100-fold magnification, Bar = 100 μm. (B) The surface expression efficiency of the HN mutant protein was detected by flow cytometry (FCM) assay. The HN mutant plasmid was transfected into BHK21 cells for 24 h, and cells were incubated with primary antibody and secondary antibody as mentioned previously, and then cell surface fluorescence intensity was measured by using a flow cytometer.

the HN protein could indeed affect the pathogenicity and virus replication of NDV.

After observing the sizes of syncytia at the cellular level, we found that NDV-Blackbird had stronger membrane fusion ability than NDV-Dove and that NDV-Blackbird was 299.6% ($P < 0.001$) better than NDV-Dove, which indicated that the virulence of NDV-Blackbird was more significant than that of NDV-Dove. Comparing the proliferation kinetics of the 2 viruses, we found that NDV-Blackbird proliferated faster than NDV-Dove. However, after 36 hpi, we observed that there were no surviving cells in the wells containing NDV-Blackbird by microscopy, but there were surviving cells in the wells inoculated with NDV-Dove, which may be the reason why the virus titers of NDV-Blackbird were lower than those of NDV-Dove after 36 hpi. These results were consistent with a previous study, which indicated that the virulence of NDV-Blackbird was obviously higher than that of NDV-Dove.

The aa sites of the F protein of the 2 viruses were completely identical, with a significant difference in virulence, so the difference in 11 aa on other proteins provided good experimental material for studying the other factors contributing to viral toxicity. The fusion promotion, receptor-binding, and NA activities of pBHN were significantly different from those of pDHN, which were 699.5%, 222.1%, and 2,991.4% ($P < 0.001$) of pDHN, respectively. These results suggested that the HN protein caused a difference in the virulence of the 2 viruses, and the difference may be caused by the 5 different sites on the HN protein.

We only showed the 3 different aa sites in the three-dimensional structure of the HN protein between the 2 viruses. However, the sites at residues 54 and 116 are not shown because the structures of 1-79 aa and 116-122 aa were not visible in the crystal structure reported by Yuan et al., 2012. However, based on the three-dimensional results, we found that the 5 aa sites were located in different regions of the HN protein, and those results showed that mutations in other regions of the HN protein also had different effects on receptor-binding and

NA activities, except for site I and site II of the globular head, while those effects ultimately led to different changes in fusion-promotion activity (Wang and Iorio, 1999; Corey and Iorio, 2007; Yuan et al., 2012; Xu et al., 2013). The stalk of the HN protein could play roles in HN-F protein interactions and head-stalk interactions (Stone-Hulslander and Morrison, 1999), and the mutations G54S and F110L in the stalk region had different effects on the fusion-promotion activity. The mutation G54S is near the transmembrane region and did not affect the cleavage of the F protein, indicating that this site may not participate in the interaction with the F protein, which ultimately led to a slight decrease in the fusion-promotion activity (78.1%) caused by a slight decrease in receptor-binding activity (77.8%). The mutation F110L led to a different change in fusion-promotion activity (564.7%), receptor-binding activity (53.4%), and NA activity (252.1%). Although the decrease in receptor-binding activity weakened the ability of the HN protein to bind sialic acid receptors, the significant increase in NA activity promoted the HN protein to hydrolyze these receptors. Therefore, when NDV infect cells, new virions could be better released to infect adjacent cells. Therefore, the mutation F110L led to an increase in fusion-promotion activity due to an increase in NA activity.

Mutations in a soft junction region (116-123 aa) between the globular head and stalk could change the biological activities of the HN protein (Adu-Gyamfi et al., 2016). The mutation G116R may alter the flexibility of the globular head, which may make the HN protein more readily associated with sialic acid receptors on the cell surface. Although the ability to hydrolyze sialic acid receptors was decreased at the same time, the final changes were beneficial for the fusion promotion of HN proteins. Therefore, the mutation G116R led to a significant increase in receptor-binding activity (133.8%), resulting in a significant increase in fusion-promotion activity (395.7%). The globular head of the HN protein has HAd and NA activity sites, and the mutations A469V and T522I are located in the β5-S1 sheet and the β6 sheet

outside these activity sites, respectively (Langedijk et al., 1997; Sun et al., 2015). They also led to different effects on biological activities. The mutation A469V led to a slight increase in fusion-promotion activity (168.6%), but the degree of increase was not major, and the increase in activity was caused by the increase in receptor-binding activity (223.2%) and NA activity (1,911.7%) but was more likely to be associated with the significant increase in NA activity. When the virus infected cells, the changes made the protein have a stronger ability to hydrolyze sialic acid receptors, making it easier to release new virions and then infect surrounding cells. Although the mutation T522I had certain effects on NA activity (279.4%) and receptor-binding activity (198.9%), those effects were not sufficient to cause a significant increase in fusion-promotion activity (149.0%). Therefore, studying these effects of the 5 sites on the virulence of the HN protein is beneficial for studying the effects of different regions on the function of the HN protein.

Simultaneous cotransfection of the HN mutant plasmid and the F plasmid resulted in no significant difference in the degree of cleavage promotion of HN mutant proteins, which suggested that the other reasons of the HN protein, not activation of the F protein, affect the virulence difference of the 2 viruses. Using an IFA and a FCM assay, we found that each mutant HN protein was expressed and that there were no significant differences in cell surface expression efficiency, indicating that their changes in activities were also not caused by protein expression efficiency.

Finally, it was determined that the difference in the 5 aa on the HN protein could indeed result in a higher membrane fusion capacity for NDV-Blackbird than for NDV-Dove. Mutation of the 5 aa sites caused different degrees of fusion-promotion activity of the HN protein, suggesting that there was some evolution between NDV-Blackbird and NDV-Dove, which may cause changes in virulence. The aa L110 and R116 were key sites in determining the different virulence between NDV-Blackbird and NDV-Dove. These results laid the foundation for subsequent studies of pathogenicity and the development of an attenuated vaccine for ND.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at http://doi.org/10.1016/j.psj.2020.04.014.

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