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Mutations in matrix protein 1 and nucleoprotein caused human-specific defects in nuclear exportation and viral assembly of an avian influenza H7N1 virus

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ABSTRACT

Nuclear exportation of influenza ribonucleoprotein is a vital step in viral replication cycle. In this study a particular H7N1 (A/ostrich/Zimbabwe/222-E3/1996) virus showed exclusively nuclear localization of the viral nucleoprotein (NP) only in human cell lines but not in cell lines of other species suggesting a human-specific nuclear exportation defect. After 10 passages in human lung cells, an adapted strain (H7N1:P10) could efficiently replicate and export viral NP in human cells. Mutations in the NP and matrix M1 gene at position 297 and 227, respectively, were found to rescue the defect. While the NP mutant showed a comparable ratio of total to NP-associated negative-sense RNA in the cytoplasm as compared to the wild type, the M1 mutant showed an increase in free negative-sense RNA in the cytoplasm. These indicated that the NP mutation might cause a nuclear export defect, whereas the M1 mutation might cause a defect in ribonucleoprotein assembly step.

1. Introduction

Aquatic birds are the natural host of influenza A viruses (Webster et al., 1992; Yoon et al., 2014). The viruses can mutate or reassort with other strains to become transmissible to other animal species including human. However, replication and transmission of avian viruses in humans is not efficient even for the highly pathogenic H5N1 avian influenza virus (Beare and Webster, 1991; Murphy et al., 1982). This indicates that there are some barriers to prevent interspecies transmission. The first barrier as the primary determinant of host range is the viral receptors, sialic acids, present on the host cell membrane. Human influenza viruses preferentially bind N-acetylneuraminic acid-α,2,6-galactose (NeuAcα2,6-Gal) on sialyloligasaccharide expressed on the epithelial cells of human upper airway and tracheobronchial tree, while most avian influenza viruses preferentially recognize NeuAcα2,3-Gal mainly expressed in duck intestine and human alveoli (Rogers and Paulson, 1983; Shinya and Kawaoka, 2006). Although majority of RNA viruses replicate their genome in host cytoplasm, influenza viruses exploit host cell machinery to replicate and transcribe their genome in the nuclei of infected cells (Deusberg and Robinson, 1967; Engelhardt et al., 2005; Plotch et al., 1978; Samji, 2009). In addition to the receptor-restricted species troprism, previous studies indicated the requirement of specific importin-α isoforms during influenza virus adaptation to human host. Importin-α is a cellular factor that regulates transportation of the viral replicase/transcriptase complex into host cell nucleus. Intriguingly, avian viruses required importin-α3 for efficient viral replication in human lung cells whereas mammalian viruses preferred importin-α7 isoform. Moreover, a reduction of viral load and restriction of virus in lung were found in importin-α7-knockout mice infected with human isolated influenza viruses (Gabriel et al., 2011).

Each segment of influenza A (vRNA) is associated with multiple copies of nucleoproteins (NP) and three subunits of RNA-dependent RNA polymerase (PB1, PB2 and PA) in order to function as viral ribonucleoproteins (vRNPs) complex (Compans et al., 1972). The newly synthesized vRNPs are subjected to transport from host cell nucleus to cytoplasm for packaging into new progeny virions in order to complete the replication cycle. Nuclear export complex of influenza vRNPs consists of nuclear export proteins (NEP), matrix proteins 1 (M1) and NP of

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Table 1
Low pathogenic avian influenza viruses used in this study.

No.	Virus name
1.	A/aquatic bird/Hong Kong/DI25/2002 (H1N1)
2.	A/wild Duck/Shan Tou/992/2000 (H2N8)
3.	A/duck/Shan Tou/1283/2001 (H3N8)
4.	A/duck/Shan Tou/461/2000 (H4N9)
5.	A/heron/Hong Kong/LC10/2002 (H6N8)
6.	A/ostrich/Zimbabwe/222-E3/1996 (H7N1)
7.	A/mallard/Alberta/242/2003 (H8N4)
8.	A/chicken/Hong Kong/G9/97 (H9N2)
9.	A/duck/Shan Tou/1796/2001 (H10N8)
10.	A/duck/Shan Tou/1411/2000 (H11N2)
11.	A/red-necked stint/Australia/5745/81 (H12N9)
12.	A/gull/MD/704/77 (H13N6)
13.	A/mallard/Gurjev/263/83 (H14N5)
14.	A/duck/Australia/341/83 (H15N8)
15.	A/shorebird/DE/172/2006 (H16N3)

viral RNP complexes. Interestingly, several mutations in the viral nuclear export complex components were founded to be responsible for influenza virus adaptation to a new host species (Hoffmann et al., 2000; McCullers et al., 2005; Finkelstein et al., 2007; Ping et al., 2011; Manz et al., 2012; Ince et al., 2013). Here we report a human-specific defect in the nuclear exportation of RNP of an avian influenza virus, which may play a role in interspecies barrier.

2. Materials and methods

2.1. Avian influenza virus

The avian influenza viruses used in this study were kindly provided by Prof. Robert G. Webster from St. Jude Children Research Hospital, USA through Prof. Pilaipan Puthavathana, Siriraj Influenza Cooperative Research Center (Siriraj ICRC), Department of Microbiolgy, Faculty of Medicine Siriraj Hospital. A list of the viruses used in this study is shown in Table 1.

2.2. Cell culture

Primary chicken embryo fibroblasts (CEF), chicken fibroblast (DF-1), human lung carcinoma (A549) and human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL®, USA). Mardin-Darby canine kidney (MDCK) and African green monkey kidney (Vero) cell lines were grown in Eagle's Minimum Essential Medium (EMEM) (Gibco BRL®, USA). Human pharyngeal carcinoma epithelial (Detroit 562) cell line was cultured in EMEM (Gibco BRL®, USA) with 1 x non-essential amino acid (Gibco BRL®, USA). All media used in this study were supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL®, USA) in the presence of penicillin, gentamicin and fungizone. All cell lines were cultured at 37 °C with 5% CO_2 and subpassaged twice a week.

2.3. Immunofluorescence assay

To detect the nuclear transport pattern of the virus, the cells of interested were plated with 10^5 cells on a sterile cover slip inside a 24-well plate. At desired time points after infection, the infected cells were washed with 1xPBS and fixed with 200 µl of 4% paraformaldehyde at room temperature for 20 min. Then the cover slip was gently pinched from the plate and the cell side was flipped upside down in order to incubate with 15 µl of 0.5% Tritox X-100 (Sigma-Aldrich, USA) on a parafilm. After 15 min incubation, the cover slip was washed in 1xPBS and incubated with 10 µl of mouse monoclonal antibody specific to influenza viral NP (Millipore™, USA). This primary antibody was diluted in blocking buffer (0.1% Tween, 1% BSA) at a dilution of 1:100. After 1 h incubation in humidified chamber, the cover slip was washed in 1xPBS and incubated further with 10 µl of Alexa® Fluor 488-conjugated anti-mouse IgG antibody (Invitrogen, USA) that was diluted at 1:250. The infected cells were counterstained with nuclear staining dye Hoechst (Invitrogen, USA) and examined under a laser scanning confocal microscope (LSM 510 Meta, Zeiss, Jena, Germany).

2.4. Sequencing method

Genomes of the parental and mammalian-adapted H7N1 viruses were sequenced by a direct dye-terminator sequencing method on RT-PCR products performed by Macrogen (Korea). The nuclear export complex genes, NS, M, and NP of the parental H7N1A/ostrich/Zimbabwe/222-E3/1996 and the adapted strain H7N1A/ostrich/Zimbabwe/222-P10/1996 were initially sequenced. However, the volume of the original stocks of both viruses were not sufficient for fully sequencing of the remaining 5 genes, we therefore propagated those viruses and rechecked their phenotypes. The parental H7N1 showed similar nuclear restricted phenotype after propagated in A549 cells. It was named as A/ostrich/Zimbabwe/222-P1/1996. However, because of its low viral titre, only the NA gene could be amplified. Therefore, we propagated A/ostrich/Zimbabwe/222-P1/1996 for an additional passage in chicken fibroblast DF1 cells. The virus retained similar nuclear export defect phenotype with a high viral titre. It was named as A/ostrich/Zimbabwe/222-DF1/1996. Thus, sequences of PB2, PB1, PA and HA was derived from the DF1-propagated virus. The H7N1 passage 9 in A549 cells showed efficient nuclear transportation between cytoplasm and nuclear of infected cells. It was named as A/ostrich/Zimbabwe/222-P9/1996 and was used for sequencing analysis.

2.5. Viral RNA amplification for generation of reverse genetic virus

Total RNA was isolated from supernatant of infected cells by using high pure viral nucleic acid kit (Roche, Switzerland) according to manufacturer instructions. Individual genomic segment of influenza A virus was amplified by one step RNA PCR Kit (TAKARA, Japan) with specific universal primer as previously described (Hoffmann et al., 2001). The amplification condition was set as follows: 50 °C for 30 s and 94 °C for 2 min, followed by 2 cycles at 94 °C for 30 s; 45 °C for 30 s; and 72 °C for 3 min, then 32 cycles at 94 °C for 30 s; 55 °C for 30 s, 72 °C for 3 min; followed by 72 °C for 10 min and a 4 °C hold. Each amplified segment of the virus was cloned into pGEM®- T easy vector and subcloned into pHW2000 vector. The pHW2000 plasmid and pHW2000 plasmids harboring each gene of A/PuretoRico/8/34 (H1N1) were kindly provided by Prof. Robert G. Webster, St. Jude Children Research Hospital, USA.

2.6. DpnI-site directed mutagenesis

After subcloning of a viral segment into pHW2000 vector, *DpnI*-site directed mutagenesis was performed in order to introduce a mutation into a gene of interest. The plasmid was denatured in order to be used as a template for *pfu* amplification (Promega, USA) by mutagenesis primer. The mutagenesis primer with A227S mutation of M1 gene of H7N1 (A/ostrich/Zimbabwe/222-E3/96) was designed by covering nucleotide position 664–694 of the gene. The primer sets for NP S297F mutation of PR8 was designed by covering nucleotide position 874–904