Influenza viral infection is a high-risk factor for developing Coronavirus disease 2019 (COVID-19)

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ABSTRACT

Coronavirus disease 2019 (COVID-19) is caused by infection with the 2019 novel coronavirus 2 (2019-nCoV, now referred to as SARS-CoV-2). COVID-19 has become a global pandemic since its outbreak at the end of Dec 2019. COVID-19 could lead to severe acute respiratory disease, especially to those who have reduced immunity. Binding of the viral Spike protein (S) to its receptor ACE2 (Angiotensin Converting Enzyme 2) on the surface of target cells has been proven to be key for virus entry and infection. Although ACE2 expression in the respiratory system is necessary for pneumonia infection by SARS-CoV-2, the regulation of *ACE2* gene expression remains poorly investigated, especially for patients that are in pre-pathological conditions. Here, by analyzing The Gene Expression Omnibus (GEO) database, we investigated the expression regulation of *ACE2* in various kinds of primary epithelial cells from the respiratory system after influenza A or respiratory Syncytial Virus (RSV) infection. Our analyses reveal that infection of influenza A, RSV or influenza vaccines greatly increased *ACE2* expression, suggesting that influenza viral infection could represent a high risk factor for developing COVID-19. We also found that the regulatory effect of influenza A virus on *ACE2* expression is associated with activation of the interferon beta-induced pathway and viral RNA-activated host response. Together, our data provide a theoretical framework for clinical classification for SARS-CoV-2 infection susceptibility and could be used for future prevention and therapy treatment for COVID-19.

KEYWORDS

COVID-19; Influenza; SARS-CoV-2; ACE2; risk factor

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Introduction

According to the estimate report from World Health Organization (WHO), COVID-19 has spread across 114 countries and caused more than 5000 death over the world by March 15th, 2020. Besides China, numerous laboratory-confirmed infections and fatal cases have been reported in several countries including Italy, Iran, South Korea, USA, France, etc. SARS-CoV-2 was found to share similar genome sequences with severe acute respiratory syndrome coronaviruses (SARS) and probably was originated from the same type of natural host [1]. The rapid spread of SARS-CoV-2 is largely related to its transmission route. Although the fecal-oral route has been indicated to be a possible mechanism for virus transmission, respiratory droplets and/or environmental contact are still the main routes for SARS-CoV-2 spreading [2]. Besides respiratory failure, multiple organ dysfunction syndromes caused by cytokine storm have been revealed to be main reasons for patient death [3]. So far, protease inhibitors, antibiotics, and corticosteroid have been used in treating COVID-19 with limited success. While effective therapies are still needed, we reason that determining risk factors that could increase the chance of SARS-CoV-2 infection or severe COVID-19 syndrome development is crucial for clinical prevention and intervention.

Similar to SARS, infection of SARS-CoV-2 is dependent on the binding between its Spike protein (S) and the host receptor Angiotensin converting enzyme II (ACE2) [1]. However, the S protein of SARS-CoV-2 exhibited a much higher binding affinity than that of SARS [4], correlating with the greatly elevated infection potential of the former. So far, SARS-CoV-2 has been detected in multiple tissues and metabolites, such as gastrointestinal tract [5], nervus centralis [6], saliva, urine, feces [7], tears [8], etc, which might be due to the widespread expression of the ACE2 protein. Hence, analyzing ACE2 expression level could provide a prediction for tissue and organ infection of SARS-CoV-2. This seems to be particularly important for those who have pre-pathological conditions that can increase or induce ACE2 expression, leading to severe COVID-19. For instance, a higher ACE2 expression was observed in colorectal cancer patients than in healthy control [9], suggesting that colorectal cancer patients might be more susceptible to COVID-19 than healthy control.

Being a type of acute respiratory disease, COVID-19 exhibits fever, cough and abnormal chest computed tomography characteristics (*i.e.*, ground-glass opacity) as dominant symptoms [2], which are similar to seasonal influenza infection. These common clinical manifestations make it hard to distinguish the patients from one to another using just these symptoms, especially when considering that COVID-19 happened to outbreak during the influenza season in certain regions. In a number of cases, patients might be co-infected with SARS-CoV-2 and Influenza A Virus, which not only complicated the clinical identification, but could have also increased the difficulty of treatment [10]. Considering that pre-pathological conditions such as diabetes, coronary heart disease, and hypertension may influence the COVID-19 morbidity [2], we wondered whether prior influenza virus infection could affect the incidence rate and the disease progression of COVID-19. To address this question, we

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evaluate ACE2 expression levels in epithelial cells from the respiratory tract including nose, airway, Bronchi and lung. Our data demonstrate that ACE2 expression was greatly elevated by infection with varies kinds of influenza viruses, especially in epithelial cells from the lower respiratory tract. Surprisingly, we noticed that live attenuated influenza vaccine (LAIV) also increased ACE2 transcription in primary human nasal epithelial cells, indicating potential risk of administrating LAIV in developing COVID-19. We further reveal that the up-regulation of ACE2 by Influenza is associated with the interferon beta (IFNβ)-induced pathway and host reactions to viral RNA.

Method

In this work, all raw data of RNA Expression profiling were extracted from NCBI-GEO database [11, 12]. RNA expression of each cell sample was measured in triple with indicated microarray platform. All raw data have been normalized to produce cross-comparable values proved with the median-centered values distribution. After data normalization, expression value was analyzed with the interactive web tool GEO2R. Fold-change and p-values were calculated for each virus infection, as compared to the mock-infected or uninfected sample. All data were analyzed by one-way ANOVA and presented as the mean ± standard error of mean (SEM) using the GraphPad Prism 8.0. Significant differences were accepted at *P<0.05 or **P<0.01. All Data source and protocols used in this study, including GSE accession numbers, cells types, virus strains, experimental protocols, etc were listed in Table 1.

Results

Influenza infection causes an increase in *ACE*2 expression in primary epithelial cells of the respiratory tract.

To reveal the effect of influenza infection on COVID-19 susceptibility, we surveyed *ACE2* expression in primary epithelial cells isolated from both the upper and the lower tract of the respiratory system. In the GEO dataset GSE83215[13], primary human nasal epithelial cells (hNECs) was infected with seasonal influenza A virus A/Victoria/361/2011 (H3N2) at the multiplicity of infection (MOI) of 1. Thirty-six hours after one-hour incubation, cells were harvested and subjected to RNA extraction. From the public database, we were able to extract the expression array data from 5 individual donors and then compared ACE2 expression levels under different treatment conditions. Interestingly, we observed a mild, yet statistically significant, increase in ACE2 expression after influenza A virus infection compared with mock infected samples in each group of cells from all donors (Fig. 1A). We then evaluated the effect of influenza A virus on epithelial cells derived from Lung and bronchus, the lower tract of the respiratory system [14]. Three strains of virus including seasonal H1N1 (BN/59) and pandemic H1N1 (KY/180 and KY/136) were used to infect primary lung bronchus epithelial cells (wd-NHBE). Microarray analysis was conducted using two independent

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probes recognizing *ACE2* transcripts. Data extracted from GSE48466 showed that both seasonal H1N1 A and pandemic H1N1 isolates caused a significant increase in ACE2 expression in wd-NHBE cells detected by two probes (Fig. 1B). We noticed that the fold of increase was much greater in lower respiratory tract cells (Fig. 1B) than in upper respiratory tract epithelial cells (Fig. 1A), consistent with the infection pattern of SARS-CoV-2. Further, the increase in the expression of ACE2 was much greater in pandemic H1N1 infected cells than in seasonal flu infected cells, suggesting that the pandemic influenza virus generate greater host response (*i.e.*, ACE2 expression) than seasonal isolates.

To further test our hypothesis that influenza infection activates *ACE2* expression, we surveyed the host response in other type of respiratory tract epithelial cells, such as primary human airway epithelial cell (hAEC) that is isolated from human mainstream bronchi [15]. hAEC was infected with H3N2 (A/Udorn/72) or rgRSV244 (Respiratory syncytial virus) at a MOI of 2 for 2 hours and then harvested 24 or 48 h post infection (hpi). Microarray analysis was performed on two individual platforms. The *ACE2* expression value of each sample was mined from dataset GSE32138 and GSE32139. Our analysis showed that H3N2 and RSV substantially increased *ACE2* expression levels compared to their corresponding mock control (Fig. 1**C**). Together, these data suggest that influenza infection in primary epithelial cells of the respiratory tract causes a significant increase in *ACE2* expression.

Live attenuated influenza vaccine infection increased *ACE2* expression

Live attenuated influenza vaccine (LAIV) has been broadly used to elicit immune responses. Several reports have showed that antigenically-matched LAIV can elicit enhanced innate immune responses as compared to WT virus [13, 16]. We wondered whether LAIV could also induce similar host response on *ACE2* expression as its antigenically-matched WT virus. To answer this question, we re-analyzed the microarray data from GEO dataset GSE83215, in which primary human nasal epithelial cells isolated from different individual donors were infected with WT seasonal influenza A virus (H3N2) or antigenically-matched LAIV at the same dose and dpi. The results show that LAIV resulted in a similar increase in ACE2 levels to the WT virus (Fig. 2). In some donors, LAIV infection caused even higher ACE2 expression than the H3N2 WT virus (Fig. 2). These results show that LAIV could also increase *ACE2* expression in primary human nasal epithelial cells, suggesting that it is likely the host cell response, but not the influenza virus itself, that caused the upregulation of *ACE2*.

The host response of ACE2 up-regulation by influenza A infection is associated with activation of the interferon beta and viral RNA-sensing pathways

As our analyses showed that both influenza A virus and LAIV induced *ACE2* expression in epithelial cells in the respiratory tract, we next sought to understand the

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cellular mechanism underlying such host response. Although the existing data are limited, we were still able to extract valuable information by mining the public database. In the dataset GSE19392 [17], Sagi *et al* evaluated the host response of primary human bronchial epithelial cells after challenged with different virus infection. Cells were infected with H1N1 (PR8) or PR8 NS1 mutant virus in which the NS1 gene, an important viral gene that regulates virus-cell interactions [18], was mutated from the PR8 genome. Meanwhile, cells were also treated with interferon beta or transfected with virus RNA separately. All infections or treatments were performed as a time course. By analyzing the relative *ACE2* expression levels in different cell samples, we found several lines of interesting results (Fig. 3): 1, Interferon-beta treatment activated the *ACE2* expression in a time dependent manner; 2, The PR8 virus with NS1 mutation elicited even stronger host response in increasing ACE2 expression than the PR8 WT virus; 3, Viral RNA only also strongly activated ACE2 expression (fold change > 10 compared to the transfection reagent or media control). Our results indicate that activation of ACE2 expression by influenza virus infection is independent of the NS1 protein in virus, but instead could be associated with the interferon beta pathway or host reactions caused by viral RNA.

Discussion

In this study, we investigated how influenza A virus infection affects ACE2 expression in various kinds of primary epithelial cells derived from the respiratory tract. We reason that focusing on primary epithelial cells but not cancer or other immortal cells lines will better mimic viral-host interactions in vivo. While we recognize that the number of cell line types and influenza A virus isolates is limited in the current study, the common host response in increasing ACE2 expression observed in these independent studies suggest that such upregulation of ACE2 could be a ubiquitous response for influenza A virus infection. The fact that RSV infection also activated ACE2 expression leads to a hypothesis that inflammation caused by virus influenza infection in the respiratory tract is a high risk factor for COVID-19. Further, we demonstrated that the relative ACE2 expression level in NHBE cells was significantly higher when infected with the pandemic influenza virus KY/180 and KY/136 than with the seasonal flu virus BN/59, suggesting that pandemic influenza might predispose an even higher risk than seasonal influenza in developing COVID-19. This result is also consistent with the previous report that H1N1 pandemic IAV could induce stronger host response than seasonal isolates [13].

Our current analyses and predication are based on the widely accepted idea that ACE2 function as the receptor for SARS-CoV-2 entry into host cells. It has also been reported that other host factors, such as cellular protease TMPRSS2, could facilitate host cell entry of SARS-CoV-2 by priming S protein [19]. During the preparation of this manuscript, furin and CD147 were also reported to be a potential receptor for SARS-CoV-2 entry [20, 21]. Therefore, it would be interesting to investigate whether influenza infection also has any effect on these invading routes in the future.

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Meanwhile, our conclusions might also be applicable to the SARS virus infection, as it has been shown that both SARS-CoV-2 and SARS use ACE2 as the receptor for host entry [1] .

Upon infection, Influenza virus triggers the innate immune response in host cells to produce Type I interferons (IFNs), which in turn inhibit viral replication. Hence, IFN could be used as a therapy choice for influenza [22]. In our current work, IFNβ treatment activated ACE2 expression in primary human bronchial epithelial cells, suggesting that the host response of ACE2 upregulation is associated with the IFN signaling pathway. Our results raise a potential concern in using anti-viral interventions such as IFNβ, as they might increase the chance of SARS-CoV-2 infection and could eventually cause COVID-19. In parallel, LAIV was broadly used to prevent the influenza outbreak. In our study, LAIV treatment activated ACE2 expression in primary human nasal epithelial cell (hNECs). In some individual donors, the activation effect of LAIV is even greater than the WT virus, indicating that likely it is the activation of the host innate immune response that triggered the upregulation of ACE2. In conclusion, our study suggests that influenza spreading and its associated therapies could exacerbate the symptoms of COVID-19 and therefore great care must be given for patients with prior influenza infection.

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Declaration of conflicting interests

The authors declared no potential conflicts of interests.

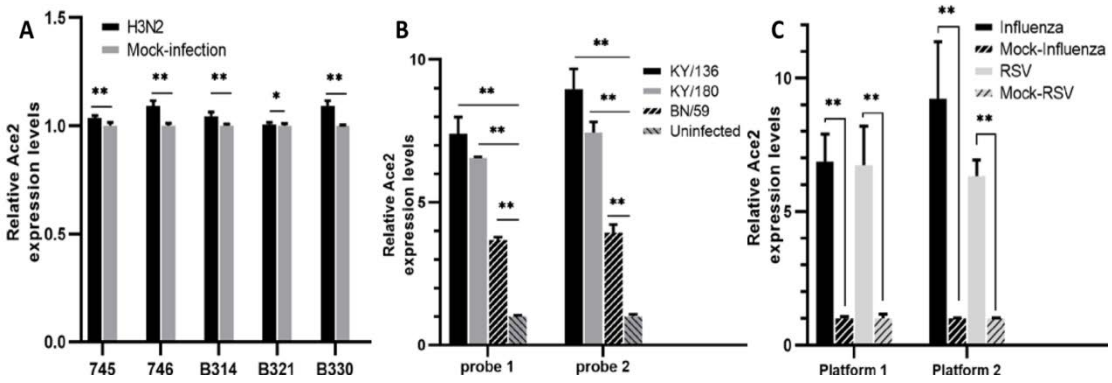

Fig. 1. Influenza infection causes an increase in ACE2 expression in primary epithelial cells of the respiratory tract. (A) Relative ACE2 expression in primary human nasal epithelial cells after infection with H3N2 or mock virus. Cells were

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separated from indicated donors. (B) Relative ACE2 expression in primary human lung bronchial epithelial cells after infection with indicated virus. Probe 1 or 2 represents different probe for microarray analysis. (C) Relative ACE2 expression in primary human airway epithelial cell was evaluated after infection with indicated virus. Two microarray platforms were utilized for analysis.

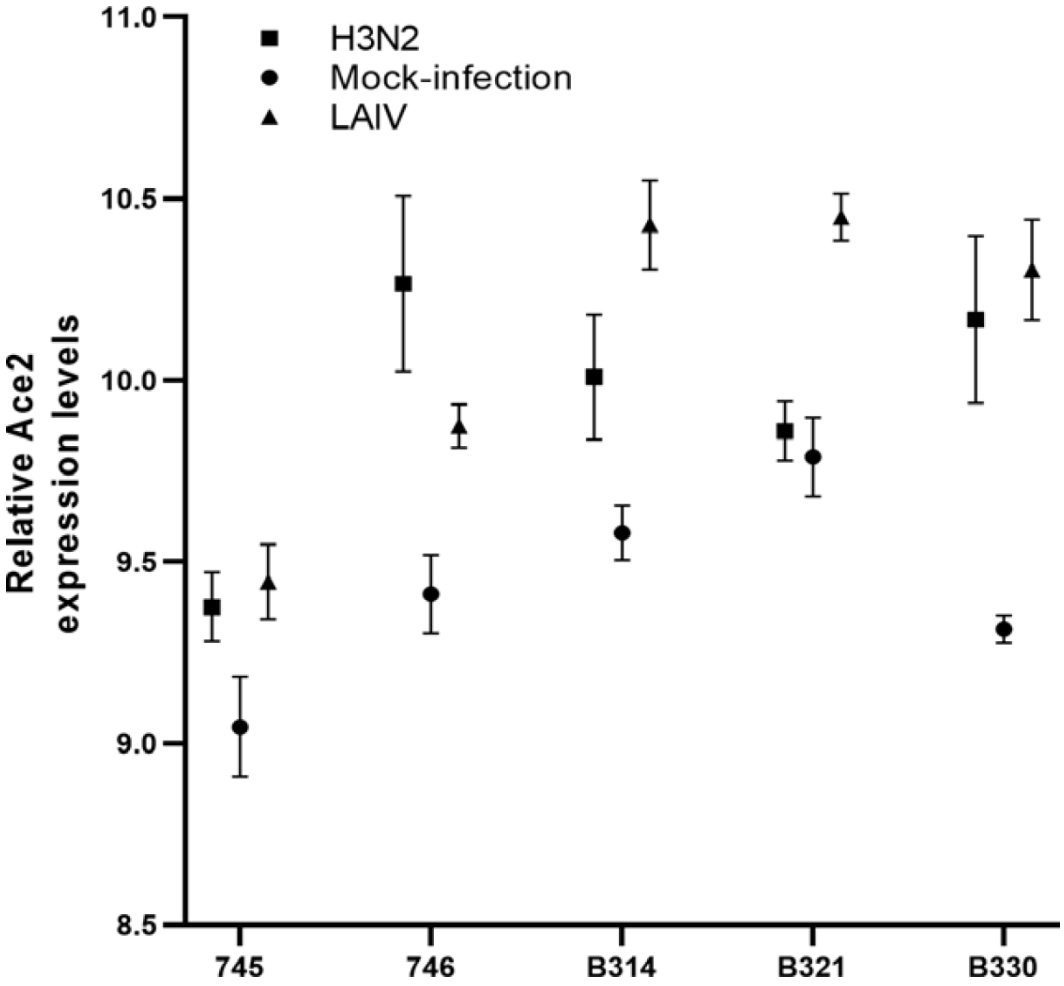

Fig. 2. Live attenuated influenza vaccine infection increases ACE2 expression. Primary human nasal epithelial cells from different donors were infected with indicated virus or vaccine and relative ACE2 expression level from each sample were evaluated.

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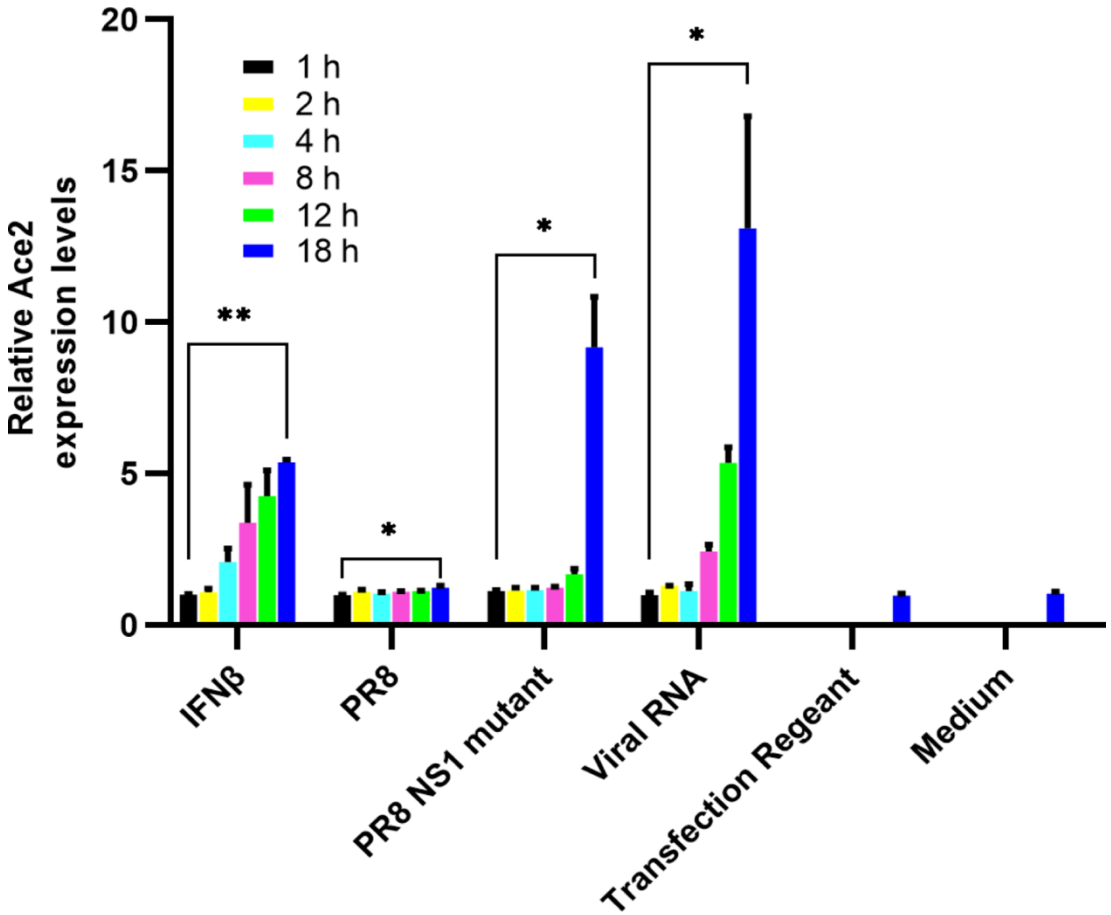

Fig. 3. Both interferon beta and viral RNA increases ACE2 expression. Primary human bronchial epithelial cells were infected with virus or treated with IFNβ or transfected with viral RNA as indicated. Relative ACE2 expression at different time point was evaluated. Transfection reagent or medium only treatment was set as control group.

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ID	Data source	Tissue	Cells Type	Virus Type	Dose (MOI)	Time Course	Experiment type	Citation
1	GSE48466	Lung and bronchus	Primary lung bronchus epithelial cells (wd-NHBE)	Seasonal H1N1 (A/BN/59/07)	MOI of 3 (1 hour)	36 hpi	Expression profiling by array	PMID: 24244384
2				Pandemic H1N1 (A/KY/180/10)				
3				Pandemic H1N1 (A/KY/136/09)				
4	GSE32138 GSE32139	Bronchi	Primary human airway epithelial cell (hAEC)	H3N2(A/Udorn/72)	MOI of 1 (2 hours)	24 hpi		PMID: 22398282
5				rgRSV244 (Respiratory syncytial virus)	MOI of 5 (2 hours)	48 hpi		
6	GSE83215	Nose	Primary human nasal epithelial cell (hNECs)	Seasonal H3N2 (A/Victoria/361/11)	MOI of 1 (1 hour)	36 hpi		PMID: 28967519
7				Live attenuated influenza vaccine (LAIV; WT HA and NA, all other proteins from A/Ann Arbor/6/1960)				
8	GSE19392	Bronchi	Primary human bronchial epithelial cells(HBEC)	H1N1 (A/PR/8/34)	MOI of 5 (15 mins)	0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 18 hpi		PMID: 20064372
9				ΔNS1 (PR8 with a deleted NS1 gene,				

Table 1 . Data source and protocols used in this stud

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