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Original article

E190V substitution of H6 hemagglutinin is one of key factors for binding to sulfated sialylated glycan receptor and infection to chickens

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34 **Abstract**

35 Avian influenza viruses (AIVs) recognize sialic acid linked α 2,3 to galactose
36 (SA α 2,3Gal) glycans as receptors. In this study, the interactions between
37 hemagglutinins (HAs) of AIVs and sulfated SA α 2,3Gal glycans were analyzed in order
38 to clarify the molecular basis of interspecies transmission of AIVs from ducks to
39 chickens. It was revealed that E190V and N192D substitutions of the HA increased the
40 recovery of viruses derived from an H6 duck virus isolate, A/duck/Hong Kong/960/1980
41 (H6N2), in chickens. Recombinant HAs from an H6 chicken virus,
42 A/chicken/Tainan/V156/1999 (H6N1), bound to sulfated SA α 2,3Gal glycans, whereas
43 the HAs from an H6 duck virus did not. Binding preference of mutant HAs revealed that
44 an E190V substitution is critical for the recognition of sulfated SA α 2,3Gal glycans.
45 These results suggest that the binding of the HA from H6 AIVs to sulfated SA α 2,3Gal
46 glycans explains a part of mechanisms of interspecies transmission of AIVs from ducks
47 to chickens.

48

49 **Keywords**

50 Avian influenza virus, Interspecies transmission; Sialic acid receptor; Hemagglutinin;
51 Sulfated glycans

52

53

54 **Introduction**

55 Migratory ducks are the natural reservoir of avian influenza viruses (AIVs) (1).
56 AIVs can infect most avian species and be transmitted to mammalian species, including
57 humans. Chickens, however, are rarely infected directly with AIVs circulating in ducks
58 and are thought to be infected by them following their adaptation to other galliform
59 species, including turkeys and quails (2). Therefore, there must be some interspecies
60 barrier between ducks and chickens for AIVs. H6 low pathogenic AIVs have circulated
61 in domestic poultry in Asian countries (3, 4) and infected humans (5). Generally, a single
62 infection of H6 AIVs does not induce clinical signs in poultry; however, the co-infection
63 of bacteria aggravates the clinical signs (6). The adaptation of H6 duck influenza viruses
64 to chickens increases the risk of economic damages.

65 Influenza viruses recognize sialylated glycans on host epithelial cells as their
66 receptor. The binding specificity of hemagglutinin (HA) of influenza viruses to
67 sialylated glycans depends on the host species from which the virus was isolated: HAs
68 of human influenza viruses preferentially bind to glycans terminating with sialic acid
69 linked $\alpha 2,6$ to galactose (SA $\alpha 2,6$ Gal), whereas the HAs of AIVs preferentially bind to
70 glycans with sialic acid linked $\alpha 2,3$ to galactose (SA $\alpha 2,3$ Gal) (7, 8). It has been reported
71 that AIVs isolated from terrestrial birds, including chickens, prefer 6-sulfo sialyl Lewis
72 X (Le^x) (Sia $\alpha 2-3$ Gal $\beta 1-4$ (Fuc $\alpha 1-3$)(6-Sulfo)GlcNAc) as receptors, to which AIVs

73 isolated from ducks do not bind (9). The result suggests that the binding of AIVs to
74 fucosylated and/or sulfated SA α 2,3Gal glycans is one of the determinants for a
75 successful infection in chickens. Previous studies have revealed that H5Nx chicken
76 influenza viruses preferentially bind to fucosylated SA α 2,3Gal glycans (10, 11).
77 However, the role of sulfation in SA α 2,3Gal glycans which are not fucosylated, in
78 infections by chicken influenza viruses is still unknown.

79 Several amino acids in the 190-helix and 220-loop in HA are known to alter the
80 receptor-binding specificity of influenza viruses; amino acid residues at the 226 and 228
81 positions of H3 HA (H3 numberings is used throughout) determine the binding
82 specificity of influenza viruses to SA α 2,6Gal glycans or SA α 2,3Gal glycans (12, 13).
83 In several studies, a relationship has been reported between fucosylated and/or sulfated
84 SA α 2,3Gal glycans and the 190-helix and 220-loop on the HA of influenza viruses; the
85 fucose moiety of the host fucosylated SA α 2,3Gal glycans is positioned close to the 220-
86 loop on the HA of the influenza virus (14). In particular, amino acid residues at positions
87 222 and 227 on the 220-loop of HA are involved in binding H5 chicken influenza viruses
88 to sialyl Le^x (11, 15). The sulfo moiety of 6-sulfo sialyl Le^x is thought to be positioned
89 close to the 190-helix on the HA of chicken influenza viruses (9); however, reports on
90 the mechanisms of the recognition of the sulfo moiety are limited. Previously, the
91 cocrystallization of HA from an H7N9 human isolate and sulfated SA α 2,3Gal glycan

92 revealed the mechanism of the recognition of the sulfo moiety: amino acid residues at
93 positions 190 and 227 of H7 HA determine the binding of HA to sulfated SA α 2,3Gal
94 glycans (16), whereas the situation for other subtypes remains unreported.

95 The distribution of sialylated glycans is highly variable in each species; SA α 2,6Gal
96 glycans are predominantly detected in human respiratory epithelial cells (17), whereas
97 SA α 2,3Gal glycans are dominant on duck cloacal epithelial cells (18). The tissue
98 tropism of influenza viruses, which circulate in each host, is consistent with the
99 distribution of their preferred glycans in hosts. In a previous study, we detected
100 fucosylated SA α 2,3Gal glycans in chicken tracheal epithelial cells, where chicken
101 influenza viruses primarily replicate, whereas this glycan structure was not detected in
102 duck colon (10). On the other hand, there are no reports on the detection of sulfated
103 SA α 2,3Gal glycans on chicken tracheal epithelial cells to date.

104 In the present study, we aimed to clarify the mechanism of interspecies transmission
105 between ducks and chickens. The growth capacity in chickens and the binding
106 specificity to sulfated glycans of AIVs isolated from chicken and duck were
107 comparatively assessed. The results indicated that E190V and N192D substitutions of
108 the HA increased the virus recovery in chickens and the binding to sulfated SA α 2,3Gal
109 glycans is one of the determinants for the increasing infectivity of duck derived AIVs in
110 chickens.

111 **Materials and methods**

112 **Viruses and cells**

113 Influenza virus A/chicken/Tainan/V156/1999 (H6N1) (Ck/Tainan) was provided
114 by the Animal Health Research Institute, Council of Agriculture, Taiwan. Influenza
115 virus A/duck/Hong Kong/960/1980 (H6N2) (Dk/HK) was kindly provided by
116 Professor Ken F. Shortridge, The University of Hong Kong, Hong Kong SAR. These
117 viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 h, and
118 the infectious allantoic fluids were used as virus stocks. Madin-Darby canine kidney
119 (MDCK) cells were maintained in Minimum Essential Medium (MEM; Nissui
120 Pharmaceutical, Tokyo, Japan) supplemented with 0.3 mg/mL L-glutamine, 100 U/mL
121 penicillin G, 0.1 mg/mL streptomycin, 8 µg/mL gentamicin, and 10% fetal calf serum
122 (FCS; Sigma Aldrich, St. Louis, MO, USA). Human embryonic kidney (HEK) 293T
123 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo
124 Fisher Scientific, Waltham, MA, USA) supplemented with the same concentration of
125 L-glutamine and antibiotics with MEM and 10% FCS (Cambrex, East Rutherford, NJ,
126 USA). HEK 293S GnT (–) cells, which lack N-acetyl-glucosaminyltransferase I (GnTI)
127 activity, were maintained in pyruvate-free DMEM (Thermo Fisher Scientific) with the
128 same supplements as DMEM.

129

130 **Reverse genetics**

131 Eight gene segments from Dk/HK were cloned into pHW2000 vector according to
132 previously described methods (19-21). Amino acid substitutions of E190V and
133 N192D in the HA of Dk/HK were generated by site-directed mutagenesis using KOD
134 -Plus- Neo (TOYOBO, Tokyo, Japan) with specific primers (Supplemental Table 1).
135 All eight plasmids were transfected into a mixed culture of MDCK and HEK 293T
136 cells and mutant viruses were rescued (Supplemental Figure). These viruses were
137 propagated in 10-day-old embryonated chicken eggs at 35°C for 48 h, and the
138 infectious allantoic fluids were used as virus stocks. Virus stocks were kept at –80°C
139 until use. All eight gene segments of rescued viruses were sequenced to confirm the
140 existence of the introduced mutations and the absence of undesired mutations.

141

142 **Virus titration**

143 Plaque assays were performed to titrate the viruses. 10-fold dilutions of viruses
144 were inoculated onto confluent monolayers of MDCK cells and incubated at 35°C for
145 1 h. Unbound viruses were removed, and the cells were washed with PBS. The cells
146 were then overlaid with FCS-free MEM containing 0.7% Bacto Agar (Thermo Fisher
147 Scientific) and 5 µg/mL Trypsin Acetylated (Sigma Aldrich). After incubation for 48
148 h at 35°C, cells were overlaid with FCS-free MEM containing 0.7% Bacto Agar and

149 0.005% neutral red. After 24 h, visible plaques were counted and expressed as plaque
150 forming unit (PFU)/mL.

151

152 **Experimental infection of chicken with viruses**

153 Four-week-old conventional chickens (*Gallus gallus*, Julia), which were free of
154 anti-influenza virus antibodies, were obtained from Hokkai Starchick, Hokkaido, Japan.

155 Each of three chickens was intranasally inoculated with $10^{5.0}$ PFU of either Ck/Tainan

156 or Dk/HK viruses. On three and five days post inoculation (d.p.i.), oral and cloacal

157 swabs were collected. Also, other three chickens were inoculated with $10^{5.0}$ PFU of

158 mutant viruses derived from Dk/HK. Tracheal and cloacal swabs and tissue samples

159 were collected at three d.p.i. The tissue samples were homogenized using a Multi-

160 Beads Shocker (Yasui Kikai, Osaka, Japan) to prepare 10% suspensions with MEM.

161 The infectivity titers of the swabs were calculated by a plaque assay. All infected

162 chickens were kept in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan).

163 Although the experiments were possibly conducted in ABSL2 condition, all the animal

164 experiments were conducted in the higher biosafety level, ABSL3 facility at the Faculty

165 of Veterinary Medicine, Hokkaido University, Japan due to the limitation of access to

166 the animal facility.

167

168 **Expression of the recombinant HAs (rHAs)**

169 The cDNAs of the HA genes of Ck/Tainan, Dk/HK and their mutants were cloned
170 into a pCD5 expression vector (22). In this vector, HA genes without transmembrane
171 regions were fused with a GCN4IL motif and Strep-tag II (WSHPQFEK; IBA,
172 Göttingen, Germany). The soluble trimeric rHA proteins were expressed in HEK293S
173 GnT (–) cells and purified from the cell culture supernatants according to a previously
174 described method (22). Briefly, rHA proteins were purified using Strep-Tactin
175 Sepharose beads (IBA). The rHA bound to Strep-Tactin beads were treated by d-
176 Desthiobiotin buffer (IBA) for 15 min at 4°C to elute the rHAs from the beads. Mutant
177 HAs were prepared by the single-site mutagenesis method used specific primers
178 (Supplemental Table 1).

179

180 **Glycan microarray**

181 Purified rHAs were analyzed according to previously described method (17). The
182 glycan microarray contains two nonsialylated glycans (glycans 1 and 2) and 56 α 2,3
183 and α 2,6 sialylated glycans (glycans 3–58). Of the 56 sialylated glycans, 5 were
184 sulfated α 2,3 sialylated glycans (glycans 3–7), 28 were nonsulfated α 2,3 sialylated
185 glycans (glycans 8–35), and 22 α 2,6 sialylated glycans (glycans 36–58) were printed
186 on the array side (Supplemental Table 2). For each glycan, the mean signal intensity
187 was calculated from six replicates. The highest and lowest signals of the six were

188 removed, and the remaining four replicates were used to calculate the mean signal
189 shown as relative fluorescence unit (RFU) and standard error (SE).

190

191 **Solid-phase direct binding assay**

192 The receptor-binding specificity of rHAs was assessed using a solid-phase direct
193 binding assay with Neu5Ac α 2-3Gal β 1-4GlcNAc- β -PEG-biotin (SA α 2,3Gal-bio) and
194 Neu5Ac α 2-3Gal β 1-4(6-Sulfo)GlcNAc- β -PEG-biotin (Su-SA α 2,3Gal-bio) (Tokyo
195 Chemical Industry Co., Ltd., Tokyo, Japan). Each sialylated glycan was serially diluted
196 from 0.039–5 μ M/well and added to each well of a Nunc immobilizer streptavidin 12
197 $\times$ 8 strips microplate (Thermo Fisher Scientific). Each well was blocked with 2%
198 bovine serum albumin (BSA) at room temperature for 1 h. After washing with PBS
199 containing 0.05% Tween 20 (PBST), the rHAs (5 μ g in PBST containing 1% BSA)
200 were added to each well, and plates were incubated at 4°C for 12 h. After washing,
201 anti-Strep-tag mouse antibody (IBA; 1,000-fold dilution in PBST containing 1% BSA)
202 was added to each well, and the plates were incubated at 4°C for 2 h. The wells were
203 then washed and incubated with goat anti-mouse IgG-HRP conjugate (Bio-Rad,
204 Hercules, CA, USA) at 4°C for 2 h. After washing, 100 μ L of the substrate solution
205 including 0.5 mM 3,3'-tetramethylbenzidine and 0.04% H₂O₂, was added to each well.
206 After incubation at room temperature for 30 min, the reactions were stopped using 50

207 μL of 2 N H₂SO₄, and absorbance at 450/630 nm was measured using a MULTISCAN
208 JX microplate photometer (Thermo Fisher Scientific). The data were presented with
209 the mean values of three technical replicates with SE.

210

211 **Amino acid sequence comparison of the H6 HA**

212 A total of 638 amino acid sequences of H6 chicken and duck influenza viruses was
213 obtained from GenBank. Sequence data were aligned with GENETYX ver. 12.01
214 (GENETYX Co., Tokyo, Japan).

215

216 **Ethics statement**

217 All *in vivo* experiments were authorized by the Institutional Animal Care and
218 Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval
219 number: 18-0037; 18-0040) and performed according to the guidelines of this committee.

220

221 **Results**

222 **Infectivity of chicken and duck influenza viruses in chickens**

223 To confirm the growth of chicken virus, Ck/Tainan, and duck virus, Dk/HK, in
224 chickens, the viral growth was compared *in vivo*. Each virus containing 10^{5.0} PFU was
225 intranasally inoculated into three four-week-old chickens. All of chickens inoculated

with each virus did not show any clinical signs. Ck/Tainan viruses were recovered from the respiratory tracts of all three infected chickens at three and five d.p.i., whereas Dk/HK was not recovered from any of the inoculated chickens (Table 1).

Preparation of E190V and N192D mutant viruses derived from Dk/HK and their viral growth in chickens

A previous study reported that 190-helix of HA is thought to be positioned close to the sulfo moiety of sulfated SA α 2,3Gal glycans (9). Therefore, the amino acid sequences of 190-helix on HA of these two viruses were compared. As a result, the amino acid properties of positions 190 and 192 are different between them. The majority of H6 AIVs isolated from ducks have amino acid residues E190 and N192 based on information from a public database (GenBank/DDBJ/EMBL). Almost one-third of H6 AIVs isolated from chickens, including Ck/Tainan, have amino acid residues V190 and D192 (Table 2). To clarify the role of amino acid residues at positions 190 and 192 of HA in the viral growth in chickens, Dk/HK viruses containing various amino acid substitutions at these positions (rgDk/HK, rgDk/HK-HA190V, rgDk/HK-HA192D, and rgDk/HK-HA190V/192D) were prepared using a reverse genetics method. To compare the virus growth in chickens, 10^{5.0} PFU of each recombinant virus was intranasally inoculated to three 4-week-old chickens. Viruses were recovered from tracheal swabs at

245 3 d.p.i from the chickens infected with rgDk/HK-190V, rgDk/HK-HA192D and
246 rgDk/HK-HA190V192D (Table 3). Viruses were also recovered from respiratory organs
247 of one of three rgDk/HK-HA190V infected chicken and of all three rgDk/HK-
248 HA190V192D infected chickens. These results suggest that both E190V and N192D
249 substitutions are involved in the replication of mutants derived from Dk/HK in chickens.

250

251 **Glycan-binding specificity of mutant viruses derived from Dk/HK**

252 To clarify the molecular basis of different viral growth in chickens, the glycan-
253 binding specificities of HAs from these two viruses were compared in a glycan
254 microarray (Fig. 1, Supplemental Table 3). The rHAs of Ck/Tainan bound to sulfated
255 SA α 2,3Gal glycans (glycans 3, 4, and 7, Fig. 2). The rHAs of Dk/HK bound to linear
256 and non-sulfated SA α 2,3Gal glycans (glycans 14 and 24–26, Fig. 2) with high RFU.
257 The rHAs of Dk/HK also bound to sulfated SA α 2,3Gal glycans with lower RFU than
258 those of the rHAs of Ck/Tainan (glycans 3, 4, and 7), indicating that the glycan-binding
259 profile of rHA of Dk/HK is different from that of rHA of Ck/Tainan. These results
260 indicated that the rHA of the chicken isolate, Ck/Tainan, selectively bound to sulfated
261 SA α 2,3Gal glycans, whereas that of the duck isolate, Dk/HK, preferentially bound to
262 linear and non-sulfated SA α 2,3Gal glycans.

263

264 **Key amino acid residues for recognition of sulfated SA α 2,3Gal glycans by HA**

265 To evaluate the contribution of amino acid residues at positions 190 and 192 of the
266 HA to the glycan-binding specificity of these viruses, mutant rHAs of Ck/Tainan and
267 Dk/HK, in which amino acid residues at positions 190 and/or 192 were substituted
268 (Ck/Tainan-HA190E, Ck/Tainan-HA192N, Ck/Tainan-HA190E/192N, Dk/HK-
269 HA190V, DK/HK-HA192D and Dk/HK-HA190V/192D), were prepared and these
270 rHAs were subjected to solid-phase direct binding assays (Figs. 3, 4). The rHAs of
271 Ck/Tainan preferentially bound to Su-SA α 2,3Gal-bio (Fig. 3A) and Ck/Tainan-
272 HA190E HAs preferentially bound to SA α 2,3Gal-bio (Fig. 3B). The introduction of the
273 192N mutation to Ck/Tainan-HA and its mutant resulted in the diminished binding to
274 both SA α 2,3Gal-bio and Su-SA α 2,3Gal-bio; yet the binding preference to Su-
275 SA α 2,3Gal-bio was slightly observed in 190V HA (Ck/Tainan-HA192N) and to
276 SA α 2,3Gal-bio in 190E HA (Ck/Tainan-HA190E/192N) (Figs. 3C, 3D). The rHAs of
277 Dk/HK bound to SA α 2,3Gal-bio (Fig. 4A), whereas Dk/HK-HA190V acquired binding
278 to Su-SA α 2,3Gal-bio (Fig. 4B). The introduction of the 192D in Dk/HK-HA resulted in
279 weaker binding to glycans without changing binding preference to SA α 2,3Gal-bio (Fig.
280 4C). The introduction of the same mutation in Dk/HK-HA190V (Dk/HK-
281 HA190V/192D) also resulted in diminished binding to the glycans, although the binding
282 to Su-SA α 2,3Gal-bio was more affected when compared with that to SA α 2,3Gal-bio

283 (Fig. 4D). These results indicate that E190V substitution of the HA determines the
284 binding of H6 AIVs to sulfated SA α 2,3Gal glycans. On the other hand, the discrepancy
285 of the 192D/N and the backbone of the HA resulted in the diminished binding to the
286 glycans.

287

288 **Discussion**

289 In the present study, the virus growth in chickens and the receptor-binding
290 specificities of viruses were analyzed to understand the mechanisms of interspecies
291 transmission of AIVs from ducks to chickens. From the growth capacity of mutant
292 viruses, E190V and N192D substitutions of the HA increased the recovery of the viruses
293 derived from the H6 duck virus, Dk/HK, in experimentally infected chickens. The H6
294 AIV isolated from a chicken, Ck/Tainan, bound specifically to sulfated SA α 2,3Gal
295 glycans, whereas Dk/HK preferred to non-sulfated SA α 2,3Gal glycans. The difference
296 of the binding specificity was thought to be related to the host range of H6 AIVs. Solid-
297 phase direct binding assay of rHAs revealed that only an E190V substitution in the HA
298 of Dk/HK altered its glycan-binding specificity from linear and non-sulfated SA α 2,3Gal
299 glycans to sulfated SA α 2,3Gal glycans. These results indicate that the amino acid
300 residue at position 190 of H6 HA is a key component for the recognition of sulfated

301 SA α 2,3Gal glycans. Furthermore, position 190 of HA might be the key for the
302 interspecies transmission of AIVs from ducks to chickens.

303 On the other hand, both V190 and D192 were important for the virus growth in
304 chickens (Table 3). Interestingly, viruses with V190/D192 were selected in chickens in
305 the field (32/101 strains) rather than those with V190/N192 (4/101 strains) (Table2).
306 These facts suggested that these two mutations, E190V and N192D, synergistically act
307 in the adaptation process of duck influenza viruses to chickens. Moreover, the mutation
308 at position 192 of the HA modulated receptor binding avidity (Figs. 3, 4). A previous
309 report on the structural analysis demonstrated that the amino acid residue at the 192
310 positioned close to penultimate Gal moiety of the sialylated glycan (23). Perhaps, the
311 mutation at position 192 might be related to the recognition of modified Gal moiety (e.g.,
312 6-sulfo-Gal), which was not analyzed in the present study.

313 Nevertheless, approximately 40% of chicken H6 viruses still have the E residue at
314 position 190 of the HA (Table 2). On the other hand, viruses possessing the V residue
315 at the same position were exceptional among duck H6 viruses; only two out of 537
316 strains possess V at the position. Interestingly, similar phenomena were observed in the
317 combination of H5 viruses and fucosylated SA α 2,3Gal (15). These facts suggest that
318 viruses recognizing linear and non-sulfated SA α 2,3Gal were selected among the duck
319 population. In addition, a previous study demonstrated that co-infection of *Mycoplasma*

320 *gallisepticum* exaggerated infection of duck influenza viruses in experimentally infected
321 chickens (24). This suggested that duck influenza viruses, which originally recognize
322 linear and non-sulfated SA α 2,3Gal are able to establish infections in chickens in the
323 field without altering their receptor-binding specificity and viruses with “chicken-type
324 receptor specificity” were selected during multiple replications and transmission events
325 in chicken flocks, rather than in the adaptation among ducks or non-chicken terrestrial
326 poultry.

327 Although rgDk/HK-HA190V, rgDk/HK-HA192D, and rgDk/HK-HA190V/192D
328 were recovered from infected chickens, the virus recovery titers from swabs at 3 d.p.i
329 were slightly lower compared with those from the chickens inoculated with Ck/Tainan.
330 These viruses contain E190V and/or N192D substitution in their HA, indicating that
331 these both mutations enhance viral infection and replication in chickens; however, the
332 backbone of the HA as well as other viral proteins remains the same as wild-type Dk/HK,
333 which could not replicate well in chickens. Therefore, some other interspecies barrier
334 between ducks and chickens for AIVs may explain these lower titers of mutant viruses
335 in chickens. Influenza viruses have eight segmented genes, and each gene encodes for
336 different proteins. Of these proteins, HA is responsible for the attachment and fusion of
337 viral and cellular membranes. Other proteins have roles for each step of viral replication.
338 It has been reported that PB2 and NS proteins are important for the interspecies

339 transmission of influenza viruses (25, 26). In a previous study, a duck influenza virus
340 adapted to chickens through mutations in its PB2 and NP proteins (27), although the
341 functional details of these mutations have not been reported.

342 The mechanisms of receptor-recognition by HA of influenza viruses were revealed
343 through the cocrystallization of HA with sialylated glycans (9). However, reports on the
344 detailed molecular interactions between HAs with modified SA α 2,3Gal glycan
345 receptors, such as fucosylated and/or sulfated receptors, are limited. One possible
346 explanation for the lack of receptor-binding of HAs from duck influenza viruses to
347 sulfated SA α 2,3Gal glycans is the negative charge of the sulfo moiety (9). Position 190
348 in the HA of Dk/HK is glutamic acid, E, whereas in Ck/Tainan, it is valine, V. The
349 negatively charged glutamic acid is perhaps not compatible with the similarly negatively
350 charged sulfo-moiety of the glycans. Therefore, the amino acid substitution to the
351 uncharged amino acid, E190V in HA must be involved in binding to sulfated glycans
352 and allowing H6 viruses to infect and replicate in chickens.

353 In conclusion, it is speculated that sulfated SA α 2,3Gal glycans are expressed on the
354 chicken trachea and binding to sulfated SA α 2,3Gal glycans contributes to the
355 interspecies transmission of H6 AIVs from ducks to chickens. There is limited
356 information on the distribution of sialylated glycans, which can be detected by lectins
357 and anti-glycan antibodies on the surface of host cells. In order to clarify the role of

358 sulfated SA α 2,3Gal glycans in the interspecies transmission, it is necessary to analyze
359 the receptor distribution on the host cells. Furthermore, to fully understand the molecular
360 mechanisms of interspecies transmission of AIVs, it is necessary to clarify the structural
361 and quantitative details of sialylated glycans used as receptors by these viruses.

362

363 **Disclosure**

364 The authors have no conflicts of interest to declare.

365

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373

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477
478

479 **List of Abbreviations:** AIV, avian influenza virus; Ck/Tainan,
480 A/chicken/Tainan/V156/1999 (H6N1); BSA, bovine serum albumin; Dk/HK,
481 A/duck/Hong Kong/960/1980 (H6N2); DMEM, Dulbecco's modified Eagle's medium;
482 d.p.i., days post inoculation; FCS, fetal calf serum; GnTI, N-acetyl-
483 glucosaminyltransferase I; HA, hemagglutinin; HEK, human embryonic kidney; Le^x,
484 Lewis X; MDCK, Madin-Darby canine kidney; MEM, Minimum Essential Medium;
485 PBST, PBS containing 0.05% Tween 20; PFU, plaque forming unit; RFU, relative
486 fluorescence unit; rHA, recombinant HA; SA α 2,3Gal, sialic acid linked α 2,3 to
487 galactose; SA α 2,6Gal, sialic acid linked α 2,6 to galactose; SE, standard error.
488

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491 Virus recovery from chickens intranasally inoculated with chicken and duck H6
492 influenza viruses.

493

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497

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499 Virus recovery from chickens intranasally inoculated with wild-type and mutant viruses
500 derived from A/duck/Hong Kong/960/1980 (H6N2).

501

502 **Fig. 1.**

503 Glycan-binding specificity of the soluble trimeric recombinant hemagglutinins (rHAs).

504 The glycan-binding specificities of rHAs from Ck/Tainan (A) and Dk/HK (B) were
505 analyzed using a glycan microarray. Nonsialylated controls (glycans 1 and 2), sulfated
506 α 2,3 sialylated glycans (glycans 3–7), nonsulfated α 2,3 sialylated glycans (glycans 8–
507 35), and α 2,6 sialylated glycans (glycans 36–58) were printed on the array. The data are

presented as the mean $\pm$ SE of triplicate experiments. The structures of glycans that were preferentially bound (glycans 3, 4, 7, 14, and 24–26) are shown in Fig. 2.

Fig. 2.

Structures of glycans that were bound by rHAs on the array. Each symbol indicates sialic acid (purple diamonds), galactose (yellow circles), *N*-acetylglucosamine (blue squares), *N*-acetylgalactosamine (yellow squares), fucose (red triangles) and sulfo (S). The schematic structures of the glycans that yielded significant signals in the glycan microarray analysis with rHA from Ck/Tainan and Dk/HK (Fig. 1) are shown in this figure.

Fig. 3.

Glycan-binding specificity of wild-type and mutant rHAs of Ck/Tainan. The glycan-binding specificities of rHAs of Ck/Tainan (A), Ck/Tainan-HA190E (B), Ck/Tainan-HA192N (C), and Ck/Tainan-HA190E/192N (D) to sialylglycoconjugates containing SA α 2,3Gal-bio (closed circles) and Su-SA α 2,3Gal-bio (open circles) were investigated using solid-phase direct binding assays. The data are presented as the mean $\pm$ SE of triplicate experiments.

527 **Fig. 4.**

528 Glycan-binding specificity of wild-type and mutant rHAs of Dk/HK. The glycan-
529 binding specificities of rHAs of Dk/HK (A), Dk/HK-HA190V (B), Dk/HK-HA192D
530 (C) and Dk/HK-HA190V/192D (D) to sialylglycoconjugates containing SA α 2,3Gal-bio
531 (closed circles) and Su-SA α 2,3Gal-bio (open circles) were investigated using solid-
532 phase direct binding assays. The data are presented as the mean $\pm$ SE of triplicate
533 experiments.

534

535 **Supplemental Table 1**

536 Primer information for single-site mutagenesis.

537

538 **Supplemental Table 2**

539 Printed glycan list for the array.

540

541 **Supplemental Table 3**

542 Result of glycan microarray for rHAs of Ck/Tainan and Dk/HK

543

544 **Supporting Figure Legend**

545 Genome structure of mutant viruses in this study. The gene constellation and amino acid
546 substitutions of parental and mutant viruses generated by reverse genetics were
547 illustrated.
548

Table 1 Virus recovery from chickens intranasally inoculated with chicken and duck H6 influenza viruses.

Viruses	Swabs (log PFU/ml)			
	Oral		Cloacal	
	3 dpi	5 dpi	3 dpi	5 dpi
Ck/Tainan	5.3	2.3	-*	-
	2.7	2.8	-	-
	3.4	-	-	3.2
Dk/HK	-	-	-	-
	-	-	-	-
	-	-	-	-

* Dash (-) indicates <1.0

dpi: days post inoculation

Ck/Tainan: A/chicken/Tainan/V156/1999 (H6N2)

Dk/HK: A/duck/Hong Kong/960/1980 (H6N2)

Table 2 Amino acid residues comparison of 190/192 motifs in HA of H6 chicken and duck influenza viruses.

Host	Amino acid residue		Number of strains
	190	192	
Chicken (101 strains)	E	N	42
	V	D	32
	A	N	8
	V	N	4
	E	E	4
	E	D	3
	G	N	2
	E	A	2
	L	E	2
	L	N	1
	R	N	1
Duck (537 strains)	E	N	454
	E	E	51
	E	A	20
	A	N	5
	E	D	4
	V	N	1
	V	D	1
	L	D	1

Amino acid sequence of H6 HA were obtained from GenBank.

Amino acid motifs of Ck/Tainan and Dk/HK are shown in bold.

Table 3 Virus recovery from chickens intranasally inoculated with wild type and mutant viruses derived from A/duck/Hong Kong/960/1980 (H6N2)

Viruses	Swabs (log PFU/ml)		Tissues (log PFU/g)		
	Tracheal	Cloacal	Trachea	Lung	Colon
rgDk/HK	.*	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
rgDk/HK-HA190V	-	-	-	-	-
	1.4	-	-	1.7	-
	1.7	-	-	-	-
rgDk/HK-HA192D	1.7	-	-	-	-
	1.5	-	-	-	-
	-	-	-	-	-
rgDk/HK-HA190V/192D	2.4	-	2.6	-	-
	2.3	-	2.5	1.8	-
	4.3	-	4.4	3.3	-

* Dash (-) indicates <1.0

rgDk/HK: A/duck/Hong Kong/960/1980 (H6N2) generated by reverse genetics

Samples were collected at 3 dpi.

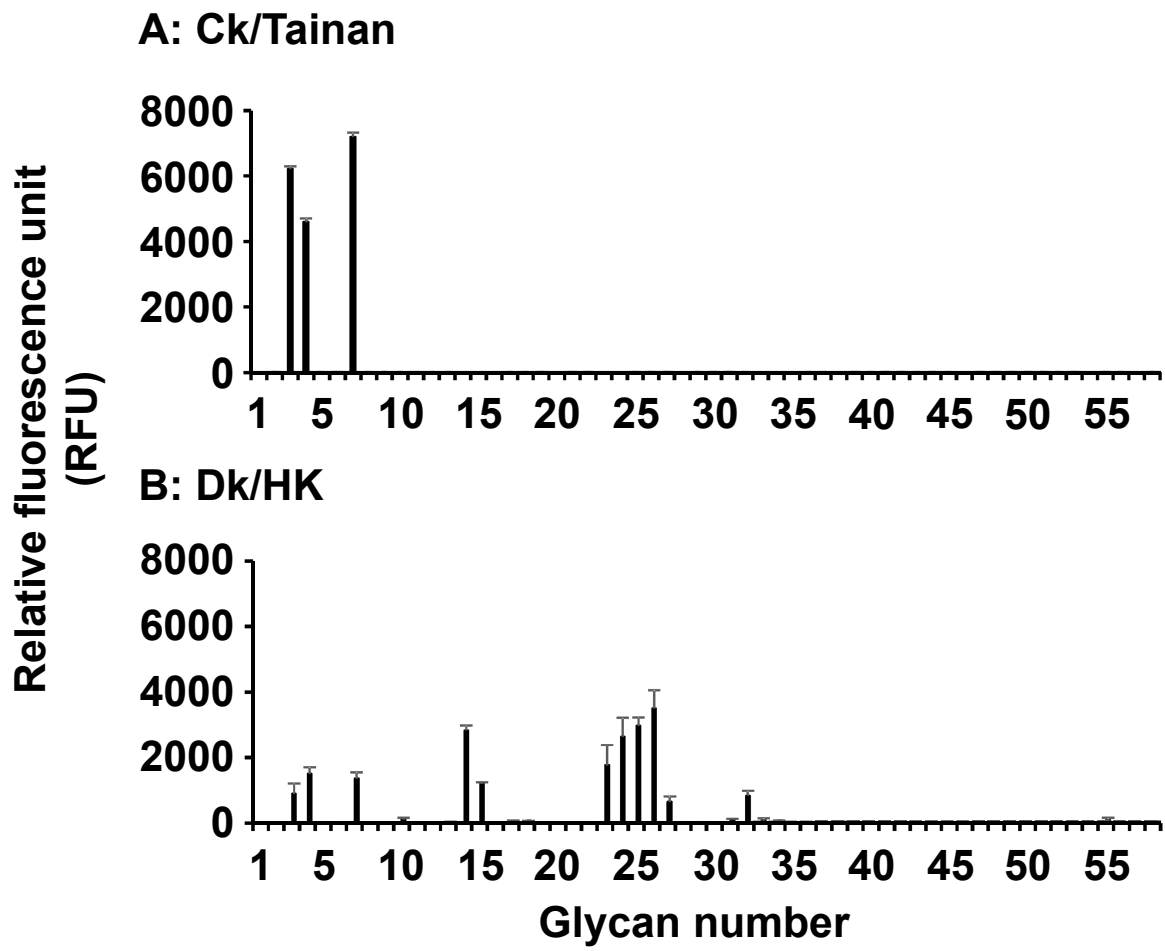


Fig. 1. Kikutani *et al.*

No	Structures
3	
4	
7	
14	
24	
25	
26	

Fig. 2 Kikutani *et al.*

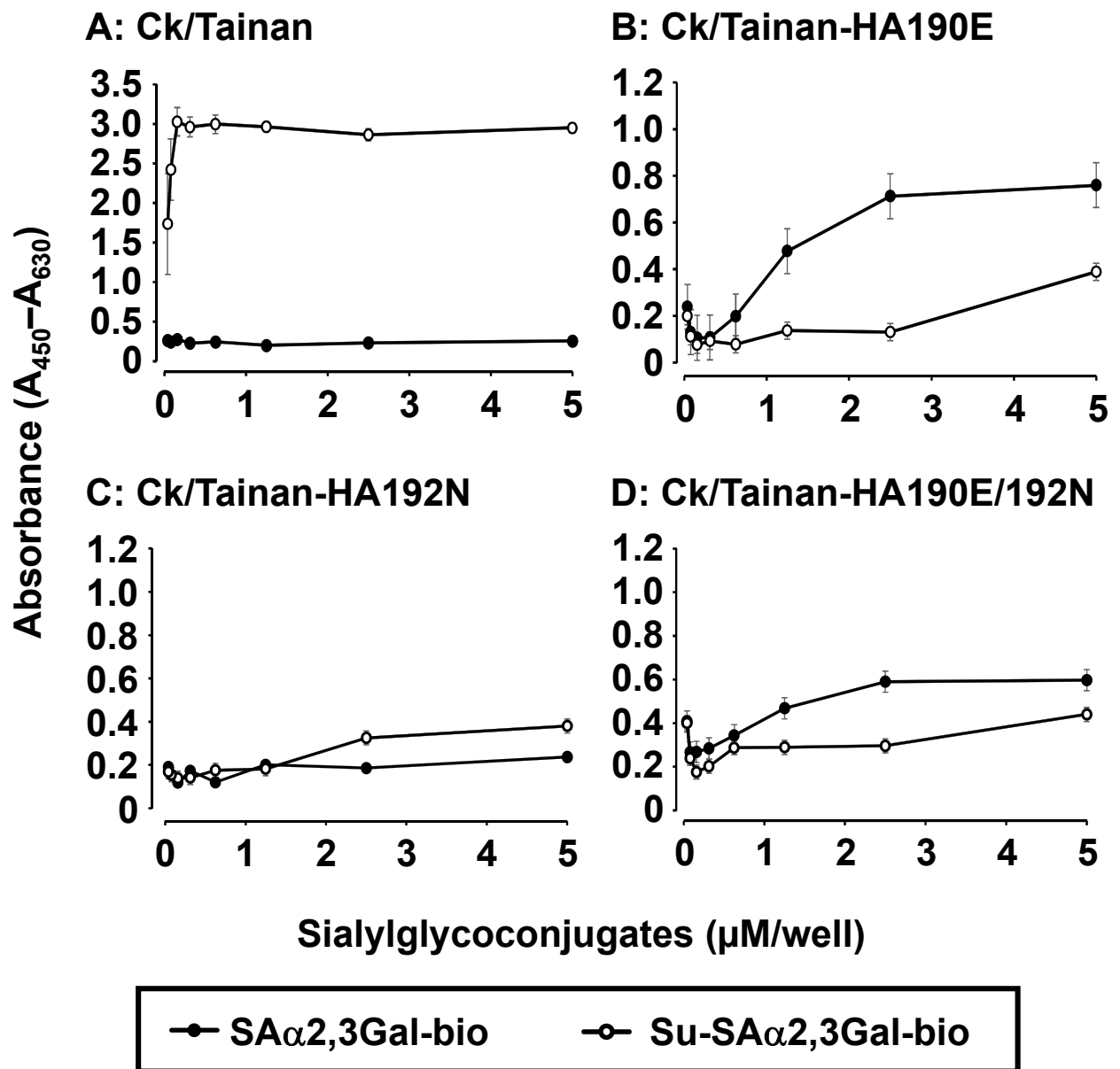


Fig. 3. Kikutani *et al.*

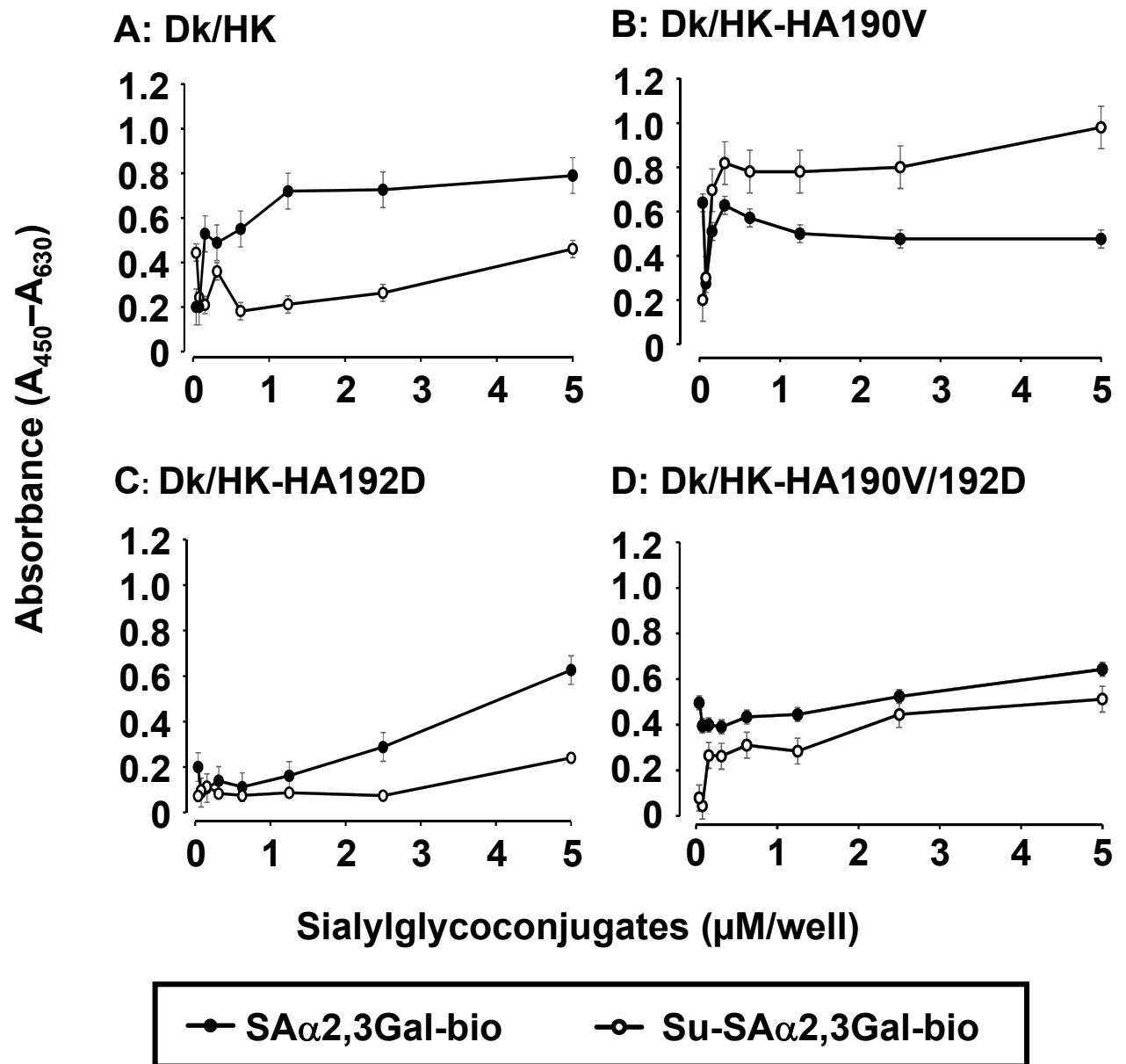


Fig. 4. Kikutani *et al.*

	PB2	PB1	PA	HA	NP	NA	M	NS
Ck/Tainan				^{190 192} V D				
Dk/HK				E N				
rgDk/HK				E N				
rgDK/HK-HA190V				V N				
rgDK/HK-HA192D				E D				
rgDK/HK-HA190V/192D				V D				

Ck/Tainan: A/chicken/Tainan/V156/1999 (H6N1), Dk/HK: A/duck/Hong Kong/960/1980 (H6N2)

Supplemental figure. Genome structure of mutant viruses in this study