



Evolution of the receptor binding phenotype of influenza A (H5) viruses

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Abstract

Receptor specificity of influenza A/H5 viruses including human 2003–04 isolates was studied. All but two isolates preserved high affinity to Sia2–3Gal (avian-like) receptors. However, two isolates (February, 2003, Hong Kong) demonstrated decreased affinity to Sia2–3Gal and moderate affinity to a Sia2–6Gal (human-like) receptors. These two viruses had a unique Ser227-Asn change in the hemagglutinin molecule. Thus, a single amino acid substitution can significantly alter receptor specificity of avian H5N1 viruses, providing them with an ability to bind to receptors optimal for human influenza viruses. Asian 2003–04 H5 isolates from chickens and humans demonstrated highest affinity to the sulfated trisaccharide Neu5Ac α 2–3Gal β 1–4(6-HSO₃)GlcNAc β (Su-3'SLN) receptor but, in contrast to 1997 isolates, had increased affinity to fucosylated Su-3'SLN. American poultry H5 viruses also had increased affinity to Su-3'SLN. These data demonstrate that the genetic evolution of avian influenza A(H5N1) viruses is accompanied during adaptation to poultry by the evolution of their receptor specificity.

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Keywords: Influenza virus; Hemagglutinin; Receptor specificity; Host range

Introduction

Influenza virus infection is initiated by interactions between the viral HA and sialic acid-containing molecules on target cells. Viruses from different host species usually demonstrate binding preference for either Neu5Ac α 2–3Gal or Neu5Ac α 2–6Gal natural disaccharide epitopes (reviewed by Paulson, 1985). Human viruses of H1, H2, and H3 subtypes recognize α 2–6-linked sialic acid, the prevalent form found on cells of the human respiratory tract (Couceiro et al., 1993). Avian viruses preferentially bind to Neu5Ac α 2–3Gal, the form that predominates in the duck enteric tract where these viruses replicate (Ito et al., 1997, 1998).

Recently, it was shown that human airway epithelium harbors α 2–3-linked sialic acids on ciliated cells; this finding explains the ability of H5 viruses to replicate in humans despite their avian virus-like receptor specificity (Matrosovich et al., 1999; 2004).

Chicken and human H5N1 viruses isolated in 1997 in Hong Kong differed from H5 viruses isolated from wild ducks by their extraordinary high affinity to sulfated trisaccharide Sia α 2–3Gal β 1–4(6-HSO₃)GlcNAc β (Su-3'SLN) (Gambaryan et al., 2004). Based on the fact that the high affinity of H5 chicken viruses to mammalian (green monkey) trachea cells was diminished after treatment with glucosamine-6-sulfatase, we suggested that the airway epithelium of mammals most likely has Su-3'SLN determinants (Gambaryan et al., 2004).

In the present study, we examined the receptor binding specificity of a broad set of H5 viruses from both American and Asian lineages, including highly pathogenic H5N1 viruses isolated from chickens and humans in 2003 and 2004 (Nguyen et al., 2005; Peiris et al., 2004; WHO, 2005). We also investigated whether the increased affinity of 1997 H5N1 isolates from chickens and humans to Su-3'SLN was accidental or a result of adaptation of avian H5 viruses to chickens.

Results

Sialoglycoconjugates with differing inner saccharide residues were used to characterize binding epitopes of H5 viruses.

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The structures of tested sialooligosaccharides are presented in Table 1. The saccharides were attached to soluble polyacrylamide and used in the competitive assay with peroxidase-labeled fetuin. SiaLe^c differs from 3'SLN by type of linkage between galactose and adjacent glucosamine (β 1–3 in SiaLe^c and β 1–4 in 3'SLN). SiaLe^x is 3'SLN fucosylated at O-3 of glucosamine. All sulfated saccharides carry sulfo-group at O-6 of glucosamine. Receptor binding characteristics of the viruses studied are presented in Table 2. Human influenza A/Aichi/1/68 (H3N2) isolate was included as control viruses with typical 6'SLN receptor binding specificity and several duck viruses of different subtypes were used as typical wild bird isolates (Table 2, top).

Affinity to Sia2–6Gal and Sia2–3Gal terminated receptors

First, we tested whether the Sia2–3Gal receptor specificity of 2003–2004 avian H5N1 viruses changed after replication in humans. Table 2 shows that most human 1997–2004 isolates preserved their high affinity to Sia2–3Gal and low affinity to Sia2–6Gal receptors. Similar receptor binding specificity was observed for avian H5 viruses from both North American and Eurasian lineages as well as for reference duck viruses of different subtypes (H1, H2, H4, and H10; Table 2). However, the receptor specificity of two human H5N1 isolates, A/Hong Kong/212/2003 and A/Hong Kong/213/2003, demonstrated a unique pattern. Both were isolated in February 2003 and both had decreased affinity to all Sia2–3Gal-terminated receptors and moderate but clear capacity to bind to 6'SLN, a Sia2–6Gal-terminated receptor that is typical for human influenza viruses.

Affinity to sulfated and non-sulfated sialosaccharides

In agreement with our previous data (Gambaryan et al., 2005), typical duck viruses of different subtypes (H1, H2, H4, and H10) did not distinguish between sulfated and non-sulfated sialosaccharides (Table 2, top part). However, the majority of H5N1 viruses isolated from humans and chickens demonstrated higher affinity to sulfated (Su-3'SLN) than to non-sulfated 3'SLN. Yet, their affinity to Su-SiaLe^c was nearly the same as their affinity to non-sulfated SiaLe^c thus indicating that the orientation of the sulfo group in relation to the receptor binding center of the hemagglutinin (HA) molecule is essential for the receptor binding activity of H5 viruses.

The pattern of receptor specificity of A/duck/Minnesota/1525/81, an American lineage H5N1 virus, was similar to that

of the reference duck viruses (Gambaryan et al., 2004). The majority of American H5 viruses isolated from chickens and turkeys had increased affinity to sulfated sialosugars, especially to Su-3'SLN (Table 2). Similar receptor binding pattern was found for A/Duck/New Jersey/117228-7/2001 (Table 2), the virus that was isolated from a poultry duck and has an extra glycosylation site at the HA position 199 that is typical for viruses isolated from chickens (Lee et al., 2004). However, two isolates – A/Turkey/Wisconsin/68 and A/Ratite/New York/12716/94 – had decreased affinity to sulfated sialosaccharides.

Asian duck H5N1 viruses, such as A/Duck/Singapore/3/97 and A/Duck/Vietnam/342/2001, had slightly higher receptor affinity to Su-3'SLN than to 3'SLN. Human H5N1 viruses isolated in 1997 (A/Hong Kong/483/97, A/Hong Kong/486/97, and A/Hong Kong/542/97) demonstrated significantly increased affinity to the Su-3'SLN receptor than to non-sulfated 3'SLN (Table 2), confirming our previous findings for other 1997 H5N1 viruses (Gambaryan et al., 2004). Highly pathogenic human viruses from 2004 (A/Vietnam/1194/2004, A/Vietnam/1203/2004, A/Thailand/16/2004) as well as many avian H5N1 viruses isolated during 2003–2004 (Table 2) also had increased affinity to Su-3'SLN.

Many H5N1 viruses that belong to other evolutionary subbranches of the Asian lineage of H5 viruses (for example, A/Goose/Vietnam/324/2001) have nearly equal affinity to 3'SLN and Su-3'SLN or sometimes decreased affinity to Su-3'SLN in comparison with affinity to non-sulfated 3'SLN (for example, A/Goose/Vietnam/113/2001 and A/Chicken/Vietnam/NCVD5/2003).

Affinity to fucosylated sialosaccharides

We evaluated the effect of the attachment of fucose to sialosaccharides on receptor recognition of H5 viruses. Affinity of all viruses tested in this study declined if fucose was attached to 3OH of the 3'SLN glycosamine ring (SiaLe^x) in comparison with their affinity to 3'SLN (Table 2). For most influenza H5 viruses, addition of the sulfate group to the SiaLe^x receptor analog increased the affinity; however, the receptor binding to Su-SiaLe^x was still lower than the binding to 3'SLN.

Affinity of H5N1 viruses to tested receptors changed dramatically after 2003. For instance, highly pathogenic human viruses isolated in Vietnam and Thailand in 2004 as well as many poultry viruses isolated during 2003–2004 had almost 20 times more affinity to Su-SiaLe^x than to non-sulfated SiaLe^x (Table 2; compare SiaLe^x and Su-SiaLe^x columns). Thus, most recent highly pathogenic isolates differ from earlier H5N1 viruses that evolved from A/Goose/Guangdong/96 in their increased affinity to fucosylated sulfo-sialosaccharides.

Discussion

The results of this study demonstrate that most recent human influenza A(H5N1) isolates, similar to human H5N1 viruses isolated in Hong Kong in 1997 (Matrosovich et al.,

Table 1
Structures of oligosaccharides attached to polyacrylamide

Structure	Abbreviation
Neu5Ac α 2–6Gal β 1–4GlcNAc β	6'SLN
Neu5Ac α 2–3Gal β 1–3GlcNAc β	SiaLe ^c
Neu5Ac α 2–3Gal β 1–3-(6-O-Su)GlcNAc β	Su-SiaLe ^c
Neu5Ac α 2–3Gal β 1–4GlcNAc β	3'SLN
Neu5Ac α 2–3Gal β 1–4-(6-O-Su)GlcNAc β	Su-3'SLN
Neu5Ac α 2–3Gal β 1–4(Fu α 1–3)GlcNAc β	SiaLe ^x
Neu5Ac α 2–3Gal β 1–4(Fu α 1–3)(6-O-Su)GlcNAc β	Su-SiaLe ^x

Table 2
Virus binding affinity (K_{aff} , μM) to sialylglycoconjugates

Virus	Subtype	Sugar determinants						
		6'SLN	3'SLN	Su-3'SLN	SiaLe ^c	Su-SiaLe ^c	SiaLe ^x	Su-SiaLe ^x
<i>Human isolate with 6'SLN specificity (control)</i>								
Aichi/2/68	H3N2	1	>100	>100	>100	>100	>100	>100
<i>Wild duck isolates from Asia, Europe, and North America</i>								
Duck/Hong Kong/717/79	H1N3	>1000	5	5	5	5	100	20
Duck/Nanchang/2-0485/2000	H2N9	>1000	20	10	10	10	>100	>100
Mallard/New York/670/78	H4N6	>1000	4	8	2	4	100	>200
Mallard/Netherlands/02/2000	H10N4	>1000	40	20	5	5	>100	>100
<i>Avian isolates from North America</i>								
Turkey/Wisconsin/68*	H5N8	>1000	5	50	3	10	>50	>100
Turkey/California/6878/79	H5N3	>1000	5	1	4	4	>50	10
Chicken/New Jersey/17169/1993	H5N2	>1000	5	1	4	4	>50	10
Ratite/New York/12716/94	H5N9	>1000	20	50	20	20	>50	>100
Chicken/Mexico/31381-3/94*	H5N2	>1000	5	2	5	3	>50	20
Chukkar/Minnesota/14591-7/98*	H5N2	>1000	5	1	4	4	>50	10
Chicken/Texas/167280-4/2002*	H5N3	>1000	5	1	4	4	>50	10
Turkey/California/DO-6/2002*	H5N2	>1000	5	1	4	4	>50	10
Duck/NJ/117228-7/2001*	H5N2	>1000	15	2	15	10	>50	10
<i>Non-pathogenic duck isolates, Asia</i>								
Duck/Singapore/3/97*	H5N3	>1000	5	2	10	5	>100	10
Duck/Vietnam/342/2001*	H5N2	>1000	5	3	5	5	>100	20
<i>Human isolates, Hong Kong, 1997</i>								
Hong Kong/486/97*	H5N1	>1000	2	0.3	3	2	>100	10
Hong Kong/483/97*	H5N1	>1000	3	0.4	4	2	>100	20
Hong Kong/542/97	H5N1	>1000	4	0.4	4	4	>100	20
<i>Highly pathogenic avian isolates, Asia, 1999–2003</i>								
Goose/Hong Kong/437-4/99	H5N1	>1000	20	5	20	10	>100	30
Duck meat/Amyang/AVL/2001*	H5N1	>1000	3	0.5	5	2	50	3
Teal/Hong Kong/2978.1/2002	H5N1	>1000	5	1	10	5	40	5
Goose/Vietnam/324/2001*	H5N1	>1000	5	5	5	5	50	30
Goose/Vietnam/113/2001*	H5N1	>1000	5	10	4	5	50	100
Chicken/Vietnam/NCVD5/2003	H5N1	>1000	5	40	3	5	40	>100
RBPochard/Hong Kong/821/2002*	H5N1	>1000	4	2	3	3	50	4
Goose/Hong Kong/739.2/2002*	H5N1	>1000	5	3	5	5	50	5
<i>Human isolates (family cluster), Hong Kong, February 2003</i>								
Hong Kong/212/2003*	H5N1	100	30	20	30	30	>100	30
Hong Kong/213/2003*	H5N1	100	30	20	30	30	>100	30
<i>Highly pathogenic isolates poultry and humans, Asia, 2003–2004</i>								
Chicken/Korea/ES/2003*	H5N1	>1000	1	0.3	1	1	50	1
Chicken/Laos/7191/2004	H5N1	>1000	2	0.2	3	2	50	1
Vietnam/1194/2004*	H5N1	>1000	6	0.5	10	8	30	2
Vietnam/1203/2004*	H5N1	>1000	6	0.5	10	8	30	2
Thailand/16/2004*	H5N1	>1000	2	0.2	4	2	50	2

Affinity constants (K_{aff}) formally equivalent to the dissociation constants of virus/receptor analog complexes. Higher values of K_{aff} correspond to lower affinities. The data were averaged from 3 sets. Standard errors did not exceed 50% of the mean values. Viruses marked by asterisks are presented in Fig. 1.

1999), preferentially bind to 2–3 linked sialosaccharides. However, two viruses isolated from a family cluster (father and son) in February 2003 in Hong Kong (Guan et al., 2004) had decreased affinity to all tested 2–3 linked receptor analogs and a clear binding to a 2–6 linked sialosaccharide. Only two amino acids are different between the HA1 sequences of these two isolates and A/Goose/Hong Kong/739.2/2002. The first one is Ser-159-Asn (H3 HA numbering). Asn159 is presented in most of American and many old Asian H5 viruses. All such

viruses tested in this and previous studies (Gambaryan et al., 2004, 2005) had strong Sia2–3Gal receptor specificity. The second amino acid difference (Ser-227-Asn) is unique for all avian H5N1 viruses, including A/Goose/Hong Kong/739.2/2002. This allows us to conclude that Asn227 is responsible for the observed shift in the receptor binding specificity of A/Hong Kong/212/2003 and A/Hong Kong/213/2003. The Asn227 is located between amino acids 226 and 228 that play a key role in receptor specificity and host range restriction of influenza A

viruses (Vines et al., 1998). Thus, our data demonstrate that a single amino acid substitution can significantly alter receptor specificity of avian H5N1 viruses, providing them with an ability to bind to receptors optimal for human influenza viruses. Also, one can speculate that the Ser-227-Asn substitution played some role in the transmission of the H5N1 virus within the family cluster.

Our findings are the first demonstration that American poultry H5 viruses have increased affinity to sulfated sialosugars, especially to Su-3'SLN. This pattern was previously demonstrated for H5N1 viruses isolated in 1997 in Hong Kong. Here, we document that Asian viruses isolated from chickens and humans in 2003–2004 preserved their increased affinity to the Su-3'SLN receptor. Unlike the 1997 isolates, however, the 2003–2004 viruses demonstrated increased affinity to a fucosylated Su-3'SLN, namely to Su-SiaLe^x.

The majority of Asian H5N1 viruses tested in this study have evolved from the A/Goose/Guangdong/96 reference virus (Guan et al., 2004; WHO, 2005). Two clades of viruses that evolved from A/Goose/Guangdong/96 (viruses from HK, 1997, and many 2004–2005 viruses from Vietnam and Thailand; Fig. 3) include highly pathogenic chicken viruses that caused infections in humans (Peiris et al., 2004; Suarez et al., 1998; Subbarao et al., 1998; WHO, 2005). All tested viruses from these two groups demonstrated very high affinity to Su-3'SLN. The other clade of the A/Goose/Guangdong/96 lineage contains viruses isolated from different hosts – geese, chickens, ducks, and some wild birds – and evolved after re-introduction of chicken viruses in these species (Fig. 3). Viruses from this clade demonstrate broad genetic variability. Thus, in contrast to highly pathogenic viruses isolated from humans in 2004–2005, the HA molecules of most of these viruses are not glycosylated at the Asn158 (Fig. 1). Many viruses from this broad clade have shortened polybasic amino acid motifs at the HA cleavage site. The neuraminidase (NA) of

viruses from this clade does not have the deletion in the stalk area of the molecule (Peiris et al., 2004; Nguyen et al., 2005; WHO, 2005). The pattern of receptor specificity within this clade of viruses is also variable although many of them, similar to wild-type duck viruses, have SiaLe^c as their most optimal receptor.

Some isolates from this clade (A/Goose/Vietnam/113/2001, A/Goose/Vietnam/324/2001, A/Chicken/Vietnam/NCVD5/2003) did not demonstrate high affinity to Su-3'SLN. Comparison of receptor affinity data (Table 2) with the HA amino acid sequences for A/Goose/Vietnam/113/2001 and A/Goose/Vietnam/324/2001 (Nguyen et al., 2005) allowed us to demonstrate (Fig. 1) that a single amino acid substitution (Lys-193-Glu) can lead to significant reduction in the affinity of H5N1 viruses to Su-3'SLN. The important role of amino acid 193 in the HA receptor binding was documented for other viruses as well. A/Turkey/Wisconsin/68 that has Glu193 also demonstrated reduced affinity to sulfated sialosugars (Table 2). The Lys-193-Ser substitution significantly decreased the ability of an equine H3N8 influenza virus, A/equine/Grosbois/1/99, to bind to erythrocytes of different animal origin (Medeiros et al., 2004). Another H3N8 equine virus, A/Equine/Miami/1/63 with Lys193 in the HA was highly adapted to Su-3'SLN (Gambaryan et al., 2004). All of these data support the idea that interaction of Lys193 with the glucosamine 6-O-Su-group enhances the binding of the H5 HA with the Su-3'SLN receptor.

We simulated the interaction between Su-SiaLe^x and the HA receptor binding site (RBS) of A/Duck/Singapore/3/97 HA by using the X-ray data of Ha et al. (2001). Fig. 2 shows that the 6-O-Su-group of Su-SiaLe^x is directed towards the amino group of Lys193, and fucose is located near Lys222. The 6-O-Su-group enhances the affinity of the receptor to the HA of the A/Duck/Singapore/3/97 virus, while the fucose residue reduces it (Table 2). There is probably an electrostatic interaction between the 6-O-Su-group and Lys193 while fucose may

	140	*	160	*	180	*	200	*	220
dkSing3/97	: SGVSSACPYNGRSFFRN	WVLI	KKN	AYPTIKRSYNN	TNQEDLLILWG	IHPNDAAEQTKLYQ	NPTTYVSVGTSTL	NQRSVPEIATRF	PKVNGQS
dkMN1525/81	:T.....M.....SN.....I.....
tuWI/68	:S.....	S.V.....	T.....V.....	E.....SN.....I.....
chuMN7/98	: ..L.....	GP.....T.....V.....SN.....I.....
chMX3/94	:	DV.R.....	T.....V.....	I.....N.....I.....
chTX4/02	:	H.....	T.....	V.R.V.T.....	V.....	I.....SN.....	K.I.V.....
tuCA6/02	:	T.....V.....	I.....SN.....I.....
dkNJ7/01	:	T.....V.....	I.....SN.S.....I.....
dkVN342/01	:	S.....	V.....
HK483/97	:	L.K.....	ST.....	V.....	I.....	L.....
HK486/97	:	L.....	S.....	V.....	I.....	L.....
dkmtAm/01	:	K.....	I.....	S.....	I.....	L.K.S.....
chVN5/03	:	Q.P.....	R.....	V.....	I.....	L.K.S.....
gsVN113/01	:	Q.KP.....	S.....	V.....	NE.....E.....	I.....	L.K.S.....
gsVN324/01	:	Q.KP.....	S.....	V.....	NE.....	I.....	L.K.S.....
gsHK739.2/02	: L.....	Q.K.....	S.....	V.....	R.....	I.....	L.K.S.....
HK212/03	: L.....	Q.K.....	V.....	R.....	I.....	L.K.S.....	N.....
HK213/03	: L.....	Q.K.....	V.....	R.....	I.....	L.K.S.....	N.....
RbpHK821/02	: L.....	Q.K.....	S.....	V.....	S.....R.....	I.....	L.K.S.....
chKOES/03	:	Q.....	S.....	V.....	R.....	I.....	L.K.S.....
VN1194/04	: L.....	Q.K.....	ST.....	V.....	I.....	L.R.S.....
VN1203/04	: L.....	Q.K.....	ST.....	V.....	I.....	L.R.S.....
Th16/04	: L.....	Q.K.....	ST.....	V.....	I.....	L.R.S.....

Fig. 1. Partial amino acid sequences of the H1 HA of H5 viruses. The 158–160 glycosylation site and amino acids 193, 222, and 227 are highlighted. See Table 2 for complete virus designations.

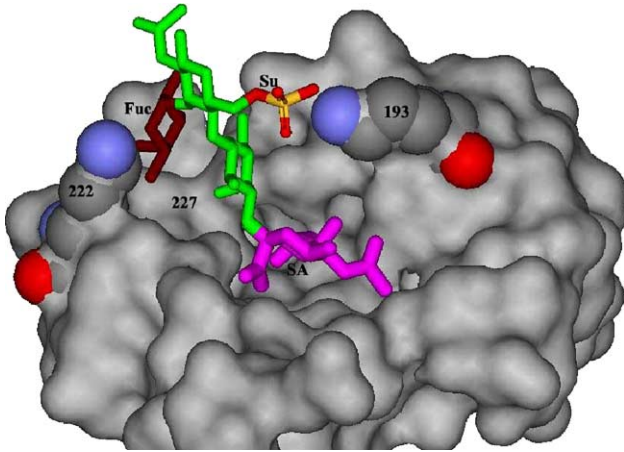


Fig. 2. Putative disposition of the 6-O-Su-SiaLe^x in the receptor binding site of the A/Duck/Singapore/3/97 HA. The molecular model of 6-O-Su-SiaLe^x was built up based on SiaLe^x (2KMB structure, Brookhaven Protein Databank, Ng and Weis, 1997). This analog was fitted into the RBS of A/Duck/Singapore/3/97 HA complexed with LSTa (1JSN structure, Brookhaven Protein Databank; Ha et al., 2001) by superimposing galactose residue of the sulfated SiaLe^x over the galactose residue of LSTa. The protein surface is colored grey, except for the multicolored Lys193 and Lys222 residues. Sialic acid is presented in pink. Receptor sugar core is shown in green, except for the fucose (brown) and sulfogroup (yellow and red). The modeling was performed using Discovery Studio ViewerPro.

sterically interfere with Lys222. We speculate that during the adaptation of H5N1 viruses to chickens, a positive contribution of the sulfo group to interaction with the HA RBS has been increasing while a negative effect of the fucose residue to this interaction has been decreasing. Several amino acid substitutions near the RBS (positions 128, 130, 133, 142, 144, 216, and

221) distinguish H5N1 viruses of 2003–2004 from those isolated in 1997 (Fig. 1) (Nguyen et al., 2005; Peiris et al., 2004; WHO, 2005). We assume that the Pro-221-Ser substitution can result in a spatial shift of Lys222, thus promoting better fit of fucose into the HA RBS. It should be assumed, however, that the presence of Lys/Arg193 and Ser221 is necessary but not sufficient for the high affinity of the HA molecule to Su-3'SLN or Su-SiaLe^x receptors. Additional compensatory amino acid changes at the top of the HA molecule can allow the RBS to recognize sulfated receptors via the Lys193–HSO₃ interaction. This could explain why the A/chicken/Vietnam/NCVD5/2003 isolate did not demonstrate affinity for Su-3'SLN and Su-SiaLe^x in spite of the presence of Lys193 and Ser221 in their HA molecule.

Because of the threat of an influenza pandemic, H5N1 viruses have been a focus of many studies since they were first isolated from humans in 1997. It is now well documented that chickens play a key role in transmission of H5N1 viruses from wild birds (ducks) to humans. It is obvious that chicken H5N1 influenza viruses have continued to evolve since 1997. Fig. 3 presents the phylogenetic relationships between the HA molecules (amino acid sequences) of H5N1 viruses evolved from A/goose/Guangdong/96 along with the receptors most optimal for different groups of Asian avian and human influenza H5 isolates. The figure demonstrates that the genetic evolution of avian influenza A(H5N1) viruses from Asia is accompanied by the evolution of their receptor specificity.

The fact that a single amino acid substitution in the receptor binding area of the H5 HA of two human isolates, A/Hong Kong/212/2003 and A/Hong Kong/213/2003, resulted in

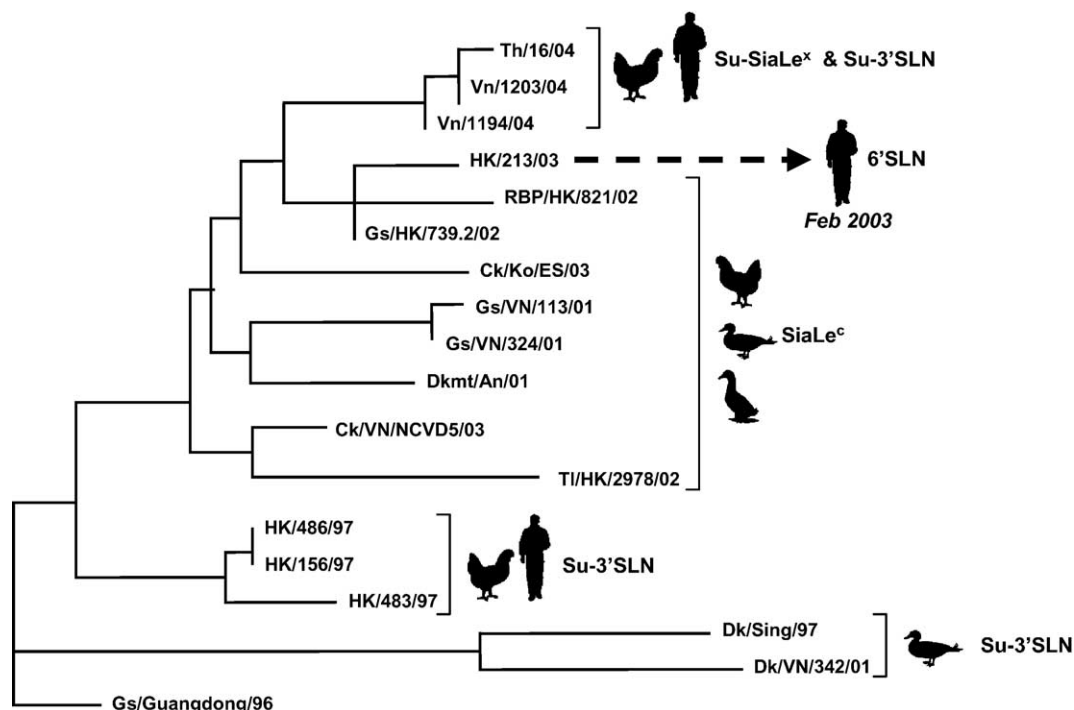


Fig. 3. The phylogenetic relationships between the HA molecules (amino acid sequences) of H5N1 viruses evolved from A/goose/Guangdong/96. Receptors most optimal for different groups of Asian avian and human influenza H5 isolates are indicated.

marked affinity to a receptor optimal for typical human viruses underscores the necessity and significance of further comprehensive surveillance for the affinity of H5N1 viruses to different receptors for better understanding of their pandemic potential.

Materials and methods

Materials

Oligosaccharides conjugated with polyacrylamide were synthesized from spacers sialooligosaccharides- ω -aminoglycosides (spacer = $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$, $-\text{OCH}_2\text{CH}_2\text{NH}_2$, or $-\text{NHCOCH}_2\text{NH}_2$) and poly(4-nitrophenylacrylate) with molecular mass of 30 kDa. Spacers oligosaccharides were either synthesized as described earlier (Pazynina et al., 2003) or obtained from the Consortium for Functional Glycomics (<http://web.mit.edu/glycomics/consortium/>). We used the following oligosaccharides: Neu5Ac α 2–6Gal β 1–4GlcNAc β (6'SLN); Neu5Ac α 2–3Gal β 1–4GlcNAc β (3'SLN); Neu5Ac α 2–3Gal β 1–4-(6-O-Su)GlcNAc β (Su-3'SLN); Neu5Ac α 2–3Gal β 1–3GlcNAc (SiaLe^c); Neu5Ac α 2–3Gal β 1–3-(6-O-Su)GlcNAc (Su-SiaLe^c); Neu5Ac α 2–3Gal β 1–4(Fuc α 1–3)GlcNAc β (SiaLe^x); and Neu5Ac α 2–3Gal β 1–4(Fuc α 1–3)(6-O-Su)GlcNAc β (6-Su-SiaLe^x).

Viruses

Viruses were grown in 9-day-old embryonated chicken eggs and were inactivated by treatment with β -propiolactone. The allantoic fluids were clarified by low-speed centrifugation; the viruses were pelleted by high-speed centrifugation, re-suspended in 0.1 M NaCl, 0.02 M Tris buffer (pH 7.2) containing 50% glycerol, and stored at -20°C . Work with highly pathogenic isolates was performed under BSL-3 enhanced conditions.

Virus binding to soluble receptor analogs

Affinity of viruses to sialylglycopolymers was evaluated in a competitive assay based on inhibition of binding by the solid-phase immobilized virus of standard preparation peroxidase-labeled fetuin (Gambaryan and Matrosovich, 1992). The data were expressed in terms of affinity constants (K_{aff}) formally equivalent to the dissociation constants of virus/receptor analog complexes. For the calculation of the constants, concentration of the sialic acid residues in solution was used. The data shown in Table 2 were averaged from the same set of experiments.

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