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Original Article

Sequence comparison between two quasi strains of H6N1 with different pathogenicity from a single parental isolate

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Background and Purpose: Relationships between gene change and virulence for hemagglutinin (HA) subtypes of avian influenza virus remain inconclusive. In this study, sequences of these nearly identical virus strains were obtained in order to elucidate the relationship between molecular determinants and virulence.

Methods: Two strains, with different virulence, of an H6N1 avian influenza virus were isolated from an infected chicken flock. Complete 8-gene fragments from the 2 strains were cloned and sequenced. Putative amino acid sequences were compared.

Results: Comparisons of the sequences from the 2 strains showed 0.65%, 0.79%, 0.28%, 1.41%, 0.80%, 0.20%, 0.43%, and 0.83% differences in PB2, PB1, PA, HA, NP, neuraminidase (NA), NS1 and NS2 proteins, respectively. The M1, M2, and PB1-F2 protein sequences from the strains were identical. The HA cleavage site of both strains contained a single R, despite their difference in virulence. Thus, the difference in virulence might be due to sequences other than the HA cleavage site. Most of the changes were in the HA2 part. The sequence immediately after the HA cleavage site was GILR in the non-virulent strain and GIFG in the virulent strain. The change from E to G at position 106 in the HA, near the receptor binding site, might influence the virulence. Other sequence changes likely to influence virulence were from K to R at position 391 (K391R) in NP protein and from P to T at position 201 (P201T) in NA protein.

Conclusion: The amino acid changes identified in this study may be important in the virulence of influenza viruses.

Key words: Amino acid sequence, chickens, influenza A virus, molecular sequence data, virulence

Introduction

Avian influenza virus (AIV) is widespread among birds throughout the world. It has been divided into 16 hemagglutinin (HA) protein subtypes and 9 neuraminidase (NA) protein subtypes [1]. AIV is further divided into high pathogenic and low pathogenic AIV according to its virulence in chickens. All high pathogenic AIVs are of H5 and H7 serotypes [2].

It is well known that virulence is related to the amino acid sequence upstream from the HA cleavage site [2]. Highly pathogenic H5 or H7 viruses contain multiple basic amino acids. Although the nature of the HA cleavage site plays an important role in determining virulence, there are viruses with multiple basic amino acids at the HA cleavage site that show variations in virulence [3]. Some H5N1 viruses with multiple basic amino acids at this site show low virulence. The most well known of these is the H5N2 virus isolated in Pennsylvania in 1983; the HA cleavage site is hindered by the presence of the N glycosylation at the HA position 11 [4], even though this virus contains multiple basic amino acids. In addition, the dogma of multiple basic amino acids relating to virulence might be true only for the H5 and H7 subtypes, as viruses of other subtypes having no such multiple basic amino acids sequence also show variations in virulence [5]. Therefore, other virulence determinants are likely to exist in viruses. Numerous studies have shown that influenza virus virulence is a polygenic trait that may require a critical gene constellation [6].

Subbarao et al reported that a single amino acid substitution, from E to K in PB2 at the position 627 was associated with host range specificity [7], whereas

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Shinya et al [8] reported that this change was associated with replication and virulence. The same mutation was found in a European H7N7 virus from a patient that died from severe pneumonia but not in the virus from patients with only conjunctivitis [9]. However, this association was not found in recent H5N1 viruses from Thailand [10]; both E and K were found from fatal viruses. The relationship between the gene change and virulence is still not conclusive.

In Taiwan, the low pathogenicity H6N1 virus has been present for many years [5]. Some viruses caused diseases of intermediate severity with systemic infection but relatively low mortality. Viruses with different virulence levels were obtained experimentally from a single H6N1 virus population, a non-virulent strain (2838N) by limiting dilution and a virulent strain (2838V) by contact infection. We investigated the molecular determinants that distinguish these 2 strains with different virulence derived from a single field isolate by comparing the complete sequences of all 8 genes to elucidate the association between sequence and virulence.

Methods

Viruses

Both non-virulent (2838N) and virulent (2838V) H6N1 AIV strains derived from a field isolate in 2000 [5] were propagated in specific pathogen-free chicken embryos (Animal Research Health Institute, Chidin, Taiwan). Aliquots were taken for RNA extraction. Their intravenous pathogenicity indexes were 0.16 and 0.48, respectively.

Cloning and analysis of virus gene fragments

Eight gene fragments from both strains were extracted with TriSolution (Genmedika Biotechnol Corp., Taipei) and amplified with specific primers by using reverse transcriptase-polymerase chain reaction (RT-PCR) for different fragments. The forward and reverse primers reported by Hoffmann et al [11] were used for amplifying PB2, PB1, PA, NP, NA, M, and NS gene fragments. P1 and P4 primers (provided by Dr. P. C. Chang at National Chung-Hsing University, Taichung) were used for the HA gene fragment. The forward primer sequence was P1: 5'-GGAAAATGATTGCAATCATT (–5-15), and the reverse primer sequence was P4: 5'-TTATATACATATCCTGCATTGCAT (1704-1681).

The RT-PCR products were cloned into yT&A cloning kit (Yeastern Biotech. Co., Taipei) and sequenced using an ABI prism cycle sequencing kit (Perkin Elmer, Banchburg, NJ, USA). Forward and reverse sequences were performed. The nucleotide sequences and the putative amino acid sequences were compared using the Vector NTI Suite 6 software (InforMax, Bethesda, MD, USA).

Results

The sequence differences for the 8 genes between 2838N and 2838V ranged from 0% to 1.41%, with HA showing the greatest difference (Table 1). RNA segment 1 of 2838N and 2838V coded for a 759-amino acid PB2 protein from MERIK to RMAIN. Comparing non-virulent with virulent strains, 5 amino acid changes were noted in this protein. The amino acid at position 627 of PB2 from both strains was E (AAPP-E-QSRM).

RNA segment 2 of both strains coded for a 757-amino acid PB1 protein from MDVNP to LRRQK. A

Table 1. Amino acid differences in different genes between 2838N and 2838V[a]

Gene (%, no. of differences/total)	Position	2838N	2838V
Gene 1: PB2 (0.65, 5/759)	106	PT-S-ST	PT-T-ST
	432	PM-H-QL	PM-Y-QL
	460	MG-M-IG	MG-I-IG
	494	ER-V-VV	ER-I-VV
	586	VP-K-AA	VP-R-AA
Gene 2: PB1 (0.79, 6/757)	171	DV-M-ES	DV-I-ES
	240	RR-A-IA	RR-E-IA
	414	FN-M-LS	FN-T-LS
	464	GV-D-RF	GV-N-RF
	475	VG-V-NM	VG-I-NM
	706	SY-R-RP	SY-G-RP
Gene 3: PA (0.28, 2/716)	350	IE-N-EE	IE-D-EE
	686	GG-Q-YE	GG-L-YE
Gene 4: HA (1.41, 8/567)	–5	LA-A-AG	LA-T-AG
	106	LI-E-SG	LI-G-SG
	304	IG-E-CP	IG-K-CP
	332-3	GI-L-RA	GI-F-GA
	381	NK-V-NS	NK-A-NS
	419	FL-E-VW	FL-D-VW
	541	MC-Q-NG	MC-S-NG
Gene 5: NP (0.80, 4/498)	253	AE-I-ED	AE-V-ED
	348	FI-G-GT	FI-R-GT
	391	RT-K-SG	RT-R-SG
	420	LP-F-ER	LP-L-ER
Gene 6: NA (0.20, 1/457)	201	II-P-DT	II-T-DT
Gene 8: NS1 (0.43, 1/230)	65	QI-V-ER	QI-A-ER
NS2 (0.83, 1/121)	100	TF-M-QA	TF-V-QA

Abbreviations: HA = hemagglutinin; NA = neuraminidase; NP = nucleoprotein; NS = non-structural protein

[a]M1, M2 from M gene and PB1-F2 protein from PB1 gene are identical between the strains.

newly discovered protein, PB1-F2, started at position 95 (+1 ORF) in the PB1 gene, which began with M and stopped at different positions in different influenza viruses, resulting in different lengths [12]. Both strains contained an identical 90-amino acid PB1-F2.

RNA segment 3 of both strains coded for a 716-amino acid PA protein from MEDFV to THALK. Only 2 amino acid differences were found between both strains.

RNA segment 4 of both strains coded for a 567-amino acid HA protein from MIAII to CRICI, with a 16-amino acid signal peptide. The main HA protein was 551 amino acids in length, with 329 amino acids of HA1 and 222 amino acids of HA2. The mature HA1 was from DKICI to QIETR. The sequences of both strains were indistinguishable through the connecting peptide region, consisting of a single R, which was QIETR↓GIL in 2838N and QIETR↓GIF in 2838V (arrows indicate the cleavage site). The sequence after the cleavage site in most influenza viruses is GLF [13]. Surprisingly, the related sequence in the present H6N1 HA sequence was GILR in 2838N and GIFG in 2838V. The latter is the same as A/chicken/Taiwan/7-5/99 (Genbank accession number: AF310983). Most of the changes were in the HA2 part. Only 1 change was found in the signal peptide and 2 in the HA1, respectively. No difference in putative predicted N-linked glycosylation in HA was found in both strains. Both had no glycosylation at position 156 near the tip of HA, a receptor binding site, suggesting increased binding affinity to Sia2-3Gal, an avian receptor [14]. Instead, both had NNT sequence and glycosylation at position 167. Whether this interferes with receptor binding is not known because no related data are available for H6 AIV. The striking finding in 2838N and 2838V was GQRSRI (223-228) at the critical HA receptor binding site (Table 2).

RNA segment 5 of both strains coded for a 498-amino acid NP protein from MASQG to EEYDN. The anchor residue of lymphocyte epitope recognized by specific cytotoxic T-lymphocytes was amino acid 383-391 SRYWAIRTR and SRYWAIRTK in 2838V and 2838N, respectively.

RNA segment 6 of both strains coded for a 457-amino acid NA protein from MNPNQ to FTVDK with a K ending. Both strains showed 12-amino acid deletion in the NA stalk. The only different amino acid in NA between the strains was P201T.

RNA segment 7 of both strains coded for a 252-amino acid M1 protein and a 97-amino acid M2 protein, respectively. Both strains had identical protein sequences.

RNA segment 8 of both strains coded for a 230-amino acid NS1 protein and a 121-amino acid NS2 protein, respectively. Both strains had identical NS1 and NS2 sequences. The position 92 of NS1 protein from both strains was D.

Table 2. Hemagglutinin receptor-binding site sequences of avian and human-adapted influenza viruses

Virus strain	Host	H subtype	Sequence (225-230)[a]
Mallard/Potsdam/178/83	Duck	H2	G-Q-G-G-RM
Victoria/15681/59	Human	H2	G-L-G-S-RM
Duck/Hokkaido/10/85	Duck	H3	G-Q-S-G-RI
Los Angeles/2/87	Human	H3	G-L-S-S-RI
2838V	Chicken	H6	G-Q-R-S-RI

[a]Amino acid change from G to S at position 228 (H3 numbering) adapts the virus from avian to humans [9,14].

Discussion

AIV shows great variation in virulence. Numerous influenza virulence studies have sought to define the roles of each viral gene in disease production. The virulence of a virus has been attributed to the presence of the multiple basic amino acids at the HA cleavage site. Gao et al have indicated that virulence in the influenza viruses is multigenically determined [3]. This paper confirms that the sequences other than the HA cleavage may play roles in virulence determination, because both non-virulent and virulent H6N1 viruses share a single R at the cleavage site.

The amino acid change from E to G at position 106 in the HA from 2838N to 2838V might be near the receptor binding site [15,16], although the exact site is not known in H6 virus. G has more conformation freedom than any other amino acid [17], and therefore may affect the ability of the virus to recognize and bind to target cells and thus influence the degree of virulence and tissue tropism [18]. This might explain our previous result that 2838V spreads to the kidney after intranasal inoculation, whereas 2838N does not [5].

At the critical HA receptor binding site (223-228), both strains contained GQRSRI. The changes in Q226L and G228S (H3 numbering, Q224 and S226 in H6) of HA transfer the virus attachment from avian to humans [15,16]. The position 228 in HA from the 2838N and 2838V was S, as in human-adapted H3 strains. This raises the possibility that H6N1 might transmit to humans.

In RNA segment 5, the anchor residue of lymphocyte epitope recognized by specific cytotoxic T lymphocytes was amino acids 383-391 SRYWAIRTR and SRYWAIRTK in 2838V and 2838N, respectively [19]. The amino acid change from R to K at position 384 of NP protein accounts for the virus abrogating major histocompatibility complex class I presentation and allowing escape from recognition by specific cytoxic T-lymphocyte. Although both 2838N and 2838V had R at this position, the amino acid at position 391 was K in 2838N and R in 2838V. This difference might account for the virulence difference, because it is the epitope recognized by the cytotoxic T-lymphocyte if the related sequence in AIV has the same function.

The N glycosylation at amino acid position 144 of NA, which is conserved due to the functional requirement [20], was also found in both strains (HSN-GT). The K at the NA C-terminus and the absence of glycosylation at position 144 (146 in N2 numbering) increases the virulence in the virus because this protein binds and sequesters plasminogen leading to a higher concentration of the ubiquitous protease and thus facilitates HA cleavage [20,21]. Both 2838N and 2838V strains contained K at the C-terminus and glycosylation at position 144. This information suggests that changes other than the presence of K at the end of the NA contribute to the virulence of AIV H6N1 strains [13].

H5N1 viruses in chickens differ from H5N1 viruses in wild birds by a 19-amino acid deletion in the NA stalk and the presence of a carbohydrate at HA globular head [22]. The length of the NA stalk affects the host range of influenza A virus [23]. Both 2838N and 2383V strains showed a 12-amino acid deletion. This deletion may be required for the adaptation of influenza viruses from wild aquatic birds to domestic chickens and raises the possibility that chickens may be a possible intermediate host in zoonotic transmission [22].

The only different amino acid in NA between the strains was P201T. P and T are very different in structure because P is the only amino acid with a cyclic structure [17]. Many site-directed mutagenesis researchers have used this change to perform functional studies. If P is in the active site for the virus enzyme, its alteration might increase or decrease the enzyme activity, and thus virulence.

The amino acid segment positions 27 to 30 of the M2 associated with amantadine resistance [24] was VIAS. The amino acid change from I to T or S at position 27 and from A to T or S at position 30 of M2 are associated with amantadine resistance. The position 30 in M2 of both strains was S. This means that these strains might be resistant to amantadine.

The position 92 of NS1 protein from both strains was D, which was sensitive to cytokine and less virulent than the highly pathogenic H5N1 [25].

Predicting from the HA, NA and M2 sequences, these viruses might have human infection potential and be resistant to amandatine. Although no reassorted viruses were available for elucidating the association of these gene sequence differences and virulence, information on amino acid changes in different proteins from non-virulent to virulent virus may be useful for future studies.

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References

1. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005;79:2814-22.
2. Klenk HD, Rott R, Orlich M, Blodorn J. Activation of influenza A viruses by trypsin treatment. Virology 1975;68:426-39.
3. Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, et al. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol 1999;73:3184-9.
4. Webster RG, Kawaoka Y, Bean WJ Jr. Molecular changes in A/Chicken/Pennsylvania/83 (H5N2) influenza virus associated with acquisition of virulence. Virology 1986;149:165-73.
5. Wang CW, Wang CH. Experimental selection of virus derivatives with variations in virulence from a single low-pathogenicity H6N1 avian influenza virus field isolate. Avian Dis 2003;47:1416-22.
6. Brown EG, Bailly JE. Genetic analysis of mouse-adapted influenza A virus identifies roles for the NA, PB1, and PB2 genes in virulence. Virus Res 1999;61:63-76.
7. Subbarao EK, London W, Murphy BR. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 1993;67:1761-4.
8. Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 2004;320:258-66.
9. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, et al. Avian influenza A virus

(H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci USA 2004;101:1356-61.
10. Puthavathana P, Auewarakul P, Charoenying PC, Sangsiriwut K, Pooruk P, Boonnak K, et al. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J Gen Virol 2005;86:423-33.
11. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 2001;146:2275-89.
12. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, et al. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 2001;7:1306-12.
13. Macken C, Lu H, Goodman J, Boykin L. In: Osterhaus ADME, Cox N, Hampson AW, eds. Options for the control of influenza. 4th ed. Amsterdam: Elsevier Science; 2001:103-6.
14. Suarez DL, Perdue ML, Cox N, Rowe T, Bender C, Huang J, et al. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol 1998;72:6678-88.
15. Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, et al. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol 2000; 74:8502-12.
16. Harvey R, Martin AC, Zambon M, Barclay WS. Restrictions to the adaptation of influenza A virus H5 hemagglutinin to the human host. J Virol 2004;78:502-7.
17. Branden C, Tooze J, eds. Introduction to protein structure. New York: Garland Publishing Inc; 2000:347-72.
18. Hulse DJ, Webster RG, Russell RJ, Perez DR. Molecular determinants within the surface proteins involved in the pathogenicity of H5N1 influenza viruses in chickens. J Virol 2004;78:9954-64.
19. Voeten JT, Bestebroer TM, Nieuwkoop NJ, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J Virol 2000;74:6800-7.
20. Saito T, Kawano K. Loss of glycosylation at Asn144 alters the substrate preference of the N8 influenza A virus neuraminidase. J Vet Med Sci 1997;59:923-6.
21. Goto H, Kawaoka Y. A novel mechanism for the acquisition of virulence by a human influenza A virus. A novel mechanism for the acquisition of virulence by a human influenza A virus. Proc Natl Acad Sci USA 1998;95:10224-8.
22. Matrosovich M, Zhou N, Kawaoka Y, Webster R. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol 1999;73:1146-55.
23. Castrucci MR, Kawaoka Y. Biologic importance of neuraminidase stalk length in influenza A virus. J Virol 1993; 67:759-64.
24. Bean WJ, Threlkeld SC, Webster RG. Biologic potential of amantadine-resistant influenza A virus in an avian model. J Infect Dis 1989;159:1050-6.
25. Seo SH, Hoffmann E, Webster RG. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 2002;8:950-4.