Virologica Sinica (2019) 34:334–337
https://doi.org/10.1007/s12250-019-00110-7

www.virosin.org
www.springer.com/12250

LETTER

Identification of a Novel Universal Potential Epitope on the Cytoplasmic Tail of H7N9 Virus Hemagglutinin

Xi Liu[1] · Li Ding[1] · Jing Yuan[3] · Jian Liao[4] · Lian Duan[4] · Wenfei Wang[5] · Weiguo Tan[2] · Weiye Yu[2] · Boping Zhou[6] · Xinchun Chen[5] · Zheng Yang[2,4]

Received: 10 July 2018 / Accepted: 5 March 2019 / Published online: 23 April 2019

Dear Editor,

H7N9 is a recently identified subtype of influenza A virus that caused a major outbreak in humans in China in 2013. According to the latest data provided by the Chinese Center for Disease Control and Prevention (http://www.moh.gov.cn/zwgk/yqbb3/ejlist.shtml, updated on October 31, 2018), the mortality rate of H7N9 infections in China amounts to 39.7% (611/1536). Thus, H7N9 poses a serious public health threat.

Influenza A viruses comprise a group of antigenically diverse pathogens that cause respiratory tract infections in both animal and human populations (Thornburg *et al.* 2016). Subtypes of influenza A viruses are characterized by their surface glycoproteins, including hemagglutinin (HA) and neuraminidase (NA). HA consists of a globular head region with a receptor-binding pocket and a conserved stem motif (Liu *et al.* 2016), and mediates the binding of influenza A virus to host cells (Melikyan *et al.* 1999). After binding, HA can be divided into three domains: an extracellular domain, a transmembrane domain, and a cytoplasmic tail (CT) (Thornburg *et al.* 2016). More than a dozen of neutralizing epitopes of HA have been identified to date (Shcherbinin *et al.* 2016), most of which are on the receptor-binding pocket (Whittle *et al.* 2011) and the stem motif (Dreyfus *et al.* 2012). However, H7-subtype inactivated virus is a weak inducer of neutralizing antibody compared to H1N1 and H3N2 (Lee *et al.* 2015), highlighting the necessity of developing novel strategies in vaccine design against H7N9.

This study aimed to identify a novel antigenic epitope on H7N9 virus HA_7. In total, 37 patients with H7N9 infection admitted to Shenzhen Third People's Hospital between December 2012 and March 2015 were included in this study. Plasma and peripheral blood mononuclear cells were separated from fresh blood samples and were stored at − 80 °C.

Full-length HA protein [of strain A/Shanghai/02/2013 (H7N9)] was fragmented into 110 peptides (P1–P110). Each peptide contained 15 amino acids (AAs), with a 10-AA overlap between two neighboring peptides. A library of 108 peptides covering the full-length HA_7 was synthesized except for P9 and P18. Plasma samples from 11 H7N9-infected patients were tested for reactivity with the 108 synthetic peptides by peptide microarray ELISA. As shown in Fig. 1A, the OD450 values for P15, P21, P30, P63, P76, P98, and P110 were significantly higher than those for other peptides, suggesting that the patients' plasma strongly reacted with these peptides, which may be candidate epitopes of HA_7. Among the seven candidates, P110 (AA sequence: FICVKNGNMRCTICI), the last 11 AAs of which compose the HA_7-CT, showed the strongest immunoreaction, whereas the specific affinities of serum antibodies to peptides on extracellular and transmembrane domains were lower. Therefore, the HA_7-CT peptide (KNGNMRCTICI) was further investigated in this study.

Xi Liu and Li Ding have contributed equally to this work.

✉ Zheng Yang
xiaobei02@163.com

[1] Department of Infectious Diseases, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai 51900, China

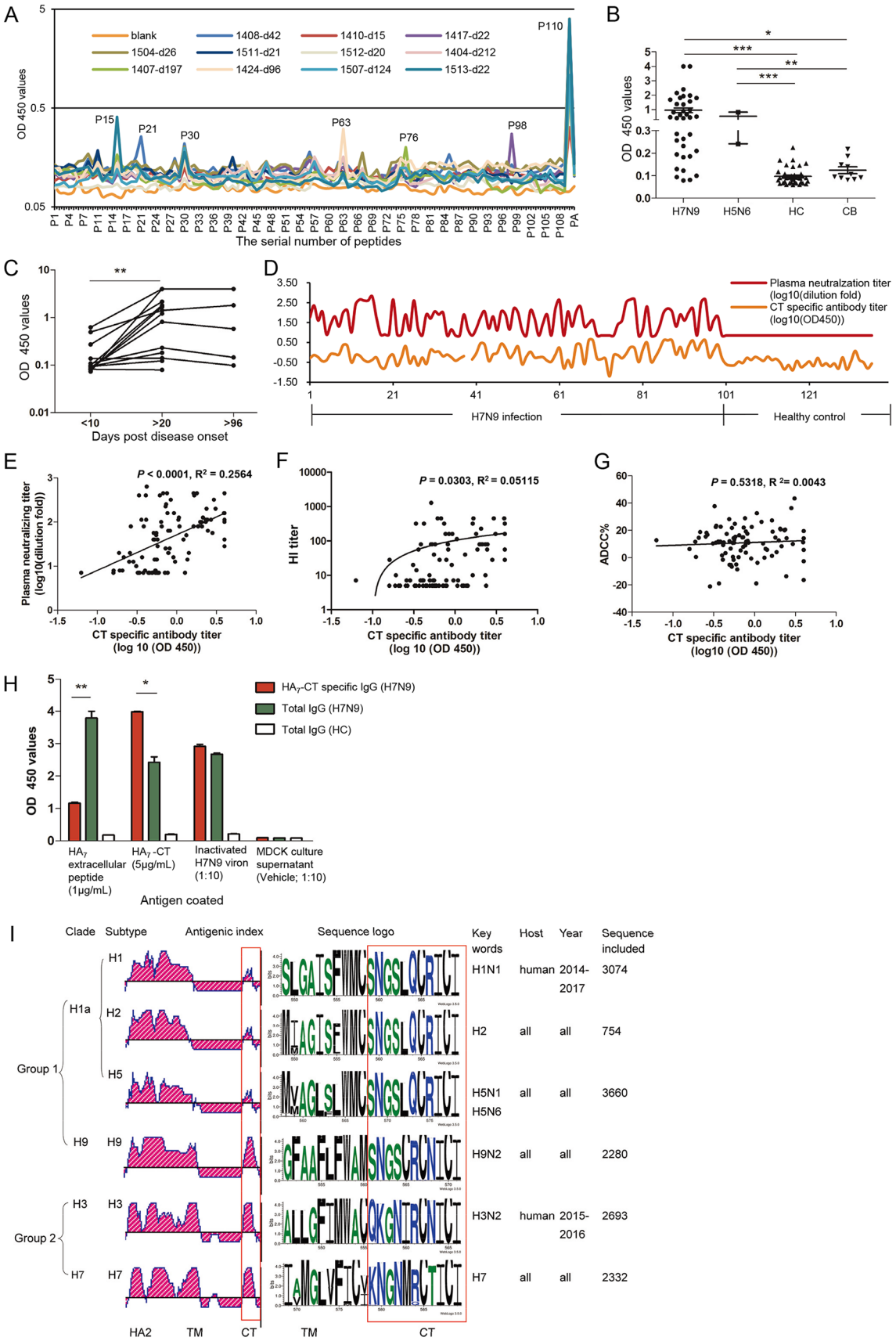
[2] Lab for Tuberculosis Research, Shenzhen Center for Chronic Disease Control, Shenzhen 518020, China

[3] Department of Infectious Diseases, The Third People's Hospital of Shenzhen (The Second Affiliated Hospital of Southern University of Science and Technology), Shenzhen 518000, China

[4] Shenzhen Key Laboratory of Infection and Immunity, The Third People's Hospital of Shenzhen (The Second Affiliated Hospital of Southern University of Science and Technology), Shenzhen 518000, China

[5] Department of Pathogen Biology, School of Medicine, Shenzhen University, Shenzhen 518000, China

[6] Longhua Branch of Shenzhen People's Hospital, Shenzhen 518000, China


A
blank
1408-d42
1410-d15
1417-d22
1504-d26
1511-d21
1512-d20
1404-d212
1407-d197
1424-d96
1507-d124
1513-d22
OD 450 values
P15
P21
P30
P63
P76
P98
P110
The serial number of peptides
B
OD 450 values
H7N9
H5N6
HC
CB
C
OD 450 values
Days post disease onset
D
Plasma neutralzation titer (log10(dilution fold)
CT specific antibody titer (log10(OD450))
H7N9 infection
Healthy control
E
P < 0.0001, R² = 0.2564
Plasma neutralizing titer (log10(dilution fold))
CT specific antibody titer (log 10 (OD 450))
F
P = 0.0303, R² = 0.05115
HI titer
CT specific antibody titer (log 10 (OD 450))
G
P = 0.5318, R ²= 0.0043
ADCC%
CT specific antibody titer (log10 (OD 450))
H
HA7-CT specific IgG (H7N9)
Total IgG (H7N9)
Total IgG (HC)
OD 450 values
HA7 extracellular peptide (1μg/mL)
HA7 -CT (5μg/mL)
Inactivated H7N9 viron (1:10)
MDCK culture supernatant (Vehicle; 1:10)
Antigen coated
I
Clade
Subtype
Antigenic index
Sequence logo
Key words
Host
Year
Sequence included
Group 1
Group 2
H1
H1a
H2
H5
H9
H3
H7
H1N1
human
2014-2017
3074
H2
all
all
754
H5N1
H5N6
all
all
3660
H9N2
all
all
2280
H3N2
human
2015-2016
2693
H7
all
all
2332
HA2
TM
CT
TM
CT

◀**Fig. 1** Immunogenicity of the cytoplasmic tail (CT) of H7N9 virus hemagglutinin (HA). **A** Full-length sequence of HA protein [of the strain A/Shanghai/02/2013 (H7N9)] was fragmented into 110 peptides (P1–P110) that were synthesized, except for P9 and P18. The plasma samples collected from H7N9-infected patients (n = 11) were labelled based on the number of patient (before the hyphen) and days post disease onset (after the hyphen). The samples (diluted at 1:100) were incubated with 5 μg/mL peptides pre-coated in the ELISA plate. OD450 values represent immunoreactivity. Peptides with $OD450_{max-} > 0.2$ were regarded epitopes of HA. **B** HA_7 CT peptide (i.e., P110) was incubated with plasma samples from H7N9- (n = 37) or H5N6-infected (n = 2) patients (collected at 21–42 days post disease onset), or healthy controls (HC; n = 40), or incubated with cord blood (CB; n = 10) samples. ELISA was conducted. **C** HA_7-CT peptide was incubated with plasma samples collected from the H7N9-infected patients at different times post disease onset. ELISA was conducted. **D** HA_7-CT antibody titer (log_{10}OD450) and neutralization titer (log_{10}dilution fold) of plasma samples from H7N9-infected patients (X axis from 1 to 99) and healthy donors (X axis from 100 to 136). Pearson correlation analysis of HA_7-CT-specific antibody titer with plasma neutralization titer (**E**), hemagglutination inhibition titer (**F**) and antibody-dependent cell-mediated cytotoxicity percentage (**G**). **H** The affinity of HA_7-CT-specific antibody pulled down from H7N9 patients' total IgG with HA_7 extracellular peptide, HA_7-CT, or purified virions was evaluated by ELISA. Total IgG of H7N9-infected patients and healthy controls (HC) were used as controls. **I** Sequence similarity and antigenic index prediction of HA-CT. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. *HA* hemagglutinin, *CT* cytoplasmic tail, *OD* optical density, *PA* the average OD450 values of all peptides to a sample, *HC* healthy control, *CB* cord blood, *TM* transmembrane domain.

The plasma samples from the 37 H7N9 patients were tested for reactivity with HA_7-CT peptide, 40 healthy plasma samples and 10 cord blood samples were used as controls. As shown in Fig. 1B, the OD values in the H7N9 group were significantly higher than those in the control groups. Plasma samples from two H5N6 patients also presented higher affinity than control samples. These findings suggested that the HA_7-CT epitope might be specifically recognized by influenza A virus-infected human plasma. Time course analysis of the immune response of plasma of different patients to the HA_7-CT epitope revealed that immune reactivity time-dependently increased from day 10 to day 20 post disease onset, peaked on day 20, and then remained constant until day 96 (Fig. 1C). Taken together, these results suggested that the HA_7-CT epitope is a potentially antigenic epitope that could be recognized by the plasma of patients infected with influenza A virus and may drive a strong immune response after the onset of virus infection.

HA-CT (Siche *et al.* 2015) plays an important role in virus replication (Imai *et al.* 2012), viral infectivity (Siche *et al.* 2015), and virus entry into host cells (Scolari *et al.* 2016). Although its functions have been well studied, little is known about the immunogenicity and sub-localization of HA-CT. Previous studies have identified a specific neutralizing antibody against the CT of human immunodeficiency virus glycoprotein 41 (Chen *et al.* 2015), which led us to hypothesize that HA-CT may function in a similar manner. To investigate the immunogenicity of HA_7-CT epitope further, we pulled down CT-specific antibody from total IgG in the plasma samples from five H7N9 patients using biotinylated HA_7-CT peptide. We analyzed the relationship between the titer of HA_7-CT-specific IgG (measured photometrically) and the total IgG titer in H7N9 patient plasma (measured by virus neutralization assay in H7N9-infected Madin–Darby canine kidney (MDCK) cells or by hemagglutination inhibition (HI) assay). As shown in Fig. 1D, the HA_7-CT-specific IgG titer showed a pattern similar to that of the plasma neutralization titer. Furthermore, the HA_7-CT-specific IgG titer was significantly positively correlated with the plasma neutralization titer ($P < 0.0001$; Fig. 1E) and the plasma HI titer ($P = 0.0303$; Fig. 1F), but not with the antibody-dependent cell-mediated cytotoxicity percentage ($P = 0.5318$; Fig. 1G). The IgG titers of the other candidate epitopes, i.e., P15, P21, P30, P63, P76, and P98 (data not shown), were not correlated with the plasma neutralization titer. These results suggested that HA_7-CT possesses an antigenic epitope that is capable of blocking virus infection by neutralization, but not of the antibody-dependent cell-mediated cytotoxic effects.

Because surface protein of the influenza virus is a major antibody target, we next sought to investigate whether HA_7-CT is displayed on the H7N9 surface and thus can be recognized by antibodies. We pulled down HA_7-CT-specific IgG from total IgG in plasma samples of H7N9 patients and tested its affinity with HA_7-CT peptide, HA_7 extracellular domain peptide, and inactivated H7N9 virion by ELISA. MDCK cell culture supernatant was used as a blank control. As shown in Fig. 1H, compared with total IgG, HA_7-CT-specific IgG showed significantly low binding affinity with the HA_7 extracellular domain peptide ($P < 0.01$), but markedly high binding affinity with the HA_7-CT peptide ($P < 0.05$), indicating that HA_7-CT-specific IgG can be specifically recognized by the HA_7-CT peptide. On the other hand, there was no difference in binding affinity for inactivated virions between HA_7-CT-specific and total IgG, suggesting that HA_7-CT-specific and total IgG can bind to whole-virus particles with similar affinity. These results suggested that HA_7-CT is displayed on the virus surface, at least occasionally.

The CT of HA consists of 11 AAs. Based on HA and CT sequences of various subtypes of influenza A virus retrieved from the influenza database (https://www.fludb.org) and weblogo3.5 (http://weblogo.threeplusone.com/), respectively, we found that the CT sequence was highly conserved, especially within virus clades. As shown in Fig. 1I, all sequences analyzed shared a CXICI motif at the

C terminus. The CT sequences of HAs in group 1 were more similar than those in group 2. Of note, H1, H2, and H5 in clade H1a had identical CT sequences. To explore whether the CTs of different influenza A subtypes commonly possess antigenicity, we analyzed the antigenic indexes of the CTs predicted by the Jameson-Wolf model. The CTs of clades H9, H3, and H7 had significantly higher antigenic index values than those of clade H1a (H1, H2, H5), consistent with our experimental results.

Currently available influenza vaccines are mainly HA- or NA-specific neutralizing antibodies (Gerhard *et al.* 2006). However, because of the high antigenic variability of HA and NA, it is difficult to produce a universal and effective vaccine to prevent epidemics (Carrat and Flahault 2007; Deng *et al.* 2015). We found more than 50% sequence similarity in the CT sequences of different HA subtypes (Fig. 1I). The highly conserved CT sequence and high antigenic index scores indicated that HA-CT may serve as a potential antigenic epitope for the development of a broad-spectrum influenza antibody.

Our study had some limitations. First, we used short linear peptides to coat the ELISA plates, and we cannot guarantee consistent coating efficiency. Second, total IgG in the plasma may recognize conformational epitopes on HA, but the linear peptide-based ELISA is incapable of detecting conformational epitope-specific antibodies. Third, we showed that HA_7-CT-specific antibody titer was positively correlated with the plasma neutralization and HI titers, but we did not present direct evidence to prove that HA_7-CT specific antibody had neutralizing ability. We hypothesize that HA_7-CT is a potential neutralizing epitope that could be utilized in universal vaccine design, but further research will be required to verify our results.

Acknowledgements This study was funded by the National Natural Science Foundation of China (#31500719 (Zheng Yang) and #81672027 (Boping Zhou)), the Science and Technology Grant of Shenzhen (#JCYJ20160427151500192 (Jing Yuan), #JCYJ20150402111430643 (Zheng Yang), and #JSGG20140701164558078 (Boping Zhou)), the Science and Technology Project of Zhuhai (#20161027E030031 (Xi Liu)) and the Medical Science and Technology Research Fund of Guangdong (#A2017037 (Xi Liu)).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement The study was approved by the ethics committees of Shenzhen Third People's Hospital. All participants provided written informed consent. Written consents were obtained from all patients or their authorizers involved in the study.

References

Carrat F, Flahault A (2007) Influenza vaccine: the challenge of antigenic drift. Vaccine 25:6852–6862

Chen J, Kovacs JM, Peng H, Rits-Volloch S, Lu J, Park D, Zablowsky E, Seaman MS, Chen B (2015) HIV-1 ENVELOPE. Effect of the cytoplasmic domain on antigenic characteristics of HIV-1 envelope glycoprotein. Science 349:191–195

Deng L, Cho KJ, Fiers W, Saelens X (2015) M2e-based universal influenza A vaccines. Vaccines (Basel) 3:105–136

Dreyfus C, Laursen NS, Kwaks T, Zuijdgeest D, Khayat R, Ekiert DC, Lee JH, Metlagel Z, Bujny MV, Jongeneelen M, van der Vlugt R, Lamrani M, Korse HJ, Geelen E, Sahin Ö, Sieuwerts M, Brakenhoff JP, Vogels R, Li OT, Poon LL, Peiris M, Koudstaal W, Ward AB, Wilson IA, Goudsmit J, Friesen RH (2012) Highly conserved protective epitopes on influenza B viruses. Science 337:1343–1348

Gerhard W, Mozdzanowska K, Zharikova D (2006) Prospects for universal influenza virus vaccine. Emerg Infect Dis 12:569–574

Imai M, Watanabe S, Kawaoka Y (2012) The cytoplasmic tail domain of influenza B virus hemagglutinin is important for its incorporation into virions but is not essential for virus replication in cell culture in the presence of compensatory mutations. J Virol 86:11633–11644

Lee AC, Zhu H, Zhang AJ, Li C, Wang P, Li C, Chen H, Hung IF, To KK, Yuen KY (2015) Suboptimal humoral immune response against influenza A(H7N9) virus is related to its internal genes. Clin Vaccine Immunol 22:1235–1243

Liu WC, Jan JT, Huang YJ, Chen TH, Wu SC (2016) Unmasking stem-specific neutralizing epitopes by abolishing N-linked glycosylation sites of influenza virus hemagglutinin proteins for vaccine design. J Virol 90:8496–8508

Melikyan GB, Lin S, Roth MG, Cohen FS (1999) Amino acid sequence requirements of the transmembrane and cytoplasmic domains of influenza virus hemagglutinin for viable membrane fusion. Mol Biol Cell 10:1821–1836

Scolari S, Imkeller K, Jolmes F, Veit M, Herrmann A, Schwarzer R (2016) Modulation of cell surface transport and lipid raft localization by the cytoplasmic tail of the influenza virus hemagglutinin. Cell Microbiol 18:125–136

Shcherbinin DN, Alekseeva SV, Shmarov MM, Smirnov YA, Naroditskiy BS, Gintsburg AL (2016) The analysis of B-cell epitopes of influenza virus hemagglutinin. Acta Nat 8:13–20

Siche S, Brett K, Moller L, Kordyukova LV, Mintaev RR, Alexeevski AV, Veit M (2015) Two cytoplasmic acylation sites and an adjacent hydrophobic residue, but no other conserved amino acids in the cytoplasmic tail of ha from influenza A virus are crucial for virus replication. Viruses 7:6458–6475

Thornburg NJ, Zhang H, Bangaru S, Sapparapu G, Kose N, Lampley RM, Bombardi RG, Yu Y, Graham S, Branchizio A, Yoder SM, Rock MT, Creech CB, Edwards KM, Lee D, Li S, Wilson IA, García-Sastre A, Albrecht RA, Crowe JE Jr (2016) H7N9 influenza virus neutralizing antibodies that possess few somatic mutations. J Clin Invest 126:1482–1494

Whittle JR, Zhang R, Khurana S, King LR, Manischewitz J, Golding H, Dormitzer PR, Haynes BF, Walter EB, Moody MA, Kepler TB, Liao HX, Harrison SC (2011) Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. Proc Natl Acad Sci USA 108:14216–14221