Original Article

Identification of Dual Receptor-binding Specific Strains of Human H5N1 Viruses in China*

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Abstract

Objective Both the 2, 6 linkage and its topology on target cells are critical for the recognition by human influenza virus. The binding preference of avian flu virus H5N1 HA to the 2, 3-linked sialylated glycans is considered the major factor limiting its efficient infection and transmission in humans. To monitor potential adaptation of H5N1 virus in human population, the surveillance of receptor-binding specificity was undertaken in China.

Methods The binding specificity of 32 human H5N1 virus strains isolated from 2003 to 2009 was tested by 2, 3-specific sialidase-treated chicken red blood cell (CRBC) agglutination assay and a solid-phase direct binding assay with synthetic sialylglycopolymers.

Results Dual binding preference to 2, 3 and 2, 6-glycans were found in two strains: A/Guangdong/1/06 (A/GD/1/06) and A/Guangxi/1/08 (A/GX/1/08). Though minor effect of short-2, 6-binding was detected in A/GX/1/08 at a low virus titer, both showed high affinity to the oligosaccharide at a high load. Notably both are of the long-2, 6-recognition, with the same topology as that of human H1N1 and H3N2 viruses.

Conclusion The findings suggest that human H5N1 virus in China likely acquired the potential human-adaptation ability. Further research and surveillance on receptor-binding specificity of H5N1 viruses are required.

Key words: Receptor-binding specificity; Human H5N1 virus; China

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INTRODUCTION

Via envelope glycoprotein hemagglutinin (HA), influenza viruses bind to cell-surface glycosylated oligosaccharides terminated by sialic acids(SA), where their linkage is cell- and species-specific. Differential receptor binding preference is a host barrier for influenza virus transmission. Although most H5N1 viruses have low affinity to Neu5Acα2, 6Gal (human-type) receptor, recent findings have suggested that the adaptation of H5N1 virus to human by mutations in the receptor-binding site (RBS) did happen and resulted in enhanced affinity to human-type receptor[1-3]. In contrast to its putative precursor, A/Gs/GD/1/96, the diverse genotypes, accelerating evolution and widespread occurrence were present in currently circulating H5N1 virus[4-5]. To date, 10 distinct phylogenetic

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clades (0-9) were identified based on H5N1 HA and the confirmed human infections were caused by clade0, 1, 2.1, 2.2, 2.3, and 7[6]. In China, human H5N1 disease was mostly caused by clade 2.3.4, which was identified in 28 isolates from 39 confirmed patients in 17 provinces since 2003. Clade 7 and clade 2.2 were responsible for the case in 2003 and 2006 respectively. Two current cases of 2009 and 2010 were due to clade 2.3.2. Now the information on receptor property was documented in some H5N1 viruses of clade1, 2.1, and 2.2[1-3,7]. Little is known about H5N1 virus of clade 2.3.4, particularly from humans.

Recently, α2, 3-specific sialidase-treated red blood cell (RBC) agglutination assay was developed and used for receptor specificity screening of H5N1 virus[3,8]. The α2, 6 or α2, 3-binding preference can be distinguished by the change of hemagglutination titer reacted with RBCs and enzymatic RBCs. Since fine receptor specificity existed in H5N1 viruses[9-10], the glycan array including sulfated-, fucosylated-, linear sialosides, di-sialosides or direct binding assay with synthetic polyacrylamide(PAA)-based sialylglycopolymers was also recommended for the receptor-specificity surveillance on H5N1 viruses. Furthermore, the long-branched α2, 6 sialylated glycans were currently identified to predominate on the upper respiratory epithelial in humans and the recognition of this topology, 6'SLN-LN is the key determinant for the human-adaptation of influenza A virus[11].

Here, we analyzed the receptor-binding specificity of human H5N1 viruses isolated in China from 2003 to 2009.

MATERIALS AND METHODS

Sampling and Virus Isolation

Since 2003, a total of 39 H5N1 infection cases have been confirmed in China from 17 provinces. The pharyngeal swabs and lower airway aspirations from the patients were collected within 12 days after disease onset, maintained in viral-transport medium and tested within 24 h. The speciments were propagated in the allantoicsac of 10-11 day embryonated chicken eggs for 33 h at 37 °C.

Hemagglutination(HA) Assay

10% CRBC suspension was treated by 625 mU 2,3-specific sialidase (Takara) at 37 °C for 15 min. Complete elimination of α2, 3-receptor on sialidase-treated CRBCs was confirmed by receptor staining and flow cytometry. HA assay of live viruses with 1% CRBC or 1% sialidase-treated CRBC were performed in BSL-3 facility.

Direct Binding Assay With Synthetic Sialylglycopolymers

Synthetic 3'SLN-PAA-Biotin(PA191), 6'SLN-PAA-Biotin(PA190), and 6'SLN-LN-PAA-Biotin(PA343) were provided by the Scripps Research Institute (TSRI). As described elsewhere with some modifications[3], generally, serial dilutions of sialyglycopolymers were coated in 96-well-flat-bottom polystryrene plates and 32 HAU live virus/well were added. Alternatively, the plates were precoated with 5 μg/mL sialyglycopolymers and then 8, 16, 32, 64, 128 HAU live virus/well influenza viruses were added. Rabbit antisera against A/AH/1/05 diluted in PBS containing 1% BSA was added into the wells. Bound antibody was detected by use of HRP-conjugated anti-rabbit IgG antibody and tetramethylbenzidine substrate solution. Each sample was determined in duplicates and the absorbance read at 450 nm.

RESULTS

Human Avian Influenza Cases (H5N1) in China from 2003 to 2009

Totally 31 H5N1 virus strains were obtained from 2003 to 2009. The name and passage history of influenza viruses used in the study are listed in Table 1. As the same sequences of eight RNA segments were detected in A/JS/1/07 and A/JS/2/07, only A/JS/2/07 was tested here. Three amantadine-resistant variants with M2 mutation of A30S, A30T, and S31N respectively were cloned from the A/HB/1/06 isolate.

Screening of Receptor-binding Preference by HA Assay

Representative results from three sets of independent experiments are shown in Table 1. Complete HA with sialidase-treated CRBCs, which were only with α2,6-receptors, was detected in human influenza virus (A/Brisbane/59/2007, H1N1) and two human H5N1 virus strains, A/GD/1/06 and A/GX/1/08.

Identification of Subtle Receptor Specificity by Direct Binding Assay

High binding of α2, 3 oligosaccharides to H5N1 viruses was detected (Figure 1A, B, and C). And enhanced

Table 1. Hemagglutination Titers of Influenza Viruses With CRBC and Sialidase-treated CRBC

Virus and Subtype	Passage History from Initial Isolate	Genetic Clade	1% CRBC (HAU/50 uL)	1% Sialidase-treated CRBC (HAU/50 uL)
A/Brisbane/59/2007 (H1N1)	Chicken Egg×4	-	512	512
A/AH/1/05 (H5N1)	Chicken Egg×2, EMDCK×2	2.3.4	64	neg
A/AH/2/05 (H5N1)	Chicken Egg ×1, MDCK×2	2.3.4	32	neg
A/JX/1/05 (H5N1)	Chicken Egg ×4	2.3.4	256	neg
A/GX/01/05 (H5N1)	Chicken Egg ×4, MDCK×1	2.3.4	16	neg
A/FJ/1/05 (H5N1)	Chicken Egg ×4	2.3.4	512	neg
A/AH/1/06 (H5N1)	Chicken Egg ×4	2.3.4	64	neg
A/HN/1/06 (H5N1)	Chicken Egg ×4, MDCK×1	2.3.4	8	neg
A/SH/1/06 (H5N1)	Chicken Egg ×4, MDCK×1	2.3.4	8	neg
A/ZJ/1/06 (H5N1)	Chicken Egg ×4	2.3.4	256	neg
A/SC/1/06 (H5N1)	Chicken Egg ×4	2.3.4	64	neg
A/SC/2/06 (H5N1)	Chicken Egg ×4	2.3.4	64	neg
A/GD/1/06 (H5N1)	Chicken Egg ×3	2.3.4	32	16
A/SC/3/06 (H5N1)	Chicken Egg ×3	2.3.4	16	neg
A/GD/2/06 (H5N1)	Chicken Egg ×3	2.3.4	1024	neg
A/XJ/1/06 (H5N1)	Chicken Egg ×3	2.2	256	neg
A/HB/1/06 (M2, A31S)(H5N1)	Chicken Egg ×4, MDCK×2	2.3.4	64	neg
A/HB/1/06 (M2, A31T)(H5N1)	Chicken Egg ×4, MDCK×2	2.3.4	32	neg
A/HB/1/06 (M2, S31N)(H5N1)	Chicken Egg ×4, MDCK×2	2.3.4	16	neg
A/BJ/1/03 (H5N1)	Chicken Egg ×7	7	256	neg
A/FJ/1/07 (H5N1)	Chicken Egg ×3	2.3.4	512	neg
A/AH/1/07 (H5N1)	Chicken Egg ×3	2.3.4	256	neg
A/JS/2/07 (H5N1)	Chicken Egg ×2	2.3.4	256	neg
A/HN/1/08 (H5N1)	Chicken Egg ×2, MDCK×1	2.3.4	128	neg
A/GX/1/08 (H5N1)	Chicken Egg ×1, MDCK×1	2.3.4	64	4
A/GD/1/08 (H5N1)	Chicken Egg ×2	2.3.4	256	neg
A/BJ/01/09 (H5N1)	Chicken Egg ×1, MDCK×1	2.3.4	64	neg
A/HN/1/09 (H5N1)	Chicken Egg ×2, MDCK×1	2.3.4	64	neg
A/SD/1/09 (H5N1)	Chicken Egg ×1, MDCK×1	2.3.4	64	neg
A/XJ/1/09 (H5N1)	Chicken Egg ×3, MDCK×1	2.3.4	64	neg
A/GZ/1/09 (H5N1)	Chicken Egg ×3, MDCK×1	2.3.4	64	neg
A/GX/1/09 (H5N1)	Chicken Egg ×2, MDCK×1	2.3.2	128	neg
A/HN/2/09 (H5N1)	Chicken Egg ×1, MDCK×1	2.3.4	32	neg

α2, 6-binding preference was also detected in A/GD/1/06 and A/GX/1/08. The α2, 6-binding was dose dependent for sialyglycopolymers and virus titer. Notably, as compared with A/GD/1/06 of both short- and long-α2, 6 recognition, A/GX/1/08 preferred to bind to long-α2, 6 oligosaccharides at low viral titer (Figure 1B, C). However, both of them showed strong affinity to short- and long-α2, 6 oligosaccharides at high viral loads (Figure 1D).

DISCUSSION

Sialoside-, galactoside-, mannoside-, and sulfo-OS-binding is the four types of carbohydrate- binding properties of influenza virus[12]. Binding of influenza virus to the α2, 3 or α2, 6-linked sialylated glycans on cell surface is important for host range restriction and the preference to α2, 3 of H5N1 virus limiting its efficient infection in human. Here, dual receptor-binding preferences were detected in A/GD/1/06 and A/GX/1/08, which are of clade 2.3.4. Although there is no direct evidence supporting the occurrence of human-to-human transmission in these infection events or the association between viral virulence and receptor-binding switching, viral systemic disseminations are indeed found in the both fatal cases (data not shown). Furthermore, with the introduction of clade 2.3.4 into the adjacent countries of China[4], the finding of H5N1 virus with 2-6-binding in human should be of concern.

Though H5N1 virus with human-type receptor-binding was isolated from one patient treated by oseltamivir and those viruses were with HA and/or

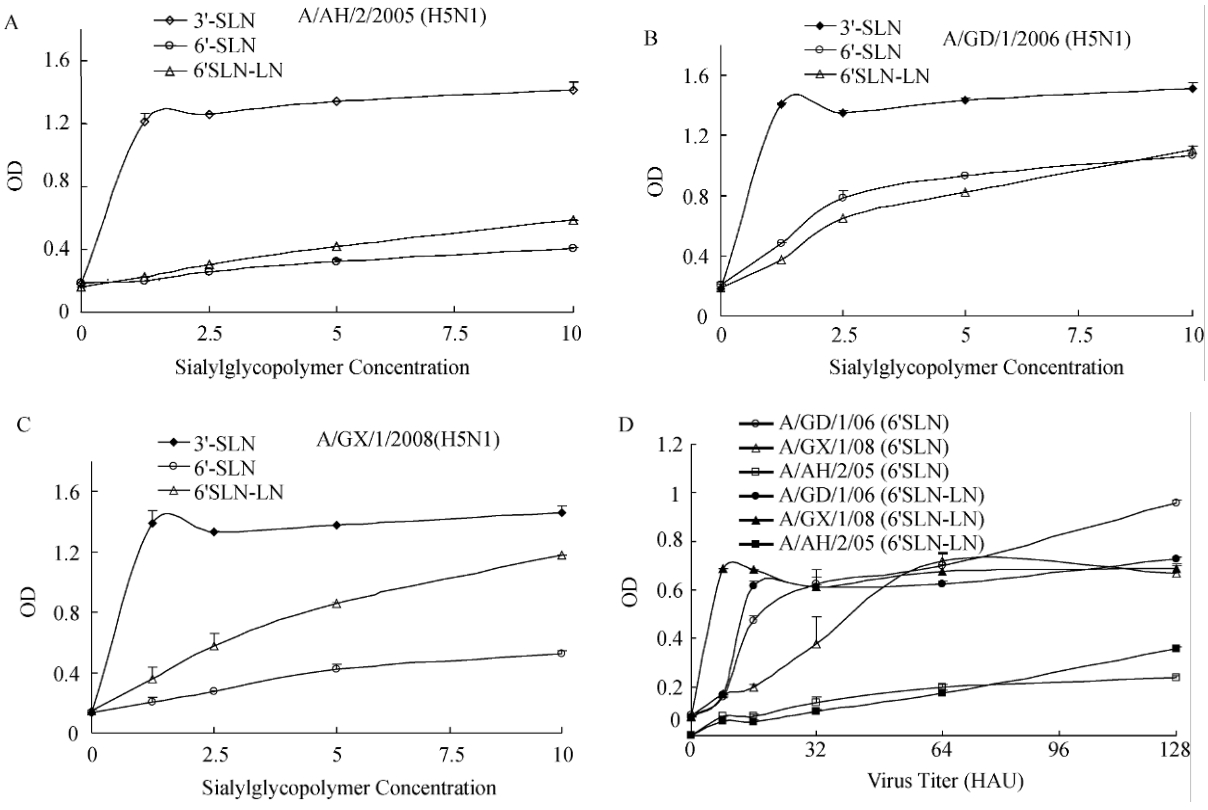

Figure 1 Direct binding assay using sialyglycopolymers. The dose-dependent direct binding tests on the three viruses (A) A/AH/2/05(H5N1) (B) A/GD/1/06(H5N1) (C) A/GX/1/08 (H5N1) were performed in the same set of experiment. (D) Binding of 6'SLN and 6'SLN-LN to A/GD/1/06, A/GX/1/08, and A/AH/2/05. The data represent mean±SEM from duplicate wells in one representative test from two sets of independent experiments.

NA substitutions[13], the issue whether the substitutions responsible for receptor specificity switching is pre-existing or selective in the human host remains unknown. Our finding that three mutant viruses bearing M2 mutations of A30S, A30T, and S31N cloned from one isolate A/HB/1/06 suggested that the resistant viruses were likely emerging in the host environment. No variation was found in their HA and NA sequence and all of them showed high affinity to α2-3-binding. Our data suggested that the binding-specificity was not affected by the mutations on viral envelope protein M2.

With the adaptation from wild aquatic birds to domestic poultry or even in human host environment, influenza virus may possess broader carbohydrate-binding spectrum or topology conformation[11,14]. We demonstrated differential α2, 6-binding property of two human H5N1 viruses, A/GD/1/06 and A/GX/1/08. Though minor effect of short-α2, 6-binding was detected in viruses A/GX/1/08 at a low virus titer, both were of high affinity to long-α2, 6 glycans, even at the low titer which are rich on apical side of human upper respiratory epithelia[11]. Notably, no evident binding preference switching was detected in the viruses isolated from the sporadic human infection cases in early 2009 in China (Table 1). However, higher affinity to the long-α2, 6 glycans was observed in A/BJ/1/09, A/GZ/1/09, and A/XJ/1/09 (data not shown). The discrepancy from the findings obtained by sialidase-treated CRBC may be associated with a limited abundance of N-linked α2-6 with long branches on CRBC as demonstrated in a recent study[11]. Therefore, glycan dose-dependent binding assay is valuable and should be applied in flu surveillance. The mechanism underlying the tendency is unknown and further research on receptor-binding specificity of H5N1 viruses is required.

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Abbreviations: 2-3'SLN, Neu5Acα2–3Gal1–4GlcNAc; 2-6'SLN, Neu5Acα2–6Gal1–4GlcNAc; 2-6' SLN-LN, Neu5Acα(2–6) (Gal1–4GlcNAcβ(1-3)2.

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