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RESEARCH ARTICLE

Origin and molecular characterization of the human-infecting H6N1 influenza virus in Taiwan

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ABSTRACT

In June 2013, the first human H6N1 influenza virus infection was confirmed in Taiwan. However, the origin and molecular characterization of this virus, A/Taiwan/2/2013 (H6N1), have not been well studied thus far. In the present report, we performed phylogenetic and coalescent analyses of this virus and compared its molecular profile/characteristics with other closely related strains. Molecular characterization of H6N1 revealed that it is a typical avian influenza virus of low pathogenicity, which might not replicate and propagate well in the upper airway in mammals. Phylogenetic analysis revealed that the virus clusters with A/chicken/Taiwan/A2837/2013 (H6N1) in seven genes, except PB1. For the PB1 gene, A/Taiwan/2/2013 was clustered with a different H6N1 lineage from A/chicken/Taiwan/A2837/2013. Although a previous study demonstrated that the PB2, PA, and M genes of A/Taiwan/2/2013 might be derived from the H5N2 viruses, coalescent analyses revealed that these H5N2 viruses were derived from more recent strains than that of the ancestor of A/Taiwan/2/2013. Therefore, we propose that A/Taiwan/2/2013 is a reassortant from different H6N1 lineages circulating in chickens in Taiwan. Furthermore, compared to avian isolates, a single P186L (H3 numbering) substitution in the hemagglutinin H6 of the human isolate might increase the mammalian receptor binding and, hence, this strain's pathogenicity in humans. Overall, human infection with this virus seems an accidental event and is unlikely to cause an influenza pandemic. However, its co-circulation and potential reassortment with other influenza subtypes are still worthy of attention.

KEYWORDS molecular characterization, phylogenetic analysis, coalescent analysis, H6N1, influenza virus, Taiwan

INTRODUCTION

On June 21, 2013, the Taiwan Centers for Disease Control formally confirmed the first human infection with an avian influenza A (H6N1) virus, and named it A/Taiwan/2/2013 (http://www.cdc.gov.tw/english/infoaspx?treeid=bc2d4e89b154059b&nowtreeid=ee0a2987cfba3222&tid=E36A5E9AB3D3A216). The patient was a 20-year-old female, diagnosed with pneumonia due to an unconfirmed type of influenza virus, which was subsequently proven to be of the H6N1 subtype. The patient has fully recovered, and no human-to-human transmission has been identified.

Avian influenza A (H6N1) virus has been circulating throughout North America (Senne, 2003) and Eurasia (Lee et al., 2006) for many years. In Taiwan, the H6N1 virus has been circulating for over 30 years and is extremely prevalent among poultry, with approximately half of the layers and 30% of the broilers having antibodies against H6N1 influenza viruses (Lee et al., 2006). In southern China, H6N1 has established itself in minor poultry species (Cheung et al., 2007) and has been proposed to be the potential progenitor of the human-infecting H5N1 influenza virus A/Hong Kong/156/97 (Hoffmann et al., 2000). Although human infection with this virus subtype was never reported prior to the Taiwanese case, H6-specific

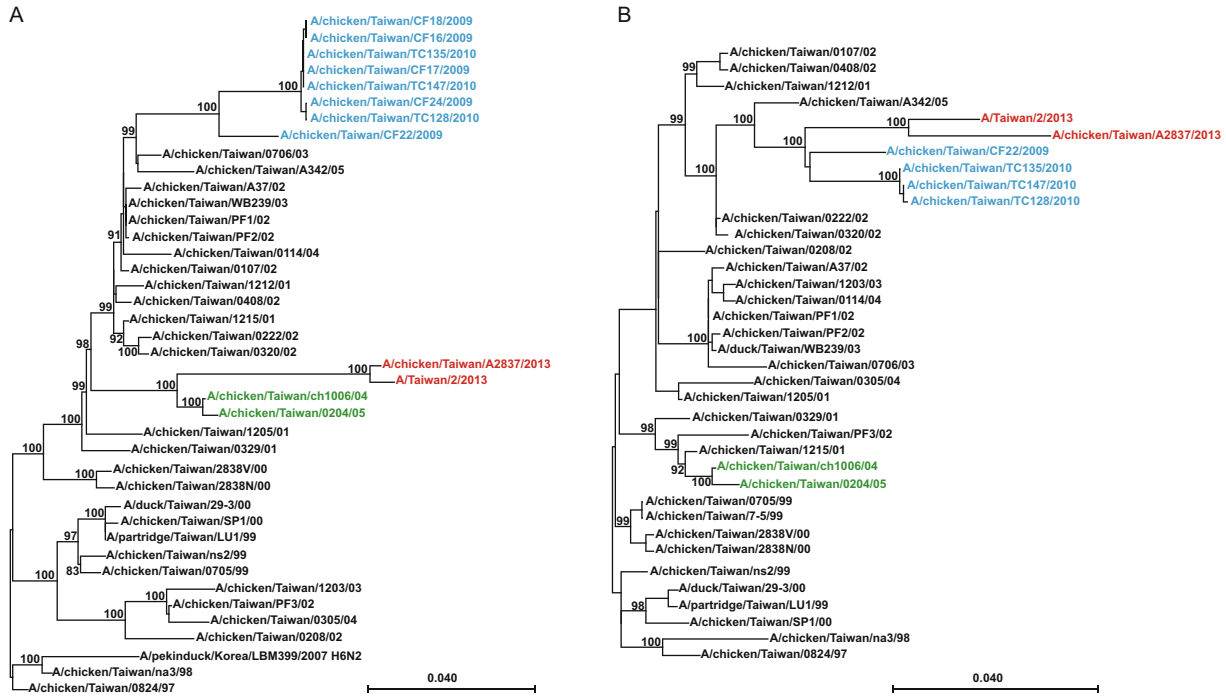

Figure 1. Maximum likelihood phylogenetic trees of HA and NA. The human-infecting H6N1 and chicken isolates from 2013 are in red, one of the 2004/05 poultry H6N1 clade viruses is in green, and the 2009/10 poultry H6N1 viruses are in cyan. (A) HA phylogenetic tree. (B) NA phylogenetic tree.

antibodies have been detected in live animal market workers in China and in veterinarians exposed to birds in the United States (Shortridge 1992; Myers et al., 2007). Furthermore, two of 11 healthy human volunteers inoculated with the H6N1 virus displayed mild upper respiratory symptoms, suggesting that this virus has the potential to infect mammals (Beare and Webster, 1991). In addition, some Taiwanese H6N1 viruses can replicate in mice without pre-adaptation (Lee et al., 2006). Therefore, clinically asymptomatic or unreported human infections of H6 viruses may have occurred.

In the present report, we performed a molecular and phylogenetic analysis of the first laboratory-confirmed human-infecting H6N1 influenza virus and closely related virus strains. Our results revealed that A/Taiwan/2/2013 is a low-pathogenicity H6N1 influenza virus whose genes may have been derived from different H6N1 lineages circulating in Taiwan.

RESULTS

The human-infecting H6N1 virus is a reassortant of different lineages of poultry H6N1 viruses circulating in Taiwan

To examine the origin of the human-infecting A/Taiwan/2/2013 H6N1 virus, we performed a phylogenetic analysis of related influenza virus genomes. Except for the PB1 gene, the phylogenetic trees of the remaining genes indicated that A/chicken/Taiwan/A2837/2013 (H6N1) is closely related to A/Taiwan/2/2013 (Figs. 1 and 2). This suggests that the H6N1 virus in poultry is the source of the human virus, though changes have occurred. From the HA phylogeny, the 2013 viruses fell into the clade of the poultry H6N1 viruses isolated in 2004 and 2005 in Taiwan (Fig. 1A). Nevertheless, the branches between the 2013 viruses and 2004/2005 viruses were quite long, indicating the lack of surveillance data. In the NA phylogenetic tree, the 2013 isolates fell into another poultry H6N1 lineage, which consisted of isolates from 2009 to 2010 in Taiwan (Fig. 1B). This lineage is distant to that of the 2004/2005 isolates in the NA tree, indicating that the 2013 H6N1 influenza viruses were possibly reassortants of varied H6N1 lineages.

A comparison of the phylogenetic trees of the internal genes showed that all but the PA gene could be grouped with the 2009/2010 isolates and close to A/Taiwan/2/2013. The closest PA gene to the 2013 isolates was that from A/chicken/Taiwan/0101/2012 (H5N2) (Fig. 2). Then, the clade clustered with earlier H6N1 strains in Taiwan.

Co-circulating H5N2 influenza viruses reassorted with H6N1 viruses in poultry

Consistent with recently published data (Yuan et al., 2013), in the PB2, PA, and M trees, the closest strains of A/Taiwan/2/2013 and A/chicken/Taiwan/A2837/2013 belonged to the H5N2 subtype rather than H6N1 (Figs. 2 and 3), with the closest strains being A/chicken/Taiwan/0101/2012 (H5N2) for PB2 and PA, and A/chicken/Taiwan/A1997/2012 (H5N2) for M.

Figure 2. Phylogenetic trees of the six internal genes. The trees were generated by the maximum likelihood methods with 1000× bootstrap analyses. The Taiwan 2013 H6N1 isolates are in red, while the 2009/2010 H6N1 isolates are in cyan. The subtypes other than H6N1 are noted.

Table 1. tMRCAs of the two H6N1 strains isolated in 2013 in Taiwan and their closely related H5N2 viruses

Gene	Sequences[a]	Models			Burn-in[b]
		Constant size	Logistic growth	Exponential growth	
PB2	a + b	Aug, 2010 (Dec, 2008, Dec, 2011)	Oct, 2009 (Feb, 2008, Jul, 2011)	May, 2010 (Oct, 2008, Dec, 2011)	4 million
	a + b + c	May, 2008 (Apr, 2006, Apr, 2010)	Jul, 2007 (Jul, 2005, Jul, 2009)	Dec, 2007 (Feb, 2006, Apr, 2010)	4 million
PA	a + b	Jan, 2011 (Dec, 2009, Dec, 2011)	Dec, 2010 (Oct, 2009, Nov, 2011)	Dec, 2010 (Jan, 2010, Dec, 2011)	2 million
	a + b + c	Jun, 2007 (Sep, 2005, Mar, 2009)	Apr, 2007 (Jul, 2005, Feb, 2009)	May, 2007 (Aug, 2005, Feb, 2009)	2 million
M	a + b	Oct, 2010 (Sep, 2008, Sep, 2012)	Aug, 2010 (Apr, 2008, Sep, 2012)	Sep, 2010 (Jun, 2008, Aug, 2012)	4 million
	a + b + d	Jun, 2008 (Jan, 2006, Feb, 2011)	Mar, 2008 (Jun, 2005, Mar, 2011)	June, 2008 (Sep, 2005, Jan, 2011)	4 million

[a] In this column, "a", "b", "c", and "d" represent A/Taiwan/2/2013 (H6N1), A/chicken/Taiwan/A2837/2013 (H6N1), A/chicken/Taiwan/0101/2012 (H5N2), and A/chicken/Taiwan/A1997/2012 (H5N2), respectively.

[b] Bayesian Markovchain Monte Carlo analysis was run for 40 million steps, but different steps were removed as burn-in.

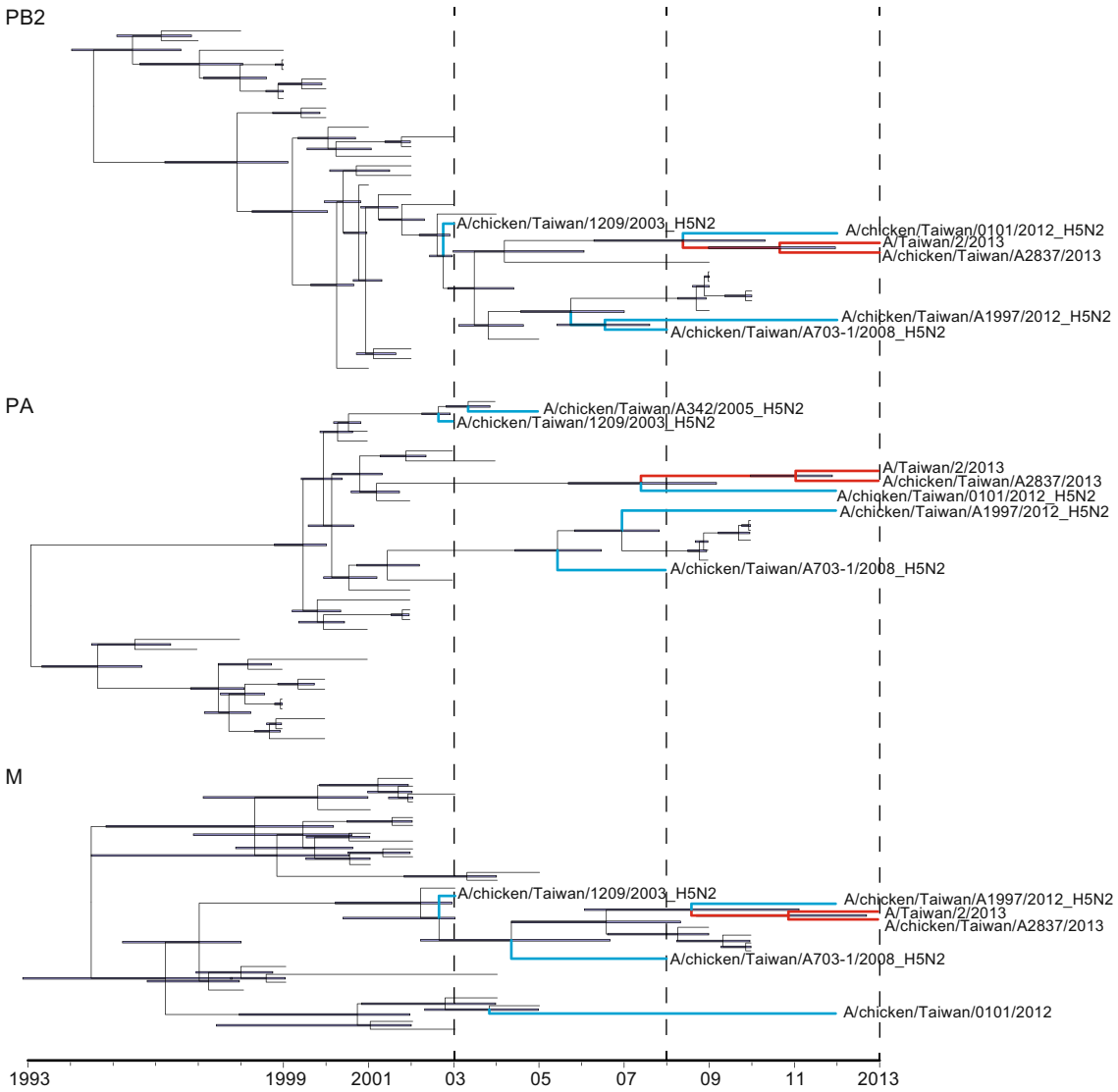

Figure 3. BEAST trees of the PB2, PA, and M genes of the human-infecting H6N1 and related viruses. The isolates from 2013 are in red. The H5 subtype viruses are in cyan. The bars represent the 95% highest posterior density of the estimation of the dates.

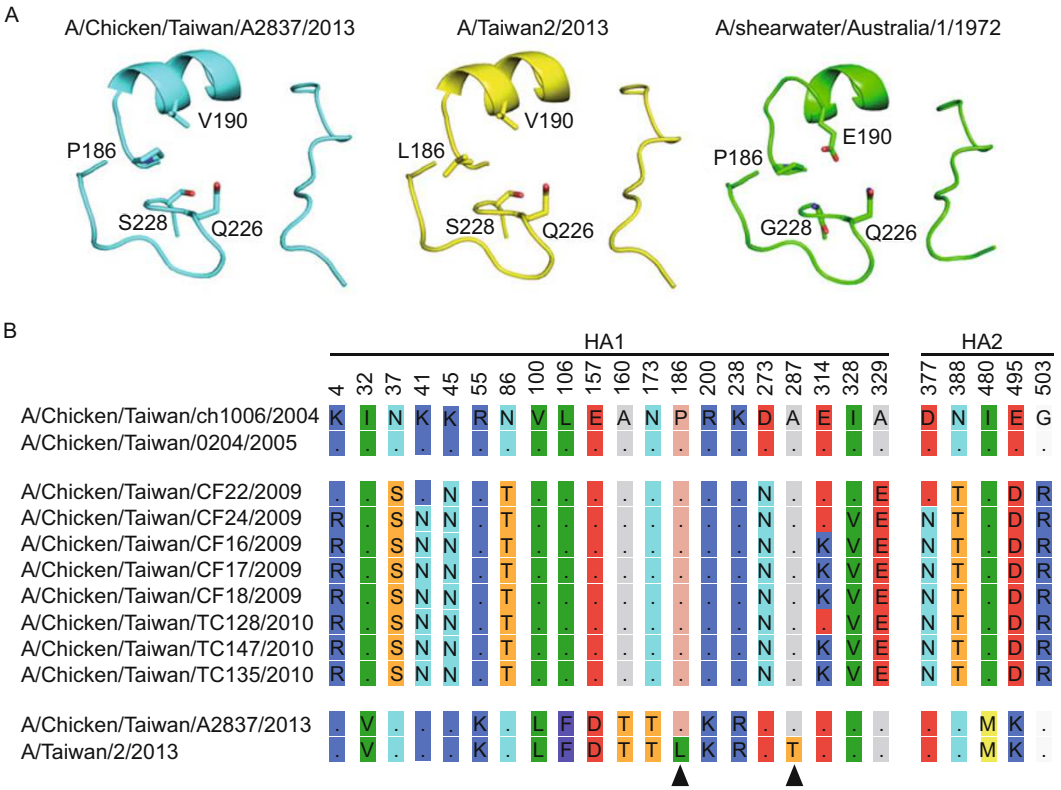

Figure 4. Structural modeling of the receptor binding site and sequence alignment of the different sites of the HA gene. (A) Receptor binding sites of two isolates from 2013 and the ancestral virus A/shearwater/Australia/1972. (B) Amino acid sites that differ from the 2004/2005 H6N1 viruses or 2009/2010 H6N1 viruses. The arrowheads point to the position in A/Taiwan/2/2013 that differs from all other strains.

However, the H5N2 viruses did not form independent lineages in the trees (Fig. 3). Instead, they scattered within H6N1 lineages that have been circulating in Taiwan for over 10 years. Moreover, the branch length between the H6N1 Taiwanese strains from 2013 and their H5N2 relatives was very long.

To further test whether the PB2, PA, and M genes of A/Taiwan/2/2013 and A/chicken/Taiwan/A2837/2013 originated from H5N2 strains, we performed a coalescent analysis and calculated the estimated time to most recent common ancestor (tMRCA) for these viruses. The tMRCA of the two H6N1 strains was estimated as early 2011 for the PA gene and before 2011 for the PB2 and M genes (Table 1), whereas the two H5N2 viruses were isolated in 2012, suggesting that they are more recent strains than the ancestor of the novel human-infecting H6N1 virus. Furthermore, the tMRCA of the two H6N1 strains and the corresponding H5N2 strains was roughly 2008 for the PB2 and M genes, and 2007 for the PA gene (Table 1), also suggesting that recent reassortment from H5N2 to H6N1 is unlikely.

The proline to leucine substitution at the receptor-binding site tends to increase the affinity to bind mammalian receptors

To investigate the molecular basis for human infectivity and pathogenicity with Taiwanese H6N1, we compared the receptor-binding sites of the 2013 isolates with an earlier avian strain A/shearwater/Australia/1/1972 (Fig. 4A). At the receptor-binding site, the human isolate had a proline to leucine substitution at position 186 (P186L, H3 numbering), which was not observed in the earlier strains or the recent circulating H6N1 lineages. Because of the stronger hydrophobicity of leucine, this substitution is hypothesized to increase the hydrophobicity at the receptor-binding site and thus make HA more likely to bind the mammalian receptor (Shi et al. 2013, Xiong et al. 2013). This may explain how this H6N1 virus was able to infect humans even though position 226 is a glutamine. The mechanism of the receptor binding switch for the H6N1 virus may be different from that for the H5N1 virus (Zhang et al. 2013). Moreover, when compared to the receptor-binding site of A/shearwater/Australia/1/1972, the 2013 isolates also contained an E190V substitution, which could also increase the hydrophobicity at the receptor-binding site. These data indicate that the 2013 isolates have evolved from an earlier avian isolate to adapt to human infection.

Aside from the P186L substitution in HA, there is only one difference at position 287 that A/Taiwan/2/2013 encodes relative to the poultry strains (Fig. 4B). However, whether the A287T substitution increases the binding to mammalian recep-

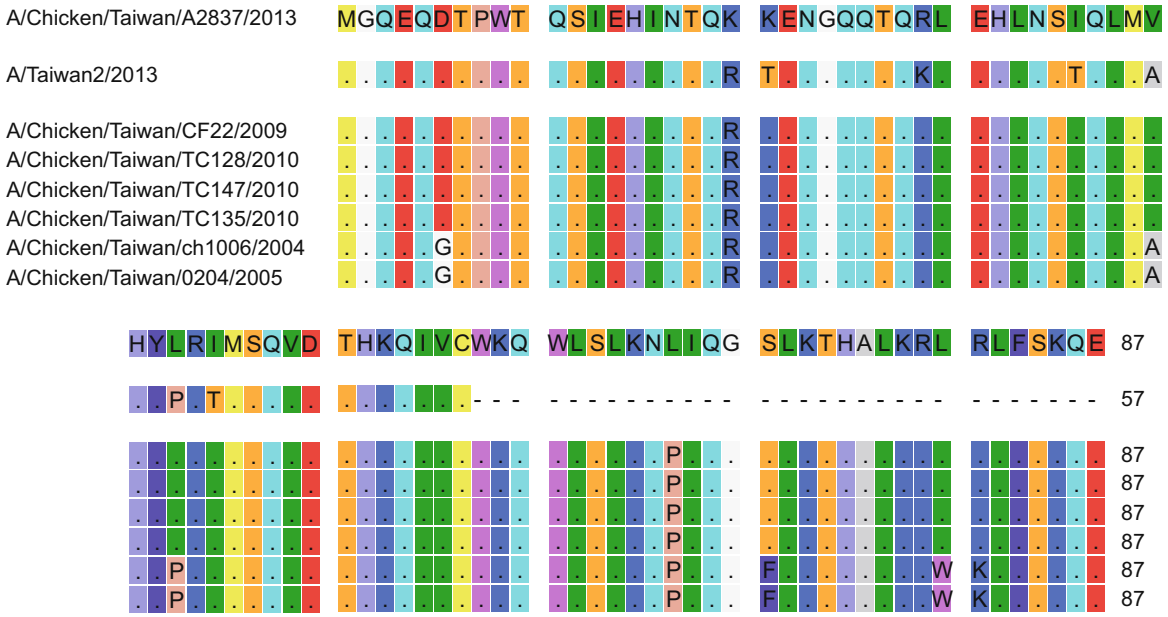

Figure 5. Sequence alignment demonstrating the truncated PB1-F2 of the A/Taiwan/2/2013 virus.

tors needs further investigation. When we compared the 2013 isolates with the relevant 2004/2005 strains and the recent 2009/10 strains, seven sites in HA1 and two sites in HA2 were different. Within these sites, position 55 is located near the antigenic sites C and E. Positions 173 and 238 are located at the antigenic site D, and positions 100, 106, and 200 are also near to it. Positions 157 and 160 are components of the antigenic site B, and the A160T substitution may have added a potential N-linked glycosylation site (NNA to NNT). All of these mutations indicate that the 2013 H6N1 viruses have greatly evolved at the antigenic sites and, thus, may have changed their antigenic and/or receptor-binding properties.

Molecular characterization of the novel human-infecting H6N1 influenza virus

The HA cleavage site of the 2013 isolates is the same as that of the 2004/2005 isolates in Taiwan, IATR (see positions 328 and 329 in Fig. 4B), whereas most of the H6N1 strains in recent years possess either VETR or IETR. The absence of multiple basic amino acids at the cleavage site suggests that the novel H6N1 strains have low pathogenicity in chickens and other avian species (Hatta et al., 2001).

In NA, two deletions, a 12-amino-acid-deletion (from position 42 to 53) and a 2-amino-acid-deletion (aa 68 and 69), in the stalk were observed. These two deletions were also found in a few H6N1 strains circulating from 2001 to 2010. This shortened NA stalk domain is a hallmark of aquatic bird viruses that become adapted to terrestrial poultry (Matrosovich et al., 1999). Another mammalian signature substitution, PB2 E627K, and the associated D701N were not observed in A/Taiwan/2/2013, suggesting that the strain is less likely to replicate well in the human upper airway and transmit well in mammalian hosts.

The R292K substitution in the NA protein has previously been reported to confer resistance to oseltamivir (Kiso et al., 2011). The strain A/Taiwan/2/2013 has an Arg at position 301 (equivalent to 292 in N2 numbering), suggesting that the virus is sensitive to oseltamivir. However, the S31N substitution in the M2 protein, which is associated with adamantine resistance (Pinto et al., 1992; Holsinger et al., 1994), was found in the two 2013 strains. In fact, most of the H6N1 strains circulating in Taiwan had this mutation.

The human-infecting H6N1 influenza virus has a truncated PB1-F2 gene

One interesting feature of the A/Taiwan/2/2013 virus strain is the presence of a truncated PB1-F2 gene (Fig. 5). The G to A substitution at position 267 (from start codon ATG) changed the codon TGG to a stop codon TAG, and thus generates a 57-amino-acid PB1-F2 protein. Compared to the full-length PB1-F2, this protein is unlikely to be functional. As a pro-apoptotic factor, PB1-F2 plays an important role in viral pathogenicity both *in vitro* and *in vivo* (Chen et al., 2001; Zamarinet et al., 2006). In addition, PB1-F2 increases susceptibility to secondary bacterial pneumonia in mice (McAuley et al., 2007). The truncation in the human H6N1 isolate might lead to the loss of its mitochondrial targeting sequence (Zell et al., 2007) and, therefore, decrease pathogenicity and result in a more localized infection (Meunier and von Messling, 2012). The detailed function of the PB1-F2 truncation in H6N1 remains unclear, and needs further examinations.

DISCUSSION

Molecular characterization of A/Taiwan/2/2013 revealed sev-

eral key features that could influence the pathogenesis of the virus, including the presence of the avian-signature Gln226 in the HA receptor-binding site, the lack of multiple basic amino acids at the cleavage site, two deletions in the NA stalk, and Glu627 and Asp701 in PB2 (avian-signatures). Therefore, A/Taiwan/2/2013 tends to be of low pathogenicity, prefers to infect avian hosts, and has adapted well within terrestrial poultry. The Arg292 in NA and Ser31 in M2 indicate that the virus is sensitive to oseltamivir but insensitive to adamantine. The molecular features of the novel H6N1 virus suggest that it is not able to replicate well in human cells and cannot be efficiently transmitted within mammalian hosts (Glu627 and Asp701 in PB2). Nevertheless, the P186L substitution has provided an increased hydrophobic environment in the sialic acid receptor-binding pocket of HA, and this change possibly enhances its ability to bind to the human receptor. Hence, the receptor binding affinity of the novel human-infecting H6N1 virus requires further examination in the future. In light of this molecular characterization it is reasonable to assume that the single human infection with this virus in Taiwan was most likely an unfortunate rare event.

A recent publication proposes that A/Taiwan/2/2013 (H6N1) originated from a reassortment between H5N2 (PB2, PA, and M) and H6N1 subtypes based on phylogenetic analyses (Yuan et al., 2013). However, the formation of the novel H6N1 influenza viruses from H5N2 viruses was not fully supported by our phylogenetic and coalescent analyses. First, based on surveillance data, the H5N2 viruses did not form independent lineages in the PB2, PA, and M trees. On the contrary, they fell within an H6N1 lineage circulating in Taiwan for approximately 10 years, indicating that the H5N2 viruses gained gene segments from H6N1 viruses at an earlier time. Second, the long branch between the H6N1 Taiwanese strains from 2013 and the H5N2 relative suggests that unknown evolutionary events may have occurred between the H5N2 to H6N1 viruses, and therefore, direct reassortment is unlikely to have occurred. Finally, coalescent analysis showed that the H5N2 viruses (isolated from 2012) were more recent strains than the ancestor of the novel human-infecting H6N1 virus. This indicates that the ancestral strain transferred the gene to both H5N2 and H6N1 viruses, and from the view of the phylogenetic trees, the ancestor strain is more likely to be an H6 than an H5 virus. Therefore, we hypothesize that A/Taiwan/2/2013 is likely a reassortant from different H6N1 lineages.

Although the emergence of human-infecting H6N1 viruses in Taiwan is unlikely to be due to direct reassortment from H5N2 viruses, the co-circulation of H6N1 and H5N2 viruses in Taiwan still requires close surveillance. Analysis of phylogenetic trees indicates that distinct H6-H5 reassortment events have occurred during the past 10 years. The reassortment of varied subtypes of influenza viruses has the potential to cause rapid adaptation and lead to an unexpected evolutionary road (Liu et al., 2013; Morens et al., 2013). Therefore, extensive surveillance is needed in the future.

MATERIALS AND METHODS

The genome sequences of A/Taiwan/2/2013 (H6N1) and A/chicken/Taiwan/A2837/2013 (H6N1) were obtained from the Global Initiative on Sharing Avian Influenza Data (GISAID) database, and closely related sequences were downloaded from BLAST searches against GenBank. Multiple sequence alignment for each gene was performed using MUSCLE (Edgar, 2004). Phylogenetic trees were reconstructed using RAxML (Stamatakis, 2006) using the GTRGAMMA model.

The tMRCAs were estimated using the Bayesian Markov chain Monte Carlo method, implemented in BEAST (Drummond and Rambaut, 2007). To investigate the extent to which dating estimates are affected by the demographic model chosen, we repeated our analyses using constant size, logistic growth, and exponential growth models, respectively. Bayesian Markov chain Monte Carlo analyses were run for 40 million steps, but different steps were removed as burn-in. Trees and other parameters were sampled every 10,000 steps.

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COMPLIANCE WITH ETHICS GUIDELINES

Weifeng Shi, Yi Shi, Ying Wu, Di Liu, George F. Gao declare that there is no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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