SOMATO PUBLICATIONS

Archives of Medical Microbiology

Research Article

Genetic and Receptor Binding Property of Seasonal Influenza A (H3N2) in Shenzhen during 2017

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Received: 24 October 2019; **Accepted:** 03 December 2019; **Published:** 05 December 2019

Citation of this article: Wu, W., Xing, X., Wang, X., Peng, B., Liu, H., Sun, Y., et al. (2019) Genetic and Receptor Binding Property of Seasonal Influenza A (H3N2) in Shenzhen during 2017. Arch Med Microbiol, 1(1): 001-006.

ABSTRACT

Influenza A (H3N2) is the most common cause of seasonal influenza, which causes acute respiratory illness in human every year. Since the summer of 2017, the number of cases with epidemic influenza-like illness increased continuously in southern areas of China with dominate infection of influenza A/H3N2 and the abrupt increase in hospitalization fatality rates caused by seasonal influenza A/H3N2 in Hong Kong Special Administrative region of China received broad media coverage worldwide. Many institutes in different regions of the world reported that the effectiveness of influenza vaccine for A/H3N2 seemed to be low. In this study, we tried to explore the phenomenon from the aspects of hemagglutinin (HA) genetic characteristics and receptor binding characteristics. Clinical samples from outpatients with influenza-like illness in monitoring hospitals in Shenzhen were collected, and conducted phylogeny analysis. Receptor binding preference of influenza viruses was tested by Enzyme-linked immunosorbent assay (ELISA). Phylogenetic analysis revealed that these influenza A (H3N2) viruses clustered into two subclades A and B. Comparative analysis with the vaccine strain (A/Hongkong/4801/2014(H3N2)), N121K and N171K mutations in epitope D are the primary molecular characteristics in group B viruses, in group A viruses, T131K and R142K in epitope are the key mutation. The HA receptor binding capability of H3N2 virus in group B showed a remarkable increase on α-2,6 receptor compared with those in group A, and a slight increase on α-2,3 receptor simultaneously. These amino acid variations in HA gene of A/H3N2 viruses isolated in Shenzhen during 2017may explain the ineffectiveness of vaccine, viral pathogenicity, and clinical features to a certain extent.

Keywords: Influenza A/H3N2, Hemagglutinin, Mutation, Epitope, Receptor binding property

Introduction

Influenza A (H3N2) is the most common cause of seasonal influenza, which causes acute respiratory illness in human every year [1]. From the summer of 2017, the number of cases with epidemic influenza-like illness increased continuously in southern areas of China with dominates infection of influenza A (H3N2). At the same time, severe and dead H3N2 infected cases were reported from Hong Kong and Taiwan [2,3]. National Center for Disease Control and Prevention reported that there was no antigenic drift for the circulating influenza A (H3N2) by serum experiment though several substitutions of amino acids were reported from studies of phylogenetic analysis [4,5]. The effectiveness of influenza vaccine for A (H3N2) seemed to be low, from 42% to 52%, evaluated and reported in many regions of the world [6-8], which may have contributed to the unusually high excess morbidity and serious epidemic in 2017.

Influenza viruses undergo frequent evolution due to genetic mutation and reassortment, resulting in the poor vaccine effectiveness and human's susceptibility. Hemagglutinin (HA), the glycoprotein

spikes covering the viral surface, is embodied with noncontiguous residuals of sialic acid binding site and is also the key region of neutralizing antibodies [9,10]. Genetic surveillance mainly focused on HA because mutation in HA is a determinant of transformation of either antigenicity or receptor binding features. As for the serious epidemic of A (H3N2), there has been no report on whether certain amino acid variants alter the HA receptor binding characteristics of H3N2 virus, which may be in corresponding with high hospitalization fatality rates.

Therefore, it's of great significance to detect the substitutions in HA and comprehend the receptor binding property of the circulating influenza A (H3N2) viruses. This present study analyzed the HA genetic characterization and evaluated the association between amino acid substitutions at epitope and the receptor binding property in Shenzhen.

Material and Methods

Sample collection and RNA extraction

Clinical samples were collected from outpatients with influenza-like illness in monitoring hospitals in Shenzhen city, Guangdong province of China in 2017. Nasal-pharyngeal or oropharyngeal swabs were collected and put in a viral-transport medium and stored in 4^0C before transferring to Shenzhen Center for Disease Control and Prevention (CDC) for detection and analysis. Collection of samples was conducted under the guidelines of the standard operation procedures (SOPs) of the China Center for Disease Control and Prevention.

RNA was extracted from samples by MagNA Pure LC2.0 (Roche, Switzerland) following the manufacturer's protocol and eluted in a volume of 100μL. H3 and N2 genes were detected by a real-time reverse transcription-polymerase chain reaction (RT-PCR) according to the SOPs of the World Health Organization (WHO) [11] in a 7500 Real Time PCR System (Applied Biosystems, California, USA).

Genetic sequencing

Haemagglutinin (HA) segments of 40 isolated positives samples selected randomly from the H3N2 RT-PCR assays were eligible for sequencing using a high-throughput sequencing strategy on an Illumina Hiseq 2500 sequencer. The detailed methods of both sequencing and data assembly were previously described [12].

Phylogenetic analysis

The sequence of reference strain, 2016-2017 northern hemisphere influenza season vaccine strain recommended by WHO were obtained from the GenBank. Sequences of HA including those from the Shenzhen influenza A (H3N2) virus strains were edited using Bioedit Sequence Alignment Editor V7.0. MEGA V6.06 and MegAlign in DNASTAR V7.1 were used to analyze the amino acid mutation as well as construct the phylogenetic tree of nucleotide sequence alignment by Neighbor-Joining method with the bootstrap analysis of 1000 replications. The amino acid residues in epitope A to E of influenza A (H3N2) was previously identified [13].

Cell culture and hemagglutination assays

Positive samples for H3N2 were propagated in Madin-Darby canine kidney (MDCK) cells and then cultured at 37 for 3 days. Hemagglutination assays were performed by adding 50μl 1% chicken red blood cells suspended in PBS to an equal volume of cultured virus prepared in V-shaped 96 well microtiter plates. The mixture in the plates was vibrated slightly to mix and then incubated at room temperature for 30 min.

Receptor binding property assay

The receptor binding preference of these viruses was tested by ELISA. The plates were coated with a mutated H3N2 virus with different interested amino acid sites in HA at 4^0C overnight. Then 5% BSA solution was used to block the ELISA plates for 1h at room temperature. Plates were washed with PBS containing 0.1% Tween 80 for two times and 50 μl stable dilution of two horseradish peroxidase (HRP)-conjugated receptor analogs (asialofetuin jointed with α-2,3 or α-2,6 sialic acid) in reaction solution were added to each well. After incubation at 4^0C for 30min, plates were washed again and 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added. 1M HCl was added to stop to TMB reaction 30min later and the absorbance value was measure by Bio-Rad iMark 14481 at 450nm. Three groups of parallel tests had been done to ensure the repeatability and the mean values were acquired for statistical analysis. **Results**

Sequencing and phylogenetic analysis

Complete HA sequences of 40 influenza A (H3N2) isolated viruses which were representative of the seasonal influenza cases during 2017 from outpatients in Shenzhen, were analyzed and compared with the 2016-2017 vaccine strain (A/Hongkong/4801/2014(H3N2)). The results showed 40 influenza A/H3N2 isolated viruses in Shenzhen during 2017 were related to the vaccine strain with identity 97.8-99.5% at nucleic acid level and 97.4-99.4% at the amino acid level.

Phylogenetic tree analysis revealed that these influenza A (H3N2) viruses clustered into two subclades A and B. N121K and N171K mutations were primary present in Group B viruses, these two amino acids located in epitope D region, and 21(52.5%) isolates fell within this clade as well as A/Hongkong/4801/2014(H3N2). The other 19 isolates in Group A had the key substitution T131K, R142K and R261Q, 131 and 142 amino acid sits located in epitope A region (Table 1). These results implied that the HA gene of the influenza A (H3N2) virus during 2017 underwent continuous evolution and the vast majority of them were divergent from the vaccine strain, although there was a high nucleic acid similarity between them.

Antigenic and receptor binding sites analysis

Comparative analysis with the vaccine strain, A/Hongkong/4801/2014 (H3N2) showed some differences between the amino acids at epitope A-E. There were T131K (19 of 40), R142K (19 of 40), R142G (5 of 40), S144K (3 of 40) and S144R (3 of 40) at antigenic site A. At antigenic site B, 20% (8 of 40) and 65% (26 of 40) of the viruses had amino acid substitutions in position 158 and 160, respectively; N121K (18 of 40) and N171K (18/40) mutations located in epitope D, In addition, D53N (5/40), H311 (15/40), S219F (4/40), E62G (4/40), and R261Q (22/40) were observed in other epitopes.

No mutation occurred in the 7 conserved amino acid residues (T-98, S-136, W-153, H-183, Y-195, I-226 and S-228) at HA receptor binding site among all the 40 isolated viruses. As for H3, I-226 and S-228 were specific for the α-2,6 linkage, and the 40 isolates had Ile and Ser at position 226 and 228 respectively as the vaccine strain.

Table 1: Amino acids mutations in HA gene for influenza A(H3N2) viruses from Shenzhen.

	Group	Codon position in HA											
		53	62	121	131	142	144	158	160	171	219	261	311
Epitope		C	E	D	A	A	A	B	B		D	E	C
A/Hongkong/4801/2014	vaccine	D	E	N	T	R	S	N	K	N	S	R	H
A/Shenzhen/03-57/2017	B	N	.	.	.	G	R	K	T	K	.	.	.
A/Shenzhen/03-58/2017	B	N	.	.	.	G	R	.	T	K	.	.	.
A/Shenzhen/1174/2017	B	N	.	.	.	G	R	.	.	K	.	.	.
A/Shenzhen/1219/2017	B	.	.	K	.	.	K	.	A	.	.	Q	.
A/Shenzhen/1131/2017	B	.	.	K	.	.	K	.	T	.	.	Q	.
A/Shenzhen/04-052/2017	B	.	.	K	.	.	K	.	T	.	.	Q	.
A/Shenzhen/01-139/2017	B	.	.	K	.	.	.	.	.	K	.	.	Q
A/Shenzhen/01-140/2017	B	.	.	K	.	.	.	.	.	K	.	.	Q
A/Shenzhen/01-141/2017	B	.	.	K	.	.	.	.	.	K	.	.	Q
A/Shenzhen/01-150/201	B	.	.	K	.	.	.	.	A	K	.	.	Q
A/Shenzhen/01-151/2017	B	.	.	K	.	.	.	.	A	K	.	.	Q
A/Shenzhen/01-152/2017	B	.	.	K	.	.	.	.	T	K	.	.	Q
A/Shenzhen/01-156/2017	B	.	G	K	.	G	.	.	.	K	.	.	Q
A/Shenzhen/01-157/2017	B	.	G	K	.	G	.	.	T	K	.	.	Q
A/Shenzhen/1144/2017	B	.	G	K	.	G	.	.	.	K	.	.	Q
A/Shenzhen/1164/2017	B	.	.	K	.	.	.	.	.	K	.	.	Q
A/Shenzhen/1185/2017	B	.	.	K	.	.	.	K	T	K	.	.	Q
A/Shenzhen/1204/2017	B	.	.	K	.	.	.	.	.	K	.	.	Q
A/Shenzhen/1213/2017	B	.	.	K	.	.	.	K	.	K	.	.	Q
A/Shenzhen/1220/2017	B	.	G	K	.	G	.	H	T	K	.	.	Q
A/Shenzhen/1225/2017	B	.	.	K	.	.	.	.	T	K	.	.	Q
A/Shenzhen/03-59/2017	A	.	.	.	K	K	.	.	.	.	.	Q	.
A/Shenzhen/04-044/201	A	.	.	.	K	K	.	.	T	.	.	Q	.
A/Shenzhen/04-045/2017	A	.	.	.	K	K	.	.	T	.	.	Q	.
A/Shenzhen/04-050/2017	A	.	.	.	K	K	.	.	T	.	.	Q	.
A/Shenzhen/04-051/2017	A	.	.	.	K	K	.	.	T	.	.	Q	.
A/Shenzhen/06-053/2017	A	.	.	.	K	K	.	.	T	.	.	Q	.
A/Shenzhen/08-073/2017	A	.	.	.	K	K	.	.	T	.	F	Q	.
A/Shenzhen/08-074/2017	A	.	.	.	K	K	.	.	A	.	F	Q	.
A/Shenzhen/08-075/2017	A	.	.	.	K	K	.	.	T	.	F	Q	.
A/Shenzhen/1082/2017	A	.	.	.	K	K	.	H	T	.	F	Q	.
A/Shenzhen/1084/2017	A	.	.	.	K	K	.	.	.	.	.	Q	.
A/Shenzhen/1111/2017	A	.	.	.	K	K	.	K	T	.	.	Q	.
A/Shenzhen/1127/2017	A	.	.	.	K	K	.	.	.	.	.	Q	.
A/Shenzhen/1147/2017	A	.	.	.	K	K	.	.	I	.	.	Q	.
A/Shenzhen/1152/2017	A	.	.	.	K	K	.	K	T	.	.	Q	.
A/Shenzhen/1168/2017	A	.	.	.	K	K	.	.	.	.	.	Q	.
A/Shenzhen/1176/2017	A	.	.	.	K	K	.	.	A	.	.	Q	.
A/Shenzhen/1178/2017	A	.	.	.	K	K	.	.	T	.	.	Q	.
A/Shenzhen/1188/2017	A	.	.	.	K	K	.	K	T	.	.	Q	.

Receptor binding property analysis

Six randomly selected H3N2 isolates of each group were submitted for ELISA receptor binding assay. All of these 12-test influenza A (H3N2) isolate strains bound to both of the two receptor analogs, with preference binding to α-2,6 receptor. These H3N2 virus with N121, N171 and Q261 in Group A were distinct compared with those with K121, K171 and R261 in Group B in their binding features.

The ELISA results showed that average OD_{450} value of the six H3N2 virus strains in Group B is higher than that of Group A with statistical significance binding to α-2,6 receptor. Meanwhile, the binding of strains with K121, K171 and R261 showed a slight increase on α-2,3 receptor compared with those with N121, N171 and Q261, though the difference between them is not significant statistically. These data demonstrated that the substitutions in amino acid sites 121, 171 and 261 of HA in current circulating influenza A(H3N2) virus affected the receptor binding property, which may help to explain why abrupt increase in hospitalization fatality rates caused by seasonal influenza A/H3N2 was taken place besides the vaccine ineffectiveness during 2017.

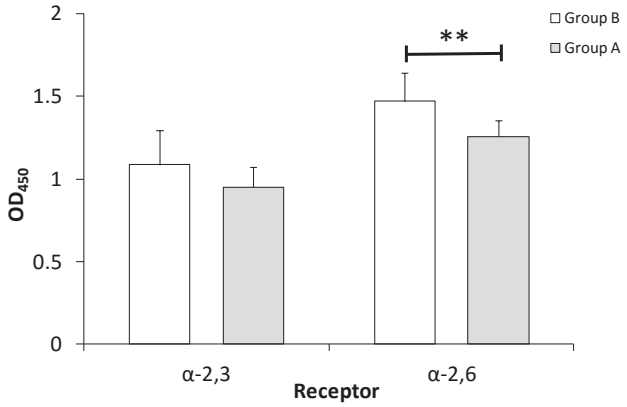

Figure 2: Receptor binding property of isolated strains in Group A and Group B.

Discussion

After high epidemic wave in 2017, influenza A (H3N2) is still in off-peak season during 2018-2019. In this study, we have explored the characteristics of influenza A (H3N2) circulating during 2017 in Shenzhen, another more comprehensive sequences analysis including Hongkong and Macao Special Administration Region from 2016 to 2017 previously were conducted [14] which showed similar evolutionary characteristics between influenza A (H3N2) viruses isolated from Shenzhen and the neighboring cities. Nucleotide sequencing and phylogenetic analysis of hemagglutinin amino acids revealed several differences as compared to the 2017 recommended vaccine strain (A/Hongkong/4801/2014(H3N2)). Substitutions at epitopes in circulating strains associated with changing receptor binding property contributed to local epidemic.

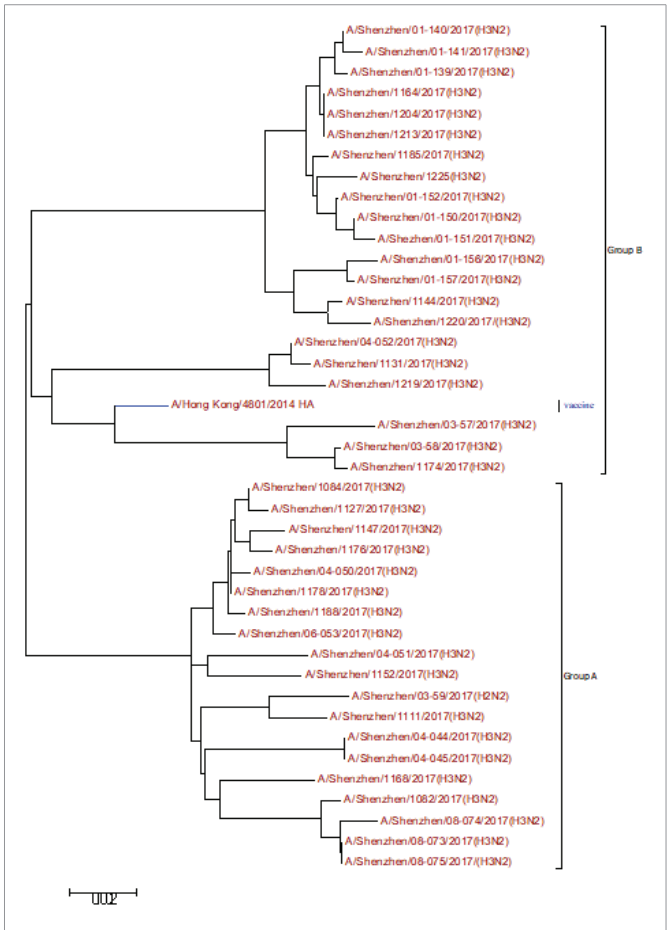

Figure 1: Phylogenetic analysis of influenza A (H3N2) viruses isolated from Shenzhen during 2017.

Phylogenetic analysis of HA showed that these isolates fell into two distinct genetic clades, with 47.5% strains being different from the vaccine clade. WHO recommended A/Hongkong/4801/2014(H3N2) strain for H3N2 strain component of the 2017-2018 influenza vaccine in northern hemisphere, which belongs to the subclade 3C.2a. In this study, 52.5% (21 of 40) isolated viruses accorded with the definition of subclade 3C.2a1 viruses with amino acid substitutions of N171K and N121K in HA1 [15], the other 47.5%(19 of 40) isolated viruses with N121, N171 and Q261 belonged to subclade 3C.2a2. 3C.2a1 and 3C.2a2 viruses showed lower neutralizing property to ferret antisera against A/Hong Kong/4801/2014 primarily inhibited [16]. Meanwhile, viruses with T131K, R142K and R261Q amino acid substitutions clustered into group A in our study. These three amino acid substitutions at 131,142 and 261 had also been observed in South Korea in 2016-2017 season and research had revealed the strong positive correlation among them by the correlation plot [17].

Mutation was found at multiple amino acid positions with epitope A-E. Nearly half of the isolates had substitutions at the HA1 positions 109 to 301 (121, 131 142, 158, 160, 171 and 261), the reported determinant for the antigenic phenotype of A/H3N2 strains [18]. And they were also the potentially key residuals that change the antibody recognition and receptor binding of H3N2 viruses. Based the variants/vaccine strains, frequent mutation at six positions (135, 158, 160, 189, 225 and 278) on HA epitopes in report [19] were not discovered in Shenzhen except N158K/H and R160T/A. But the substitution R261Q, rarely shown in other studies, occurred in 55% (22 of 40) strains in our study.

Amino acid at 53, 144, and 192 were frequently changeable

sites in H3N2 HA evolution, but only few strains with substitutions at 53 and/or 144 in epitope C or A ,respectively, were observed in Shenzhen isolates. None of the 40 viruses had mutation at 192, a site being contained in a receptor binding site. Among them, the substitutions at 158 and 160 occurred in Europe 2014/15 season and has been prevalent worldwide later [20,21]. The impact of K160T HA mutation has been explored by series experiments, and another publication revealed the T160A hemagglutinin substitution affects not only receptor binding property but also transmissibility of H5N1 clade 2.3.4 avian influenza virus in guinea pigs [22,23]. For all that, more studies are needed to confirm the detailed effects of other mutations in H3N2 viruses.

Interestingly, when receptor binding affinity assays were carried out using the HAs of these 131, 171 and 261 variants, all interactions were relatively stronger. The receptor binding properties of the viral hemagglutinin surface glycoprotein are a fundamental determinant for transmission and host adaptation, and the affinities to specific types of sialic acid containing glycan receptors affect the viral transmission properties and pathogenicity. It was reported that receptor binding property of influenza HA could change in virtual of certain amino acid substitutions within receptor binding region. Although the seven conserved amino acid residues at HA receptor binding site were unchanged and the two amino acids at 226 and 228 position that were specific for the α-2,6 linkage showed identical amino acids to the vaccine strain, some isolated strains presented alteration in sialic acid receptor binding validity following other amino acids substitutions. We found the viruses with K121, K171 and R261 acquired a better affinity to sialic acid receptors of both α-2,3 and α-2,6 linkage in comparison to those with N121, N171 and Q261. This finding emphasizes the potential correlation between receptor binding property and amino acids substitutions at position 121, 171 and 261 in the HA protein of influenza A (H3N2). Influenza A (H3N2) predominated in southern China during the season of influenza in 2017, presenting unusual epidemic and more severe symptoms and even dead cases than that in the past few years [5,24]. The increased binding property to α-2,6 and α-2,3 receptor that distributed with a relatively high density in human upper respiratory track could enhance the viral transmission property and consequently result in a increase of morbidity. In the meanwhile, better affinity to α-2,3-linked glycans contained in lower respiratory track may aggravate viral infection to lung and lower respiratory track, resulting in severe clinical symptoms to infected patients. The largely mutated influenza A (H3N2) virus with enhanced binding to the two receptors could explain the clinical feature of patients to some extent.

The study in Hong Kong revealed that the summer peak in Hong Kong was associated with the low population serum microneutralization antibody titer against the predominating influenza A(H3N2) N121K virus in 2017 [5]. Strains with N121K substitution were found in 45% isolates, all of whom clustered in group B, subclade 3C.2a1, and the low protection induced by vaccine against them could also partly explain the unusual H3N2 influenza. Together with the substitutions of N121K, N171K and R261 in group B that increase the receptor affinity of HA, the characteristics of seasonal influenza A (H3N2) in Shenzhen during 2017 could be better understood.

In conclusion, this study is significant to survey the A/H3N2 influenza variations in amino acid features, receptor binding property circulating in Shenzhen in 2017, which may be beneficial for better understanding the viral pathogenicity, and explain the epidemiologic and clinical features of infected in some way. Continuous monitoring and further study are needed to better understand mutations of clinical relevance that result in alteration in receptor binding properties.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81871631), Special Foundation for the Development of Strategic Emerging Industries of Shenzhen (JCYJ20180307102005105, and JCYJ20170817110434640), Research Project for the Health and Family Planning System of Shenzhen (201607057, and 201602002), and Medical Science and Technology Foundation of Guangdong Provincial (A2018269).

Author Contributions

WW and XX conceived and wrote the paper. XW performed most computational analyses and revised the paper. BP, HL, YS, XT performed the experiments in the study, and SF provided advice and supervised the work. All authors read and approved the final version of the manuscript.

Conflicts of Interest

There are no conflicts of interest related to this research.

Ethics Approval

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

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