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Effects of HA and NA glycosylation pattern changes on the transmission of avian influenza A(H7N9) virus in guinea pigs

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ABSTRACT

Avian influenza H7N9 virus has posed a concern of potential human-to-human transmission by resulting in seasonal virus-like human infection cases. To address the issue of sustained human infection with the H7N9 virus, here we investigated the effects of hemagglutinin (HA) and neuraminidase (NA) N-linked glycosylation (NLG) patterns on influenza virus transmission in a guinea pig model. Based on the NLG signatures identified in the HA and NA genetic sequences of H7N9 viruses, we generated NLG mutant viruses using either HA or NA gene of a H7N9 virus, A/Anhui/01/2013, by reverse genetics on the 2009 pandemic H1N1 virus backbone. For the H7 HA NLG mutant viruses, NLG pattern changes appeared to reduce viral transmissibility in guinea pigs. Intriguingly, however, the NLG changes in the N9 NA protein, such as a removal from residue 42 or 66 or an addition at residue 266, increased transmissibility of the mutant viruses by more than 33%, 50%, and 16%, respectively, compared with a parental N9 virus. Given the effects of HA-NA NLG changes with regard to viral transmission, we then generated the HA-NA NLG mutant viruses harboring the H7 HA of double NLG addition and the N9 NA of various NLG patterns. As seen in the HA NLG mutants above, the double NLG-added H7 HA decreased viral transmissibility. However, when the NA NLG changes occurred by a removal of residue 66 and an addition at 266 were additionally accompanied, the HA-NA NLG mutant virus recovered the transmissibility of its parental virus. These demonstrate the effects of specific HA-NA NLG changes on the H7N9 virus transmission by highlighting the importance of a HA-NA functional balance.

1. Introduction

It had been known that an avian influenza A virus could not readily infect human beings due to the difference of receptor binding specificity between avian and human influenza HA proteins [1–3], one of the host barriers of influenza A viruses [4]. Since its first index case of H5N1 infection reported in Hong Kong 1997 [5], however, avian influenza viruses have been considered not only the agent causing severe respiratory diseases in humans but also that of possible human-to-human transmission. It was then demonstrated in a ferret model that a H5N1 virus might diffuse within the human population by aerosol transmission [6,7].

An avian influenza A(H7N9) virus was also reported for its first invasion to humans in China 2013. Since then, the virus has attacked people in the mainland China every season, like other human seasonal influenza viruses, by resulting in more than 30% case-fatality rate (CFR) [8]. Despite some differences of transmissibility demonstrated in previous studies [9], the seasonality and higher CFR of the H7N9 virus demand our preparedness by underlining a pandemic potential of the virus.

N-linked glycosylation (NLG) is one of the biochemical reactions in the cells of eukaryotes and archaea [10]. For influenza viruses, it works not only for a way of immune evasion against host immunity but also as the determinants of viral antigenicity, virulence, and so forth [11,12]. Intriguingly, it was reported by two different studies that the presence or absence of NLG in the H5 HA appeared to affect aerosol transmission of a H5N1 virus [6,7], and these two studies reported different amino acid mutations identified in the viral genomes of aerosol transmissible H5N1 viruses. Of the mutations reported, it was the two HA mutations (N158D and T160A; H3

Abbreviations: HA, hemagglutinin; NA, neuraminidase; NLG, N-linked glycosylation.

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numbering), one from each study, that drew our attention because either mutation would disrupt a sequential NLG sequon (N-X-A/T: N, asparagine; X, any amino acid except for proline; and A/T, alanine or threonine), which might eventually remove a NLG from the HA globular head region. It was one of the various mutations that endowed inter-mammalian transmissibility to a H5N1 virus. However, given the essential binding role of the HA protein to cellular receptors [13], NLG removal from the distal tip of HA protein might be a determinant of avian influenza viruses to obtain transmissibility among human beings. In this regard, we here scrutinized the effects of NLG pattern changes in the HA and NA proteins on the transmission of the H7N9 virus in a guinea pig model. To evaluate viral transmissibility, we generated NLG mutant viruses using the HA and/or NA genes of a human-infecting H7N9 virus, A/Anhui/01/2013 (AH01), on the genetic backbone of a 2009 pandemic H1N1 virus, A/Korea/01/2009 (K09). Then, we investigated transmission efficiency of the NLG mutant viruses based on the viral titers determined using the nasal wash samples collected from the transmitted guinea pigs.

2. Materials and methods

2.1. Ethics

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Animal, Plant, and Fisheries Quarantine and Inspection Agency of Korea. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Korea University College of Medicine (permit number: KUIACUC-2014-249).

2.2. Viruses and cells

The 2009 pandemic H1N1 influenza K09 virus was obtained from the Korea Centers for Disease Control and Prevention (KCDC). Madin-darby canine kidney (MDCK) and human embryonic kidney (293T) cells were obtained from the American Type Culture Collection (ATCC) and maintained with Eagle's minimum essential medium (Lonza, Basel, Switzerland) and Dulbecco's modified eagle medium (Gibco, Waltham, MA), respectively, supplemented with 10% FBS and antibiotics (penicillin/streptomycin). The cells were then cultured at 5%, 37 °C.

2.3. NLG pattern analysis

NLG modification patterns of the HA and NA proteins of H7N9 viruses were analyzed based on the genomic sequences registered into the GenBank database. 359 HA and 289 NA sequences were downloaded and aligned using a MAFFT (v7.130b) program [14]. Based on the patterns observed from the alignment, NLG mutant plasmids were constructed, and the NLG mutant viruses were generated by reverse genetics.

2.4. Plasmid-based reverse genetics

The eight genetic plasmids of 2009 pandemic H1N1 influenza K09 virus (NCBI taxonomy ID: 644289) virus were cloned into ambisense pDZ plasmids (kindly provided by Peter Palese, Mount Sinai School of Medicine, New York, NY) and used as the backbone virus for reverse genetics in this study. The HA and NA genes of AH01 virus were commercially synthesized (Cosmogenetech, Seoul, Republic of Korea) and used for the templates of NLG modification and for reverse genetics. 6:2 and 7:1 recombinant viruses of HA and/or NA NLG mutants were generated on the backbone of K09. Briefly, the eight genetic plasmids of each combination were transfected into co-cultured 293T and MDCK cells. After 48–72 h of transfection, cell supernatants were examined by a hemagglutination assay. A single viral clone of positive samples was then purified by a plaque assay in MDCK cells and inoculated into 10 days old fertile chicken eggs. The recombination and NLG mutation of each virus were confirmed by sequence analysis after reverse transcription PCR.

2.5. Viral transmission study in a guinea pig model

Female guinea pigs (250–300 g; Orient Bio Inc, Seongnam, Republic of Korea) were used for a contact transmission study. Briefly, a guinea pig was infected intranasally with 10^5 plaque forming unit (PFU) of each virus. Next day, each infected guinea pig was co-caged with a naïve one for 14 days. Nasal wash sample was then collected using 1 ml of PBS (supplemented with 0.3% BSA and penicillin/streptomycin) every other day. The collected samples were centrifuged, and the supernatants were used for the virus titration in MDCK cells by the plaque assay.

3. Results

3.1. HA and NA NLG patterns of H7N9 viruses

Using the HA and NA sequences downloaded from the GenBank database, NLG signatures of H7N9 viruses were investigated. For the HA, seven potential glycosites at residues 30, 46, 141, 167, 249, 421, and 493 were identified, and of them, residues 141, 167, and 249 were found within the HA globular head region (Fig. 1A). For the NA, 10 potential glycosites at residues 42, 52, 63, 66, 87, 147, 202, 266, 346, and 459 appeared to have been utilized, but NLG at residues 266, 346, and 459 was identified only from one case out of 289 sequences (for NLG at residue 266, A/Nanjing/3/2013; for NLG at residue 349, A/Shenzhen/SP26/2014; and for NLG at residue 459, A/guinea fowl/Nebraska/17096/2011) (Fig. 1B). Given the roles of NLG in the HA head and NA stalk regions [15,16], we selected three glycosites (for HA, residues 141, 167, and 249; for NA, residues 42, 52, and 66) from each protein for the generation of mutant viruses using the strategy of NLG addition or removal. The four HA NLG mutants were rK09/H7+141 (NLG addition at HA residue 141), rK09/H7+167 (NLG addition at HA residue 167), rK09/H7-249 (NLG removal from HA residue 141), and rK09/H7+141+167 (NLG addition at HA residues 141 and 167). Similarly, the four NA NLG mutants of rK09/N9-42, rK09/N9-52, rK09/N9-66, and rK09/N9+266 were generated (Supplemental Tables S1 and S2). Of the three NA glycosites identified from one genetic sequence, we only used the residue 266 for the generation of a NA NLG mutant virus because NA residues 349 and 459 were located around the inhibitor binding sites [17]. rK09, rK09/H7, and rK09/N9 viruses were generated for their use as control viruses.

3.2. Transmissibility of H7 HA NLG mutant viruses in guinea pigs

We first evaluated transmission efficiency of rK09 virus in a guinea pig, contact transmission model. As demonstrated previously, rK09 exhibited approximately 66.7% transmission efficiency (Fig. 2A) [15]. Based on this efficiency, we determined the effects of NLG changes on viral transmission. rK09/H7, a 7:1 reassortant virus between the AH01 HA and seven other genes of K09, exhibited enhanced rates (88.9%) of transmission (Fig. 2B). It might indicate a comparable or better functional balance between H7 HA and N1 NA proteins than that between K09 HA and NA proteins. However, all of the four NLG mutant viruses, rK09/H7+141, rK09/H7+167, rK09/H7-249, and rK09/H7141+167, appeared to be less transmissible in guinea pigs. They only resulted in 55.6%, 55.6%, 44.4%, and 44.4%

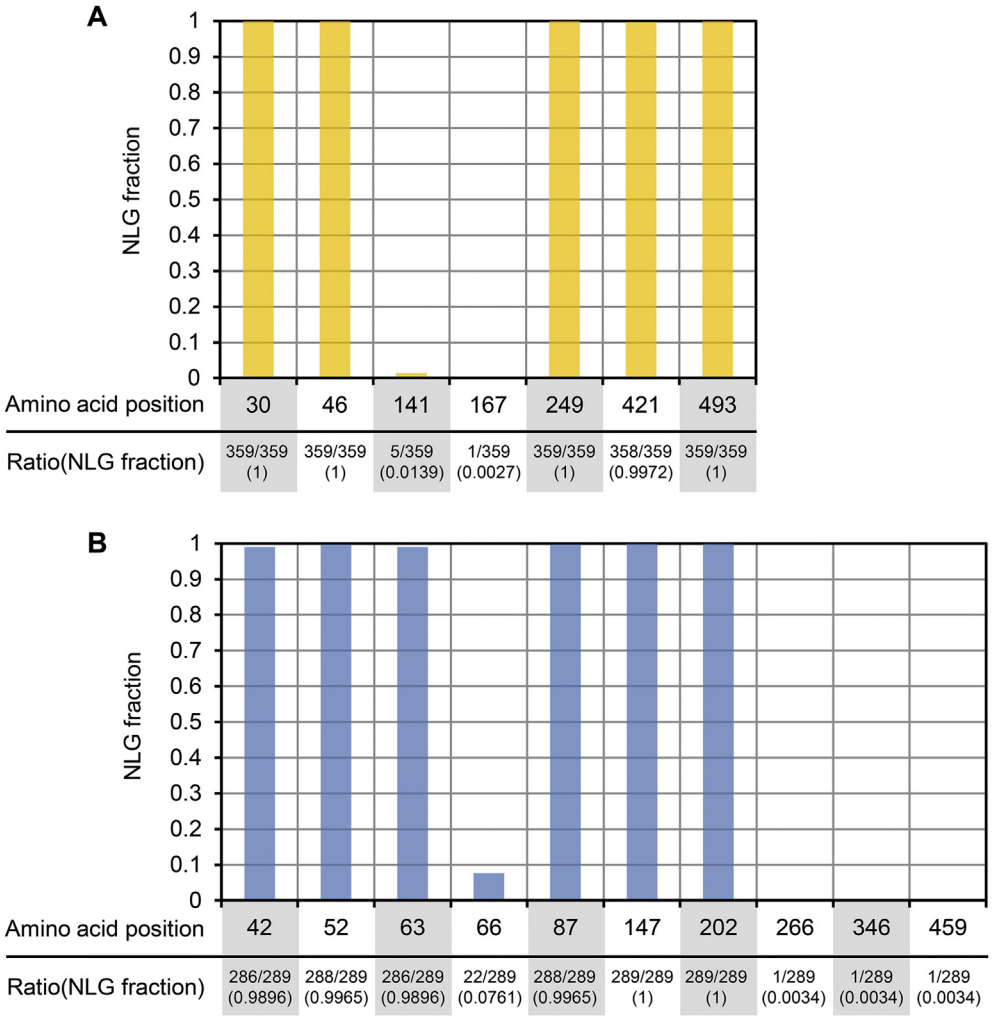

Fig. 1. NLG patterns of the HA and NA protein genes of H7N9 viruses. Using the genetic sequences (HA, n = 359 and NA, n = 289) downloaded from the GenBank database, NLG patterns of the HA (A) and NA (B) proteins of H7N9 viruses were analyzed. Ratios of NLG modification at certain residues were presented by a fraction (the number of sequences harboring NLG divided by the total number of sequences). Residue numbers are indicated based on the HA and NA sequences of A/Shanghai/02/2013 (GenBank accession number: KF021597 and KF021599, respectively).

transmission rates, respectively (Fig. 2C–F). Given the reduced transmission efficiency of the H7 HA NLG mutant viruses, the NLG pattern changes tested in this study might impede an essential interaction between the HA and its cellular receptors because the viral titers of the transmitted guinea pigs in the four NLG mutant groups were lower than those of rK09 and rK09/H7 groups. Only the dual NLG mutant virus, rK09/H7+141+167, exhibited relatively high replication titers in the transmitted guinea pigs at 2 and 4 days after transmission (Fig. 2F).

3.3. Transmissibility of N9 NLG mutant viruses in guinea pigs

We then evaluated transmission efficiency of the four N9 NA NLG mutant viruses in the same guinea pig model. Unlike the transmission rate of rK09/H7 group (Fig. 2B), N9 NA did not exhibit its compatibility to H1 HA. As seen in Fig. 3A, rK09/N9 only resulted in a 33.3% rate of transmission. NLG removal from NA residue 52 (rK09/N9-52) exhibited even worse transmission efficiency (Fig. 3C). However, NLG addition at residue 266 and removal from residue 42 resulted in approximately 17% and 33.33% efficiency increases, respectively, compared with that of rK09/N9 (Fig. 3B and E). Intriguingly, NLG removal from residue 66 showed the highest increase of transmission rate by letting the rK09/N9-66 virus transmit to five guinea pigs out of six tested (Fig. 3D). Given the location of glycosites 42, 52, and 66 in the NA protein, the highest transmission rate of rK09/N9-66 might suggest not the number of glycosites in the NA stalk but the glycosite position is of importance in terms of viral transmissibility, which would be eventually affected by viral replication property as demonstrated in the highest viral titers recorded in the transmitted guinea pigs of rK09/N9-66 group.

3.4. Effects of HA and NA NLG patterns on the transmission of H7N9 viruses in guinea pigs

To investigate the overall effects of HA-NA NLG pattern changes on the transmission of the H7N9 virus, we additionally generated 6:2 reassortant viruses using the AH01 HA protein gene harboring additional NLG at residues 141 and 167 (H7+141+167), based on the viral replication titers in the transmitted guinea pigs, and the AH01 NA protein gene harboring various NLG changes (N9-66, N9-42-66, N9-52-66, and N9-66+266) on the K09 backbone (Supplemental Table S3). The 6:2 reassortant rK09/H7,N9 virus exhibited a 90% transmission rate in guinea pigs (Fig. 4A), which is

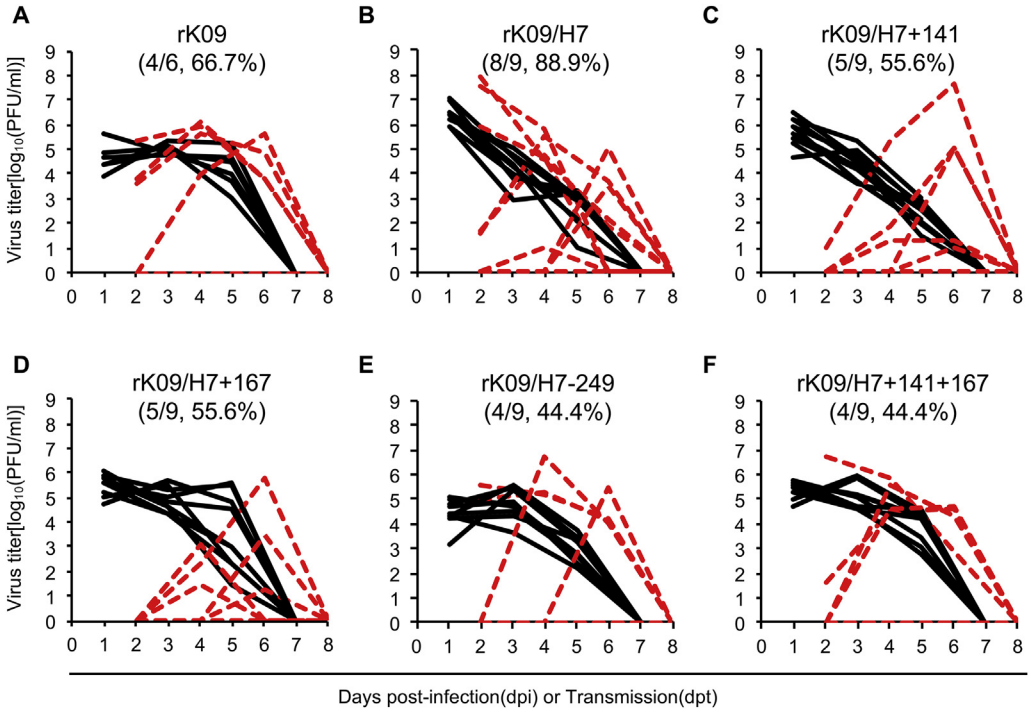

Fig. 2. Contact transmission of HA NLG mutant viruses in guinea pigs. Contact transmission efficiency (the number of transmitted guinea pigs/the number of infected guinea pigs, %) of H7 HA NLG mutant viruses (C, rK09/H7+141; D, rK09/H7+167; E, rK09/H7-249; and F, rK09/H7+141+167) was evaluated in guinea pigs. Black solid lines indicate the viral titers of nasal washing samples of the infected guinea pigs, and red dotted lines indicate those of the transmitted guinea pigs. rK09 (A) and rK09/H7 (B) were used as a control of the genetic backbone and the H7 NLG mutant viruses, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

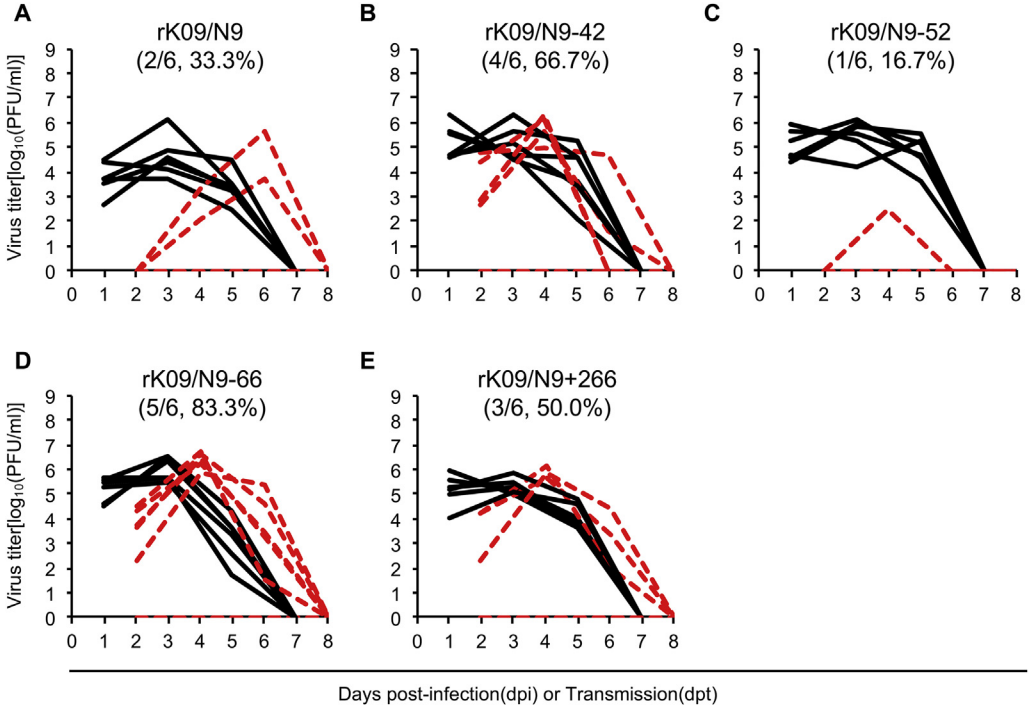

Fig. 3. Contact transmission of NA NLG mutant viruses in guinea pigs. Contact transmission efficiency (the number of transmitted guinea pigs/the number of infected guinea pigs, %) of N9 NA NLG mutant viruses (B, rK09/N9-42; C, rK09/N9-52; D, rK09/N9-66; and E, rK09/N9+266) was evaluated in guinea pigs. Black solid lines indicate the viral titers of nasal washing samples of the infected guinea pigs, and red dotted lines indicate those of the transmitted guinea pigs. rK09/N9 (A) was used as a control of the N9 NLG mutant viruses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the highest transmissibility among the tested viruses. However, addition of NLG at HA residues 141 and 167 appeared to be less compatible with the AH01 NA protein because rK09/H7+141+167,N9 transmitted only to two guinea pigs out of ten

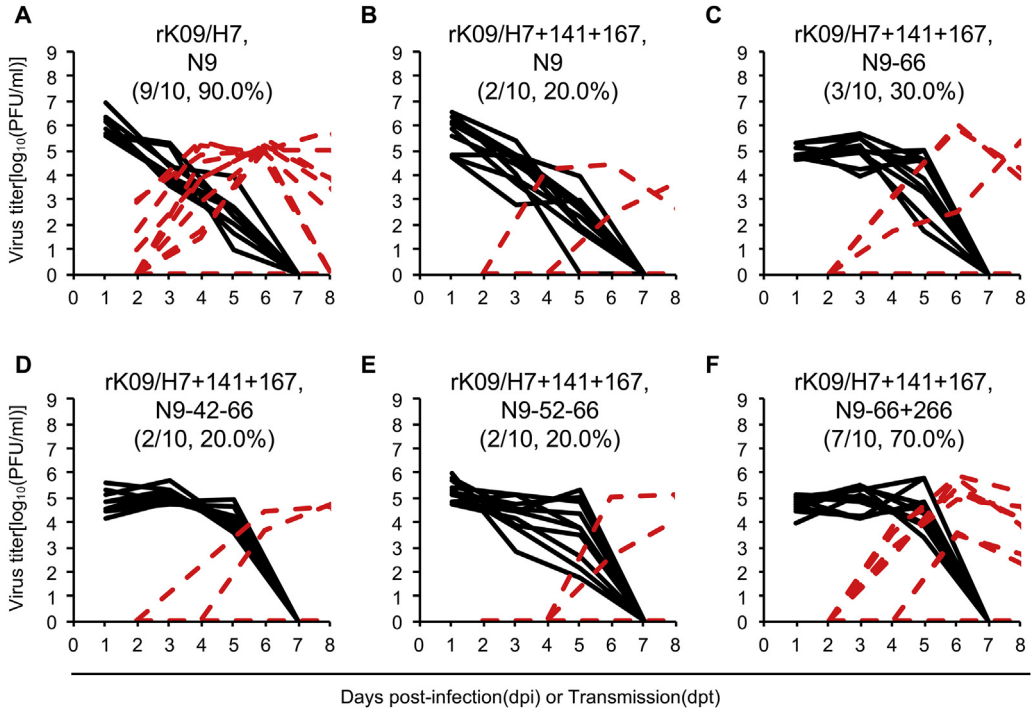

Fig. 4. Effects of HA and NA NLG modification on the transmissibility of a H7N9 virus. Effects of NLG modification on the transmissibility of a H7N9 virus was evaluated using the mutant viruses harboring various HA and NA NLG patterns (B, rK09/H7+141+167,N9; C, rK09/H7+141+167,N9-66; D, rK09/H7+141+167,N9-42-66; E, rK09/H7+141+167,N9-52-66; and F, rK09/H7+141+167,N9-66+266) in guinea pigs. Black solid lines indicate the viral titers of nasal washing samples of the infected guinea pigs, and red dotted lines indicate those of the transmitted guinea pigs. Contact transmission efficiency of the NLG mutant viruses was presented in parenthesis (the number of transmitted guinea pigs/the number of infected guinea pigs, %). rK09/H7, N9 (A) was used as a control of the NLG mutant viruses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4B). NLG removal from NA residue 66, which exhibited the best transmission efficiency among the NA NLG mutant viruses (Fig. 3D), could not increase a transmission rate of rK09/H7+141+167,N9, either (Fig. 4C). Additional NLG removal from either NA stalk residue 42 (rK09/H7+141+167,N9-42-66) or 52 (rK09/H7+141+167,N9-52-66) also failed to compensate the transmission rate of rK09/N9-66 (Fig. 4D and E). Only rK09/H7+141+167,N9-66+266 regained the transmissibility of rK09/H7,N9 by reaching up to a 70% transmission rate (Fig. 4F). These suggest the NLG patterns of the HA and NA proteins should be well-matched in terms of their functional balance. Otherwise, NLG variants may be purified away due to their low transmissibility.

4. Discussion

Transmissibility of influenza viruses in a certain host species appeared to be determined by a polygenic trait [18,19]. Receptor binding specificity of the HA protein has been suggested for its role in the transmission of a virus between different hosts [20,21]. PB2 and M proteins were also suggested as viral transmission determinants [22,23]. In this regard, it is of importance to understand which molecular changes may confer sustained human-to-human transmissibility to the virus. Of various effects of NLG changes on viral characteristics [11,12], we focused on their role for efficient viral transmission as demonstrated in the experimental results of H5N1 aerosol transmission studies [6,7].

NLG is one of the essential post-translational modifications in the cells of eukaryotes and archaea [10]. For influenza viruses, it is often considered a way of viral evasion against host immune responses because it may mask the HA or NA antigenic area by harboring a long glycan side chain in an asparagine residue [11]. Due to the fitness cost of NLG addition, however, only a limited number of residues have been utilized as NLG anchors [24,25]. For the HA and NA proteins of H7N9 viruses, the five and six residues have been identified as main NLG anchors with more than 98% NLG rates (Fig. 1). Of the three glycosites at residues 141, 167, and 249 located within the HA head region, only the residue 249 appeared to have had a 100% NLG sequon. To investigate why the other two glycosites have been excluded in most H7N9 viruses, we generated the HA NLG mutant viruses and determined their effects based on the transmission rates in guinea pigs (Fig. 2). However, no HA NLG mutations did benefit viral transmission. Rather, they all decreased transmission rates. Considered the viral titers in the transmitted guinea pigs, rK09/H7+141+167 looks interesting because it resulted in relatively high infectivity (Fig. 2F), compared with the delayed or lower infectivity observed in the other HA NLG mutant viruses (Fig. 2C–E). However, the same NLG changes in the H7 HA produced a completely different result in the case of rK09/H7+141+167,N9, which exhibited a very low transmission rate (20%). These may suggest a well-balanced function between the HA and NA proteins would be of great importance in terms of viral transmissibility. Similarly, a 90% transmission rate of rK09/H7,N9 may also indicate that the AH01 virus itself retains the well-matched HA and NA proteins (Fig. 4A).

To find out a well-matched HA and NA NLG mutant combination, we also changed NLG patterns in the N9 NA protein (Fig. 3). Of the four NA NLG mutants (rK09/N9-42, rK09/N9-52, rK09/N9-66, and rK09/N9+266), the three viruses (rK09/N9-42, rK09/N9-66, and rK09/N9+266) exhibited better transmission efficiency than the parental rK09/N9 (Fig. 3A, B, D, and E), and NLG removal from residue 66 resulted in the most enhanced efficiency (Fig. 3D). Based on these, we used NLG removal from residue 66 as a base of additional NLG changes in the N9 NA protein. As described above, rK09/H7+141+167,N9 exhibited less competent transmissibility

(Fig. 4B). We then changed the NA protein of this mutant virus with the NA harboring no NLG at residue 66 (rK09/HA+141+167,N9-66). Unfortunately, however, this virus did not exhibit a significant increase of transmission efficiency, either (Fig. 4C). Additional NLG removals from residue 42 or 52 also barely changed the low transmission rate of rK09/H7+141+167, N9 (Fig. 4D and E). Intriguingly, not an additional NLG removal but an addition of NLG at residue 266 (rK09/H7+141+167,N9-66+266) exhibited a 70% transmission rate (Fig. 4F), which increased the low transmission rate of rK09/H7+141+167,N9 by 50%. These may indicate the HA and NA NLG changes are well-balanced in the combination of the HA of H7+141+167 and the NA of N9-66+266.

In this study, we evaluated the effects of HA and NA NLG pattern changes on the transmission of the H7N9 virus. Even though we only provided the transmission results of various HA and NA NLG mutant viruses in guinea pigs, our results demonstrated NLG changes in the HA and NA proteins should be well-matched in terms of their functional balance, which was understood by the changes of transmission rates of each NLG mutant viruses. Based on these, we suggest that NLG addition or removal from the H7 HA head region may reduce viral fitness unless it is accompanied with the N9 NA protein exhibiting a balanced function. If a H7N9 virus retains the well-balanced HA and NA NLG mutants, it may have transmission benefit over currently circulating strains due to their ability of immune evasion.

Conflict of interest

The authors declare no conflict of interest.

One sentence summary

Glycosylation effects on the transmission of avian influenza A(H7N9) virus.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.09.024.

Appendix A. Supplementary data

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