Respiratory virus 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 varies of respiratory viruses infection such as influenza A virus (IFV), respiratory syncytial virus (RSV) and human rhinovirus (hRV). Our analyses reveal that infection of multiple kinds of respiratory viruses or influenza vaccines greatly increased *ACE2* expression, suggesting that respiratory viruses 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; respiratory virus; SARS-CoV-2; ACE2; risk factor

Introduction

According to the estimate report from World Health Organization (WHO), COVID-19 has spread across 216 countries and regions and caused more than 917,417 deaths over the world by September 13, 2020. Besides China, numerous laboratory-confirmed infections and fatal cases have been reported in several countries including USA, Brazil, India, Russia, South Africa, Mexico, France, Italy, 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 controls [9], suggesting that colorectal cancer patients might be more susceptible to COVID-19 than healthy people.

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 respiratory viruses 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 several cases reported, patients might be co-infected with SARS-CoV-2 and influenza A virus or

other kinds of respiratory virus, which not only complicated the clinical identification, but could have also increased the difficulty of treatment [10-13].

Considering that pre-pathological conditions such as diabetes, coronary heart disease, and hypertension may influence the COVID-19 morbidity [2], we wondered whether prior respiratory virus infection could affect the incidence rate and the disease progression of COVID-19. To address this question, we 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 respiratory viruses. 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 revealed that the up-regulation of ACE2 by Influenza is associated with the interferon beta (IFNβ)-induced pathway and host reactions to viral RNA.

Materials and Methods

In this work, all raw data of RNA Expression profiling were extracted from NCBI-GEO database [14,15]. 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 ACE2 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 [16], 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 (Figure 1(A)). We then evaluated the effect of influenza A virus on epithelial cells derived from lung and bronchus, the lower tract of the respiratory system [17]. 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 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 (Figure 1(B)). We noticed that the fold of increase was much greater in lower respiratory tract cells (Figure 1(B)) than in upper respiratory tract epithelial cells (Figure 1(A)), 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 [18]. 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 (Figure 1(C)). Together, these

data suggest that influenza infection in primary epithelial cells of the respiratory tract causes a significant increase in *ACE2* expression.

Human Rhinovirus (hRV) Infection Causes An Increase in ACE2 Expression in Respiratory Epithelial Cells in Vitro or in Vivo

Besides IFV and RSV, hRV is another common viral infectious agent in human respiratory tract. hRV was reported to be responsible for more than 50% common cold and the symptoms include fever, cough, sneezing, etc. [19]. We then wondered whether hRV infection in respiratory epithelial cells could cause similar host response, such as *ACE2* activation with IFV and RSV. To figure out this issue, we surveyed *ACE2* expression in respiratory epithelial cells or tissues after hRV infection in several separate experiments [20-22]. In two separate experiments primary human bronchial epithelial cells derived from different donors were infected with hRV at MOI of 1. Twenty four hours later, RNA was extracted then subjected for transcriptome microarray. Interesting, in each experiment, *ACE2* expression was activated in hRV infection sample compared to in control, no matter which donors these cells come from (Figure 1(D) and 1(E)). These results from *in vitro* study showed hRV infection in primary human bronchial epithelial cells could activate *ACE2* expression, which is further strengthened by an *in vivo* experiment. Nasal epithelial scrapings were collected from 31 patients pre- or 2 days post-infected with hRV then microassay analysis was performed. By analyzing the GEO datasets, we also found an elevated *ACE2* in hRV infection groups (Figure 1(F)). Taken together,

these data suggest that similar with other types of respiratory viruses, hRV infection could also cause an increase in *ACE2* expression in respiratory epithelial cells *in vitro* or *in vivo*.

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 [16,23]. 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 (Figure 2). In some donors, LAIV infection caused even higher *ACE2* expression than the H3N2 WT virus (Figure 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 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 [24], 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 [25], 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 (Figure 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 negatively regulated by NS1 protein in virus; meanwhile, it's could be also associated with the interferon beta pathway or host reactions caused by viral RNA.

Epidemics of Influenza and SARS-CoV-2 Occurred in Nearly the Same Period

To detect the potential relationship of respiratory viruses infection and COVID-19, we

analyzed the time course curve of positive test cases of influenza and SARS-CoV-2 virus in the period covering the COVID-19 outbreak and spread (Figure 4). Considering the uneven distribution status of influenza cases in North and South China, we collected the statistic data of confirmed influenza A and B cases in South China from Chinese National Influenza Center since the twenty 1st week of 2019 to 10th week of 2020. From the collected data per week, a mild curve was observed until the fourty-9th week (confirmed cases less than 1000 per week). Beginning from fiftieth week of 2019 (1577 positive cases and 31.7% positive ratio), influenza cases number increase dramatically to the peak at the 2nd week of 2020, indicating the entry of influenza Season in South China. We then surveyed the number of confirmed COVID-19 cases in Hubei province, the outbreak Center in China. The whole epidemiological curve of COVID-19 in Hubei is just behind the influenza curve in South China. One month later after the peak of influenza cases, confirmed COVID-19 cases number increase to summit at 6^{th} in Hubei (more than 18,000 lab-confirmed cases). Considering the delay of detection and test for SARS-CoV-2 virus, we deem the near co-occurrence of influenza and SARS-CoV-2 infection in Hubei, indicating a potential association of influenza and SARS-CoV-2 infection.

Discussion

In this study, we investigated how respiratory virus infection affects *ACE2* expression in various kinds of primary epithelial cells or specimens 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. 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 [16].

The fact that RSV or hRV 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, especially when we observed a increased *ACE2* expression after IFNβ only treatment. These lines make us present a hypothesis that multiple respiratory pathogens have the potential to activate *ACE2* expression since they all could trigger the innate immune response and the COVID-19 patients could also be infection with another respiratory virus. Interesting, several groups also reported similar observations. Recently, a preliminary data reported by Ian Brown et al. in Stanford School of Medicine also suggested that COVID-19 patients are often infected with other respiratory viruses [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 [26]. Recent study suggests that some tumor markers, including CD147, can also provide a new entry for SARS-CoV-2 infection through binding to the viral S protein [27,28]. Therefore, it would be interesting to investigate whether influenza infection also has any effect on these invading routes in the future. 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]. While whether a more efficient entry of the SARS-CoV-2 or SARS virus dependent on the increased *ACE2* expression still need to be further confirmed.

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 [29]. 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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Compliance with ethical standards

Conflict of interest The authors declared no potential conflicts of interests.

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

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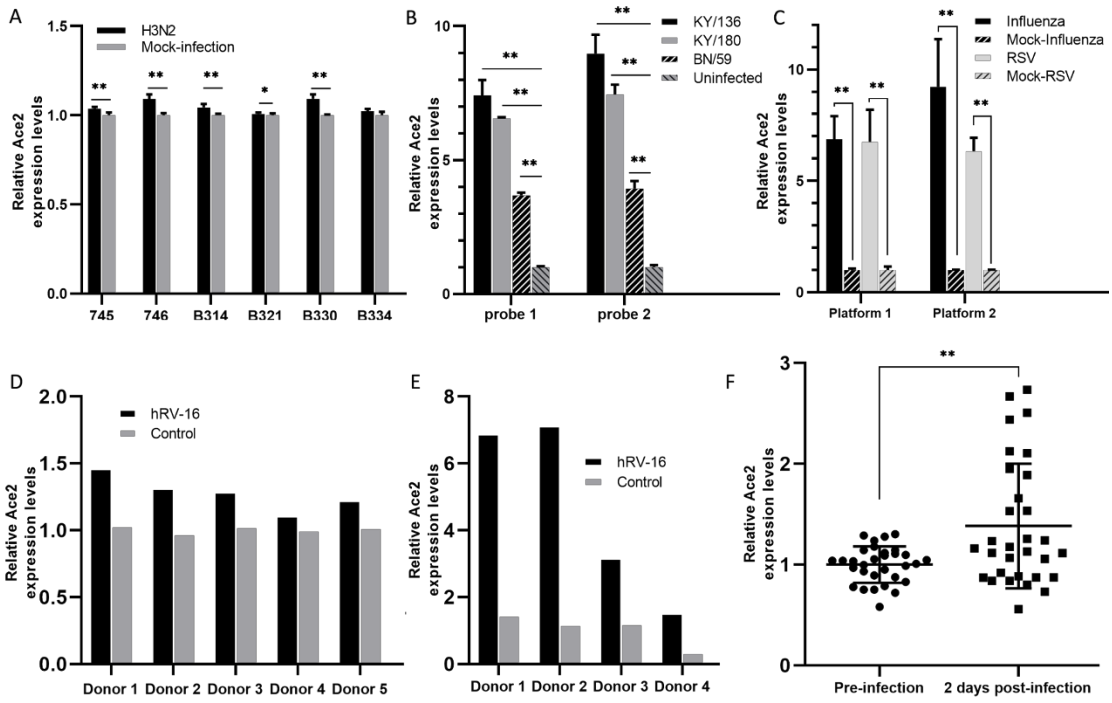
381 **Table 1.** Data source and protocols used in this study.

Data source	Data source	Virus Type	Dose (MOI)	Time course (hpi)	Citation (DOI)
GSE48466	Primary lung bronchus epithelial cells (wd-NHBE)	Seasonal H1N1 (A/BN/59/07) Pandemic H1N1 (A/KY/180/10) Pandemic H1N1 (A/KY/136/09)	3	36	10.1371/journal.pone.0078912
GSE32138 GSE32139	Primary human airway epithelial cell (hAEC)	H3N2 (A/Udorn/72) rgRSV244 (Respiratory syncytial virus)	1 5	24 48	10.1128/JVI.06757-11
GSE83215	Primary human nasal epithelial cell (hNECs)	Seasonal H3N2 (A/Victoria/361/11) LAIV (HA, NA and all other proteins from A/Ann Arbor/6/1960)	1	36	10.1128/JVI.06757-11
GSE19392	Primary human bronchial epithelial cells (HBEC)	H1N1 (A/PR/8/34) Δ NS1 (PR8 with a deleted NS1 gene)	5	0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 18	10.1016/j.cell.2009.12.018
GSE70190	Primary human bronchial epithelial cells	Human rhinovirus (HRV-16)	1	24	10.1371/journal.pone.0040762
GSE27973	Primary human bronchial epithelial cells	Human rhinovirus (HRV-16)	1	24	10.1371/journal.pone.0040762
GSE11348	Nasal epithelial scrapings	Human rhinovirus (HRV-16)	No available	48	10.1164/rccm.200805-670OC

382 **Figure 1**



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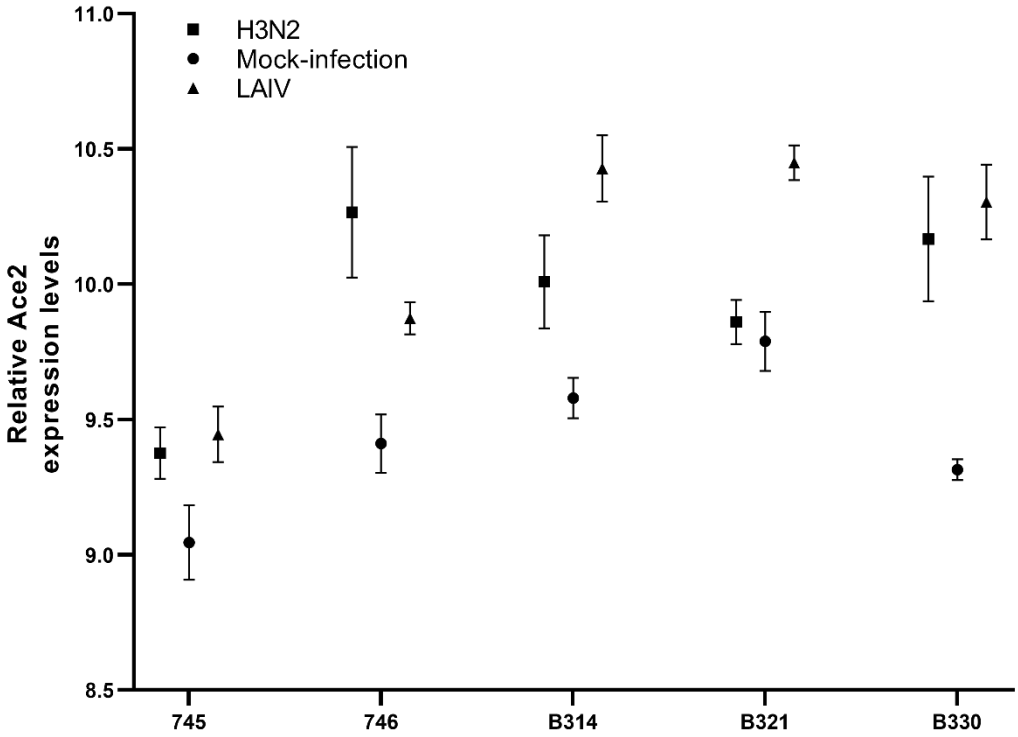
385 **Figure 2**



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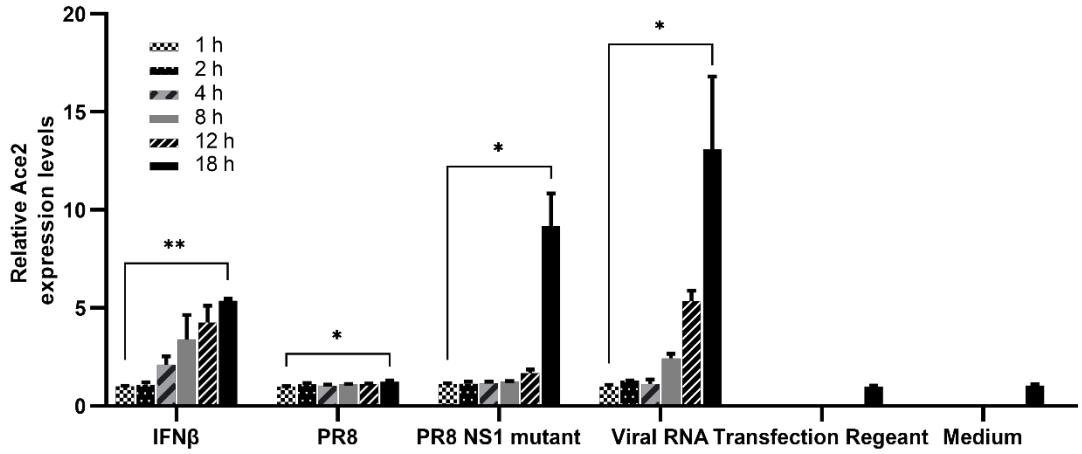
388 **Figure 3**



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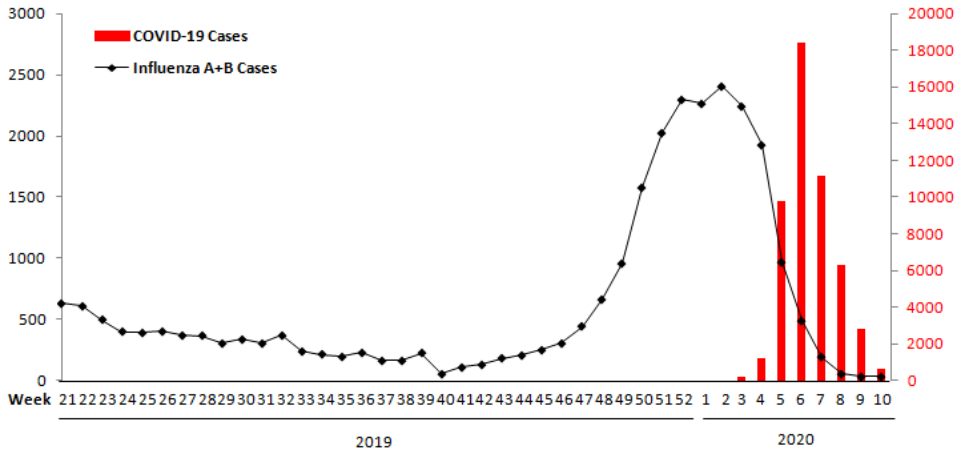
391 **Figure 4**



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394 **Legend**

395 **Figure 1.** Influenza infection causes an increase in ACE2 expression in primary
396 epithelial cells of the respiratory tract. (A) Relative ACE2 expression in primary human
397 nasal epithelial cells after infection with H3N2 or mock virus. Cells were separated
398 from indicated donors. (B) Relative ACE2 expression in primary human lung bronchial
399 epithelial cells after infection with indicated virus. Probe 1 or 2 represents different
400 probe for microarray analysis. (C) Relative ACE2 expression in primary human airway
401 epithelial cell was evaluated after infection with indicated virus. Two microarray
402 platforms were utilized for analysis. (D,E) Relative ACE2 expression in primary
403 human bronchial epithelial cells after infection with hRV-16. Data from two separate
404 experiments. (F) Comparing the ACE2 expression in nasal epithelial scrapings from
405 cohorts before HRV-16 infection or 2 days after infection.

406

Figure 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.

Figure 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.

Figure 4. The tendency chart of new cases of comfirmed COVID-19 in Hubei and influenza A and B patients in South China. The x axis represents the time of COVID-19 and influenza cases (influenza A and B). The y axises in the left and right represents numbers of influenza and COVID-19 cases, respectively. The data of laboratory-confirmed COVID-19 cases and influenza cases in South China are collected from National Health Commission of the People's Republic of China and Chinese National Influenza Center, respectively.