Effects of Smoking on ACE2 Expression Pattern:
Risk and Severity of SARS-CoV-2 Infection

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SUMMARY

Background: Respiratory epithelium expressing angiotensin-converting enzyme 2 (ACE2) is the entry for novel coronavirus (SARS-CoV-2), pathogen of the COVID-19 pneumonia outbreak, although a few recent studies have found different ACE2 expression in lung tissue of smokers. The effect of smoking on ACE2 expression and COVID-19 is still not clear. So, we did this research to determine the effect of smoking on ACE2 expression pattern and its relationship with the risk and severity of COVID-19.

Methods: The clinical data of COVID-19 patients with smoking and non-smoking were analyzed, and ACE2 expression of respiratory and digestive mucosa epithelia from smoker and non-smoker patients or healthy subjects were detected by immunohistochemical (IHC) staining.

Results: Of all 295 laboratory-confirmed COVID-19 patients, only 24 (8.1%) were current smokers with moderate smoking or above, which accounted for 54.2% of severe cases with higher mortality than non-smokers (8.3% vs. 0.4%, p = 0.018). Data analysis showed the proportion of smokers in COVID-19 patients was lower than that in general population of China (Z = 11.65, P < 0.001). IHC staining showed ACE2 expression in respiratory and digestive epithelia of smokers were generally downregulated.

Conclusions: The proportion of smokers in COVID-19 patients was lower, which may be explained by ACE2 downregulation in respiratory mucosa epithelia. However, smoking COVID-19 patients accounted for a higher proportion in severe cases and higher mortality than for non-smoking COVID-19 patients, which needs to be noted.


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INTRODUCTION
Coronavirus disease 2019 (COVID-19) caused by the novel coronavirus named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a worldwide pandemic disease. The epidemic developed rapidly in the past eight months and caused a total of 36,361,054 confirmed infections and 1,056,186 deaths by 3:36 pm CEST, 9 October 2020, reported to the World Health Organization (WHO) (https://covid19. who.int/). So far, COVID-19 has been reported by almost all countries in the world, and has become a huge health, social, and economic problem.

SARS-CoV-2 infects host cells by binding to the angiotensin-converting enzyme 2 (ACE2) receptor through the viral Spike protein, which is similar to SARS-CoV [1]. Single cell analysis found that ACE2 was highly expressed in type II alveolar epithelial cells (AT2), esophageal stratified epithelial cells, and absorptive enterocytes from ileum and colon. The main clinical manifestations of confirmed patients are fever (87.9%) and cough (67.7%). Besides, a few patients have gastrointestinal symptoms such as nausea and vomiting (5.0%) as well as diarrhea (3.7%). Severe patients have respiratory distress and multiple organ damage. The mortality caused by SARS-CoV-2 is about 1.4% to 3.06% [2]. Interestingly, COVID-19 patients seem to have a lower proportion of smokers without data correction [3-5]. Although a few recent studies have found different ACE2 expression in lung tissue of smokers [6-9], the effect of smoking on ACE2 expression in different issues is still unclear, especially the respiratory mucosa and digestive mucosa. In the current study, we explored the effect of cigarette smoking on ACE2 expression pattern by analyzing a clinical case series of COVID-19 patients and detecting ACE2 expression of tissues from smoking and non-smoking patients and healthy subjects.

MATERIALS AND METHODS
Clinical data of smoking and non-smoking COVID-19 patients
Clinical data of the laboratory-confirmed patients with COVID-19 between Jan 25, 2020 and Feb 20, 2020, from the Third Affiliated Hospital of Sun Yat-sen University, Huanggang Central Hospital, Hainan General Hospital, Guangdong Second General Hospital, and Hubei Medical Team of the Third Affiliated Hospital of Sun Yat-sen University, were collected. The patients were grouped into smokers and non-smokers as current smokers with SI > 200 (moderate smoking or above) and never smoking, respectively [10]. The COVID-19 patients were stratified as severe or non-severe cases based on the severity on admission according to international guidelines [11]. The data were analyzed to investigate the effect of smoking on SARS-CoV-2 infection, clinical features, and outcomes. Considering the small sample size of the above data, in order to assess the risk of SARS-CoV-2 infection in smokers and non-smokers, we compared the proportion of smokers in COVID-19 patients, routine viral pneumonia, and the general Chinese population on the basis of information reported in the related literature [12].

Detection of ACE2 expression in human tissues from smokers and non-smokers
Eight nasal mucosa samples from chronic hypertrophic rhinitis patients were collected during nasal endoscopic surgery. The tissues of pathologically negative incised margin of lung, esophagus, stomach, duodenum, small intestine, and colon mucosa were collected from 66 patients with lung cancer, gastric cancer, and colon cancer. The specimen providers were also grouped into smokers and non-smokers as described above. The expression of ACE2 was detected by immunohistochemistry. For immunohistochemical staining, 4 μm paraffin sections were prepared and the slides were subsequently dewaxed, rehydrated, heated in citrate buffer for antigen retrieval, and incubated in 3% hydrogen peroxide in methanol for 10 minutes to quench endogenous peroxidase. Next, the sections were treated with 10% goat serum (Bioss, Beijing, China) to block the non-specific binding and incubated with rabbit polyclonal antibody against ACE2 (1:400 dilution, Abcam, Cambridge, CB, UK) at 4°C overnight. Equal concentrations of species- and subtype-matched antibodies were used as negative control. The slides were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:100 dilution, Bioworld, Bloomington, MN, USA) at 37°C for 60 minutes. All the sections were stained with 3,3'-diaminobenzidine (DAB) solution (Bioss, Beijing, China), and counterstained with hematoxylin (Solarbio, Beijing, China). Finally, the slides were observed by two blinded experimenters. Five randomly selected high-power fields (HPF) were analyzed to quantify the ACE2 expression by using image pro-plus 6.0 analysis software (Media Cybernetics, Inc., Silver Spring, MD, USA), and the results were presented as average optical density value per unit area.

Ethical requirements
The study including the clinical data and samples collection was approved by Ethics Committee of the Third Affiliated Hospital, Sun Yat-sen University.

Statistical analysis
Continuous variables were tested for normality using the Shapiro-Wilk test and are expressed as means ± standard deviation (SD), or medians with interquartile range (IQR) according to normality test results. Categorical variables were presented as counts and percent-
ages. Quantitative data were analyzed using t-test or Mann-Whitney test, qualitative data using χ²-test (Bonferroni correction used, if necessary), and proportion using Z-test. p-value < 0.05 indicated statistical difference, < 0.01 indicated significant difference. Statistical analysis was performed using SPSS 25.0 statistical software (IBM Corp. USA).

RESULTS

The proportion of smokers in COVID-19 patients was lower than general population of China

Of all 295 laboratory-confirmed cases recruited between Jan 25 and Feb 20, 2020, 24 (8.14%) were smokers and 271 (91.86%) were non-smokers. The clinical manifestations of smoking or non-smoking COVID-19 patients were different, as shown by Table 1. Fever was the main symptom, similar in the two groups. Although there were less patients with cough in smokers than non-smokers (37.50% vs. 61.62%, p = 0.021), the proportion of dyspnea cases in smokers was higher than non-smokers (50.00% vs. 28.04%, p = 0.024). Importantly, the data indicated that the proportions of severe cases as well as death cases in COVID-19 patients with cigarette smoking were higher than non-smokers (54.17% vs. 25.09%, p = 0.002; 8.33% vs. 0.37%, p = 0.018, respectively), consistent with the conclusion of the latest meta-analyses that current smokers were at higher risk of more severe COVID-19 than non-smokers [18,19].

According to the smoking rate and age stratification information of the general population, the proportion of smokers in COVID-19, routine viral pneumonia, and general population in China from literature [3-5,12] were compared, as shown by Table 2. The results suggested that the proportion of current smokers in the COVID-19 patients was lower than that in the general population (9.80% vs. 28.62% respectively, p < 0.001), and also lower than that in routine viral pneumonia (9.80% vs. 17.69%, p = 0.003), which were similar to our case series.

Smoking down-regulates ACE2 expression in the respiratory and digestive mucosal epitheliums

In nasal mucosa, ACE2 was positively expressed in epithelial cells and inflammatory cells in the lamina propria. It was significantly lower in smokers than non-smokers (p = 0.036) (Figure 1A). Alveolar epithelial cells and macrophages in the alveolar cavity in non-smokers showed significant ACE2 immunostaining, while ACE2 expression in smokers was significantly down-regulated (p = 0.030) (Figure 1A). In contrast, ACE2 was mainly expressed in the ciliated columnar epithelial cells of bronchial mucosa, which showed no difference between smokers and non-smokers (p = 0.737) (Figure 1A). We further compared the expression of ACE2 in different anatomic positions of the respiratory epithelium, and found that there was no difference between nasal mucosa, bronchial mucosa, and pulmonary alveoli in smokers (p = 0.100 between nasal mucosa and bronchial mucosa, p = 0.057 between nasal mucosa and pulmonary alveoli, p = 0.531 between bronchial mucosa and pulmonary alveoli) (Figure 1B). Interestingly, the ACE2 expression in pulmonary alveoli was significantly higher than that in nasal mucosa and bronchial mucosa in non-smokers (p = 0.039 between pulmonary alveoli and nasal mucosa, p = 0.007 between pulmonary alveoli and bronchial mucosa, p = 0.173 between nasal mucosa and bronchial mucosa) (Figure 1C).

Besides, in digestive tracts, ACE2 was widely expressed in mucosal epithelial cells of esophagus, small intestine and colon, and in glands of stomach and duodenum. The expression of ACE2 in stomach, duodenum, and small intestine was lower in smokers than non-smokers (p = 0.016, p = 0.003, p = 0.004, respectively) (Figure 2A). In esophagus and colon, ACE2 expression also showed a down-regulation trend in smokers, although not statistically significant (p = 0.057, p = 0.072, respectively) (Figure 2A). Moreover, smokers and non-smokers showed different expression patterns of ACE2 in different segments of digestive tract. In smokers, ACE2 expression in duodenum was highest, which was significantly higher than esophagus, stomach, small intestine, and colon (p = 0.036, p = 0.032, p < 0.001, p = 0.004, respectively) (Figure 2B). While in non-smokers, ACE2 expression in stomach and duodenum was highest, both of which were significantly higher than that in esophagus, small intestine, and colon (p < 0.001 between stomach and esophagus, p < 0.001 between stomach and small intestine, p < 0.001 between stomach and colon, p = 0.002 between duodenum and esophagus, p < 0.001 between duodenum and small intestine, p = 0.001 between duodenum and colon) (Figure 2C).

DISCUSSION

Due to the continuous human-to-human transmission, the number of confirmed and death cases of COVID-19 continues to increase worldwide. So far, COVID-19 has developed into a global pandemic disease, the health systems of countries around the world are still facing unprecedented pressure, and the WHO calls on the world to strengthen cooperation and jointly respond to the epidemic [2,13]. In terms of clinical characteristics, some patients showed upper respiratory symptoms such as sore throat and nasal mucus and congestion, and a few patients showed gastrointestinal symptoms such as diarrhea, vomit, and anorexia [2,13]. The vast majority of COVID-19 patients manifested as fever and cough. As the condition worsened, some patients soon developed acute respiratory distress syndrome, heart failure, even multiple organ failure, and the risk of death was greatly increased. It is worth noting that the current consolidated epidemiological data worldwide have indicated the fairly low proportion of active smokers among all the COVID-19
### Table 1. Clinical characteristics of 295 laboratory-confirmed COVID-19 cases.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Smoker (n = 24)</th>
<th>Non-smoker (n = 271)</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58.5 (37 - 69.75)</td>
<td>55 (45 - 67)</td>
<td>0.953</td>
</tr>
<tr>
<td>Gender - male</td>
<td>23 (95.83%)</td>
<td>120 (44.28%)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical manifestation</th>
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<tbody>
<tr>
<td>Fever</td>
<td>15 (62.50%)</td>
<td>208 (76.75%)</td>
<td>0.119</td>
</tr>
<tr>
<td>Cough</td>
<td>9 (37.50%)</td>
<td>167 (61.62%)</td>
<td>0.021</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>12 (50.00%)</td>
<td>76 (28.04%)</td>
<td>0.024</td>
</tr>
<tr>
<td>Fatigue</td>
<td>6 (25.00%)</td>
<td>86 (31.73%)</td>
<td>0.495</td>
</tr>
<tr>
<td>Rhinorrhea</td>
<td>1 (4.17%)</td>
<td>26 (9.59%)</td>
<td>0.494</td>
</tr>
<tr>
<td>Sore throat</td>
<td>1 (4.17%)</td>
<td>15 (5.54%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Headache</td>
<td>1 (4.17%)</td>
<td>12 (4.43%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>2 (8.33%)</td>
<td>34 (12.55%)</td>
<td>0.750</td>
</tr>
<tr>
<td>Nausea/vomit</td>
<td>2 (8.33%)</td>
<td>9 (3.32%)</td>
<td>0.222</td>
</tr>
<tr>
<td>Anorexia</td>
<td>5 (20.83%)</td>
<td>48 (17.71%)</td>
<td>0.781</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>1 (4.17%)</td>
<td>4 (1.48%)</td>
<td>0.348</td>
</tr>
<tr>
<td>Myalgia</td>
<td>3 (12.50%)</td>
<td>20 (7.38%)</td>
<td>0.415</td>
</tr>
</tbody>
</table>

| Comorbidities                 |                  |                      |         |
| Hypertension                  | 6 (25.00%)       | 54 (19.93%)          | 0.597   |
| Coronary heart disease        | 2 (8.33%)        | 18 (6.64%)           | 1.000   |
| Diabetes                      | 6 (25.00%)       | 28 (10.33%)          | 0.044   |
| COPD                          | 2 (8.33%)        | 4 (1.48%)            | 0.078   |
| Hepatitis                     | 3 (12.50%)       | 8 (2.95%)            | 0.051   |
| Kidney disease                | 1 (4.17%)        | 2 (0.74%)            | 0.225   |
| Cancer                        | 0 (0.00%)        | 7 (2.58%)            | 1.000   |
| Others                        | 0 (0.00%)        | 10 (3.69%)           | 1.000   |

| Clinical classification       |                  |                      |         |
| Severe                        | 13 (54.17%)      | 68 (25.09%)          |         |
| Non-severe                    | 11 (45.83%)      | 203 (74.91%)         |         |

| Clinical outcome              |                  |                      |         |
| Response                      | 20 (83.33%)      | 260 (95.94%)         | 0.025   |
| No response                   | 2 (8.33%)        | 10 (3.69%)           | 0.254   |
| Death                         | 2 (8.33%)        | 1 (0.37%)            | 0.018   |

† Statistics using t-test for age, χ²-test for others.

### Table 2. Proportion of smokers in confirmed COVID-19 cases compared with viral pneumonia and general population in China.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>COVID-19</th>
<th>Viral pneumonia</th>
<th>General population</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>1,623</td>
<td>147</td>
<td>98,058</td>
</tr>
<tr>
<td>Proportion of smokers</td>
<td>9.80%</td>
<td>17.69% ↑</td>
<td>28.62% ‡</td>
</tr>
</tbody>
</table>

† - Compare to COVID-19 using χ²-test (corrected by Bonferroni), χ² = 8.966, p = 0.003.
‡ - Compare to COVID-19 using Z-test, Z = 16.69, p < 0.001.
Figure 1. Angiotensin-converting enzyme 2 (ACE2) expression in the respiratory tract mucosa was down-regulated in smokers.

A. Representative photomicrographs (100 x and 400 x) showing immunohistochemistry staining of ACE2 in the nasal mucosa, bronchial mucosa, and pulmonary alveoli in smokers (n = 3, 3, 4, respectively) and non-smokers (n = 5, 5, 7, respectively). Red arrows show epithelial cells, and black arrows show inflammatory cells. The protein expressions of ACE2 were quantified and differences between smoker and non-smoker groups were analyzed by using t-test or Mann-Whitney test. B. Differences of ACE2 expressions between nasal mucosa, bronchial mucosa, and pulmonary alveoli were analyzed by using t-test or Mann-Whitney test. Data are shown as means ± standard deviation (SD), or medians with interquartile range (IQR).

Consistently, we found that the proportion of smokers in COVID-19 patients was significantly lower. However, both literature reports [15] and our data analysis have suggested that smokers may have more severe outcomes in COVID-19. To explore the underlying mechanism of this situation, we investigated the viral receptors ACE2 in human respiratory and digestive tissues.

ACE2 was identified as an important functional receptor for SARS-CoV [16]. The receptor-binding domain of the viral Spike protein (S-RBD) can bind to the N-terminal helix of ACE2, then cause endocytosis, which is essential for viral infection [17]. SARS-COV-2 belongs to lineage B β-coronavirus and shares high sequence identity with that of SARS-CoV according to phylogenetic analysis [18]. Based on SARS-CoV S-RBD-ACE2 complex structure, researchers quantified the interactions of SARS-COV-2 S-RBD with human receptor ACE2 and found that ACE2 bound to the 2019-nCoV S ectodomain with much stronger affinity compared with the SARS-CoV RBD [19,20]. Therefore, researchers have tried to block the binding of SARS-COV-2 S-RBD and ACE2 using monoclonal antibody or recombinant fusion protein for treatment of COVID-19 [21,22].

ACE2 is usually localized on the luminal surface of res-
Figure 2. ACE2 expression in the digestive tract mucosa was down-regulated in smokers.

A. Representative photomicrographs (100 x and 400 x) showing immunohistochemistry staining of ACE2 in the esophagus, stomach, duodenum, small intestine, and colon in smokers (n = 3, 4, 5, 6, 5, respectively) and non-smokers (n = 4, 5, 4, 6, 5, respectively). Red arrows show epithelial cells, and yellow arrows show glands. The protein expression of ACE2 were quantified and differences between smoker and non-smoker groups were analyzed by using t-test or Mann-Whitney test. B. Differences of ACE2 expressions between esophagus, stomach, duodenum, small intestine, and colon were analyzed by using t-test or Mann-Whitney test. Data are shown as means ± SD, or medians with IQR.
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Declarati on of Interest:

The author reports no conflicts of interest in this work.

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