Smoking Enhances the Expression of Angiotensin-Converting Enzyme 2 Involved in the Efficiency of Severe Acute Respiratory Syndrome Coronavirus 2 Infection

Rigel Suzuki[1,#], Yuki Ono[2,#], Koji Noshita[3,4,#], Kwang Su Kim[5], Hayato Ito[1], Yuhei Morioka[1], Tomokazu Tamura[1], Daisuke Okuzaki[6], Tetsuzo Tagawa[2], Tomoyoshi Takenaka[2], Tomoharu Yoshizumi[2], Teppei Shimamura[7], Shingo Iwami[5,8,9,10,11,12], and Takasuke Fukuhara[1,*]

[1]Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Sapporo, 060-8638, Hokkaido, Japan
[2]Department of Surgery and Science, Graduate School of Medicine, Kyushu University, Fukuoka, 819-0395, Fukuoka, Japan
[3]Department of Biology, Kyushu University, Fukuoka, 819-0395, Fukuoka, Japan
[4]Plant Frontier Research Center, Kyushu University, Fukuoka, 819-0395, Fukuoka, Japan
[5]Interdisciplinary Biology Laboratory (iBLab), Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya, 464-8602, Aichi, Japan
[6]Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, Suita, 565-0871, Osaka, Japan
[7]Division of Systems Biology, Graduate School of Medicine, Nagoya University, Nagoya, 464-8602, Aichi, Japan
[8]Institute of Mathematics for Industry, Kyushu University, Fukuoka, 819-0395, Fukuoka, Japan
[9]Institute for the Advanced Study of Human Biology (ASHBi), Kyoto University, Kyoto, 606-8501, Kyoto Japan
[10]NEXT-Ganken Program, Japanese Foundation for Cancer Research (JFCR), Koutou, 135-8550, Tokyo, Japan
[11]Interdisciplinary Theoretical and Mathematical Sciences (iTHEMS), RIKEN, Wako, 351-0198, Saitama, Japan
[12]Science Groove Inc., Fukuoka, 810-0041, Fukuoka, Japan

#These authors contributed equally to this work.

*Corresponding Author: Takasuke Fukuhara
Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Sapporo, 060-8638, Hokkaido, Japan
Tel: +81-11-706-6905
Fax: +81-11-706-6905
E-mail: fukut@pop.med.hokudai.ac.jp (T.F.)

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This study is retrospective study. Enrolled patients were not required to give informed consent to this study because the analysis used specimens acquired from surgery. We applied opt-out method on this study by announcing this research on website of Kyushu University.

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Co-authors:
Rigel Suzuki, rigels@pop.med.hokudai.ac.jp
Yuki Ono, i.l.b.o.c.0704@gmail.com
Koji Noshita, noshita@morphometrics.jp
Kwang Su Kim, kwangsu815@gmail.com
Hayato Ito, ito.hayato.c4@elms.hokudai.ac.jp
Yuhei Morioka, ym6831@icloud.com
Tomokazu Tamura, tomokazu.tamura@pop.med.hokudai.ac.jp
Daisuke Okuzaki, dokuzaki@biken.osaka-u.ac.jp
Tetsuzo Tagawa, t_tagawa@surg2.med.kyushu-u.ac.jp
Tomoyoshi Takenaka, takenaka.tomoyoshi@gmail.com
Tomoharu Yoshizumi, yoshizumi.tomoharu.717@m.kyushu-u.ac.jp
Teppei Shimamura, shimamura@med.nagoya-u.ac.jp
Shingo Iwami, iwamishingo@gmail.com
Takasuke Fukuhara, fukut@pop.med.hokudai.ac.jp

Abstract

Smoking is one of the risk factors most closely related to the severity of COVID-19. However, the relationship between smoking history and SARS-CoV-2 infectivity is unknown. In this study, we evaluated the *ACE2* expression level in the lungs of current smokers, ex-smokers, and non-smokers. The *ACE2* expression level of ex-smokers who smoked cigarettes until recently (cessation period shorter than 6 months) was higher than that of non-smokers and ex-smokers with a long history of non-smoking (cessation period longer than 6 months). We also showed that the efficiency of SARS-CoV-2 infection was enhanced in a manner dependent on the ACE2 expression level. Using RNA-seq analysis on the lungs of smokers, we identified that the expression of inflammatory signaling genes was correlated with *ACE2* expression. Notably, with increasing duration of smoking cessation among ex-smokers, not only *ACE2* expression level but also the expression levels of inflammatory signaling genes decreased. These results indicated that smoking enhances the expression levels of *ACE2* and inflammatory signaling genes. Our data suggest that the efficiency of SARS-CoV-2 infection is enhanced by smoking-mediated upregulation of *ACE2* expression level.

Keywords: ACE2; COVID-19; inflammation; SARS-CoV-2; smoking.

Abbreviations: ACE2, angiotensin-converting enzyme 2; COVID-19, coronavirus disease 2019; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; $TCID_{50}$, 50% tissue culture infective doses; TMPRSS2, transmembrane protease serine 2; COPD, chronic obstructive pulmonary disease;

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the genus *Betacoronavirus* in the family Coronaviridae is the causative agent of the global pandemic of severe respiratory disease, coronavirus disease 2019 (COVID-19) [1]. The virus was initially discovered in Wuhan, China, in late December 2019 [2-4] and has spread worldwide. As of August, 8, 2022, more than 585 million COVID-19 cases have been confirmed in over 180 countries, and more than six million deaths have been reported (https://covid19.who.int/).

To enter host cells, SARS coronavirus binds its spike protein to a host cell receptor, ACE2 (angiotensin-converting enzyme 2); a recent study has also reported that SARS-CoV-2 uses ACE2 for cell entry [5]. Moreover, the spike protein needs to be activated and cleaved by host cell enzyme proteases such as transmembrane protease serine 2 (TMPRSS2) and FURIN [6,7]. Upon examining plasma ACE2 during hospitalization due to COVID-19, elevated baseline plasma ACE2 in COVID-19 patients was significantly associated with increased disease severity during the hospitalization period [8]. Therefore, ACE2 would be a risk factor for a more serious COVID-19 presentation, and the levels of the above-mentioned proteins involved in the infectious pathway should have significant implications for clinical outcomes. Identifying the background of populations with high expression of the *ACE2* gene may be important not only for public health but also for prevention and treatment of COVID-19.

Smoking is one of the risk factors for respiratory infectious disorders, including viral infections [9]. Inflammatory cells infiltrate into the mucosa and glandular tissue due to smoke exposure, which leads to the excessive production of mucus. This has additional harmful effects, such as epithelial-cell hyperplasia, prevention of tissue repair, thickened small bronchioles, and emphysema [10]. A previous investigation reported that a significantly higher proportion of patients with a history of smoking exhibited a rapid deterioration in health during their admission for COVID-19 compared with non-smokers (27% versus 3%, $p = 0.018$), suggesting that smoking may have a harmful effect on COVID-19 prognosis [11]. Moreover, smoking history was found to be associated with a severe condition of COVID-19 among young adults [12], and a meta-analysis revealed that smoking is a risk factor for the progression of COVID-19, with smokers having 1.91 times the odds of deteriorating COVID-19 severity than never-smokers [13]. Biologically, cigarette smoking reportedly increases the expression of ACE2 in the lower airways [14]. Although smoking has been confirmed to confer a risk of severe COVID-19, the relationship between smoking history and SARS-CoV-2 infectivity is unknown.

In this study, we evaluated the association between smoking history and the *ACE2* expression of lung tissue. We revealed a correlation between the period of smoking cessation and the expression of *ACE2*. Virological and mathematical analyses showed that ACE2 expression level is important for the efficiency of SARS-CoV-2 infection. In addition, RNA-seq analysis showed that the expression of inflammatory signaling genes also correlates with the period of smoking cessation and *ACE2* expression level. These results suggest that smoking upregulates the *ACE2* expression level that is involved in the efficiency of SARS-CoV-2 infection.

2. Materials and Methods

2.1. Lung tissue sample collection and RT-qPCR

Lung tissue was obtained from patients who underwent surgery at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, between January 2013 and December 2019. Clinicopathological features were examined: sex, blood type, smoking history, and respiratory disease [chronic obstructive pulmonary disease (COPD) or interstitial pneumonia (IP)]. If the period of smoking cessation before surgery was less than 6 months, this was categorized into 1-month intervals. Patients who still smoked or who had a cessation period shorter than 6 months were defined as current smokers, while patients with a cessation period longer than 6 months were defined as ex-smokers. Clinical information and follow-up data were obtained from the patients' medical records. Tissue samples were immediately flash-frozen in liquid nitrogen after resection and stored at −80°C until the preparation of cDNA from RNA extracted from the samples. Total RNA of lung tissue was extracted using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan, #315-02504). The concentration of total RNA was measured using a DS-11 Series Spectrophotometer (DeNovix, Wilmington, USA). Total RNA (1 μg) was reverse-transcribed into cDNA using Super Script III First-Strand Synthesis Super Mix (Invitrogen, Thermo Fisher Scientific, Inc., Massachusetts, USA, #11752050), in accordance with the manufacturer's protocol. qPCR was performed with Applied Biosystems StepOnePlus real-time PCR system (Life Technologies, California, USA). TaqMan gene expression assays (Applied Biosystems, Massachusetts, USA) for *ACE2* (Hs00965485_g1), *TMPRSS2* (Hs01122322_m1), and *FURIN* (Hs00965485_g1) were used, while *GAPDH* (Hs01122322_m1) was used as an internal control. Relative expression of *ACE2*, *TMPRSS2*, and *FURIN* was calculated using the ΔΔCT method. This study was approved by Kyushu University Institutional Review Board for Clinical Research (2020-807).

2.2. Statistical analysis and normalization

All assays were performed independently at least two times. The data were summarized in box plots. The statistical significance of associations of *ACE2*, *TMPRSS2*, and *FURIN* expression levels with clinical background factors was tested by the Mann–Whitney *U*-test. The trend for changes of *ACE2* expression levels with increasing duration of smoking cessation was tested by the Jonckheere–Terpstra test.

2.3. Cells

TMPRSS2-expressing Vero E6 (VeroE6/TMPRSS2) cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB1819) and maintained in low-glucose Dulbecco's Modified Eagle's Medium (low-glucose DMEM) (Sigma-Aldrich, St. Louis, USA, #D6046) containing 10% fetal bovine serum (FBS) (Biowest, Bradenton, France, #S1810) and G418 (Nacalai Tesque, Kyoto, Japan, #09380-44). The HEK293-3P6C33 (HEK293/tet-ACE2) cells[15] were a gift from Dr. Matsuura at Osaka University, and maintained in high-glucose Dulbecco's Modified Eagle's Medium (Nacalai Tesque, #08459-35) containing 10% FBS and blasticidin (solution) (10 μg/ml) (Invivogen, California, USA, #ant-bl-1), and the exogenous expression of ACE2 and TMPRSS2 was induced by the addition of doxycycline hydrochloride (1 μg/ml) (Sigma-Aldrich, #D5207). All of the above cells were cultured at 37°C under 5% CO_2.

2.4. Plasmids

Full-length cDNAs for *TMPRSS2* were amplified by PCR from a cDNA library derived from HEK293/tet-ACE2 cells. The PCR fragment was cloned into the pCSII-EF-based vector with a C-terminal HA tag. This expression vector was used for experiments after verification of the sequence of inserted DNA.

2.5. SARS-CoV-2 preparation and titration

B1.1 (GISAID ID: EPI_ISL_408667), B.1.1.7 variant (GISAID ID: EPI_ISL_804007), B.1.351 variant (GISAID ID: EPI_ISL_1122890), and P.1 variant (GISAID ID: EPI_ISL_833366) were obtained from the National Institute of Infectious Diseases. All viruses were amplified in VeroE6/TMPRSS2 cells and the culture supernatants were harvested and stored at −80°C until use. The infectious titers in the culture supernatants were determined by the 50% tissue culture infective doses ($TCID_{50}$). The culture supernatants of cells were inoculated onto VeroE6/TMPRSS2 cells in 96-well plates after 10-fold serial dilution with DMEM containing 2% FBS, and the infectious titers were determined at 72 h post-infection (hpi). All experiments involving SARS-CoV-2 were performed in biosafety level-3 laboratories, following standard biosafety protocols approved by Hokkaido University.

2.6. SARS-CoV-2 infection

HEK293/tet-ACE2 cells (200,000 cells) treated with 0.05–1000 ng/ml doxycycline for 24 h were seeded

into a 12-well plate. One day prior to infection, TMPRSS2-HA was transfected into the cells with Trans IT LT-1 (Mirus, Wisconsin, USA, #MIR2306), following the manufacturer's protocol. Twenty-four hours later, SARS-CoV-2 was inoculated and incubated at 37℃ for 1 h. The cells were washed and new culture medium was added. After 18~19 h of incubation, the infected cells were harvested and subjected to western blotting and RT-qPCR.

2.7. Human lung tissue extracts

Human lung tissue (~10 mg) was ground using BioMasher II (Nippi, Tokyo, Japan, #320203) in RIPA buffer containing 50 mM Tris-HCL pH 7.6, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM NaF, and 25 μM MG-132. The lysates were kept on ice for 30 min and sonicated using Bioruptor® II (Sonicbio Co., Ltd., Nagoya, Japan). After centrifugation at 10,000 g for 20 min at 4℃, the supernatants were mixed with SDS sample buffer (Bio-Rad, Callfornia, USA, #1610737).

2.8. Western blotting

Whole-cell lysates or the lung extracts were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride transfer membranes (Millipore, Massachusetts, USA, #IPVH00010). The membranes were then immunoblotted with specific antibodies as indicated and subsequently incubated with horseradish peroxidase-conjugated antibody against mouse or rabbit immunoglobulin (Jackson ImmunoResearch Laboratories, Pennsylvania, USA, #115-0035-003 and #111-035-003), followed by detection with ECL western blotting detection reagents (Cytiva, Massachusetts, USA, #RPN2106). The following antibodies were used in this study: anti-SARS-CoV Nucleoprotein (Sino Biological, Beijing, China, #40143-MM05), anti-ACE2 (Proteintech, Rosemont, USA, #21115-1-AP), anti-GAPDH (Wako, Osaka, Japan, #NBP2-27103H) and anti-HA (BioLegend, SanDiego, USA, #902301).

2.9. RT-qPCR from cell

Total RNA was extracted from the SARS-CoV-2-infected cells using a RNeasy Mini Kit (QIAGEN, #74104). The sample was used as the template for RT-qPCR performed in accordance with the manufacturer's protocol using the One Step PrimeScript™ III RT-qPCR Mix (Takara, #RR600B) and the following primers and probe: Forward, 5′-CAC ATT GGC ACC CGC AAT C-3′; Reverse, 5′-GAG GAA CGA GAA GAG GCT TG-3′; Probe, FAM-ACT TCC TCA AGG AAC AAC ATT GCC A-BHQ. These primers target the *nucleocapsid* gene of SARS-CoV-2. Fluorescent signals were acquired using a StepOnePlus™ Real Time PCR System (Applied Biosystems).

2.10. Quantifying viral RNA corresponding to ACE2 expression level

We evaluated the dependence of viral RNA replication on *ACE2* expression using the Hill function:

$$V = \frac{1}{1 + \left(\frac{A}{EC_{50}}\right)^{-m}}, \qquad (1)$$

where V is the amount of viral RNA normalized by the maximum viral RNA for each strain, A is the *ACE2* expression level, EC_{50} is the *ACE2* expression level that achieves 50% of maximum expressed viral RNA, and m is the Hill coefficient (corresponding to the steepness of the curve). We used the least-squares regression approach to fit Eq. (1) to viral RNA data and estimated the values of EC_{50} and m.

2.11. Non-negative matrix factorization

To extract representative patterns of expression profiles and investigate the relationship between these patterns and clinical background, we used non-negative matrix factorization (NMF) for RNA-seq data of 23 normal lung tissues. We first selected the top 8,000 genes ranked by variance in read counts. In NMF, for the given expression matrix X for 23 samples and 8,000 genes, we sought matrices W and H such that

$$X \approx WH$$

where W is a $23 \times k$ contribution matrix, H is a $k \times 8{,}000$ excitation matrix, and k is the number of factors. NMF generates factors with significantly reduced dimensions compared with the original matrix. The key property of NMF is that W and H are constrained to be positive matrices. The (n, l)-element $w_{n,l}$ of matrix W can be interpreted as the contribution to factor l of sample n, and the (l, k)-element $h_{l,k}$ of matrix H can be interpreted as the relative abundance of the k-th gene given factor l. In our analysis, W and H are estimated using a variational Bayesian inference [16] and the number of factors was selected by the evidence lower bound (ELBO).

2.12. Pathway enrichment analysis

To identify key pathways that are related to each factor in NMF, pathway enrichment analysis using Fisher's exact test was performed on the top 500 genes for each factor ranked by coefficients, as supplied by columns of W. The gene lists of 321 pre-annotated pathways collected from KEGG (http://www.genome.jp/kegg/) were used for enrichment analysis and pathways with a p-value $< 10^{-5}$ were selected as significant.

3. Results

3.1. ACE2 expression level is associated with smoking

To investigate the associations of *ACE2*, *TMPRSS2*, and *FURIN* expression with clinical background, we analyzed the expression levels of these genes by RT-qPCR using normal human lung tissues. We tested *ACE2* (Hs00965485_g1, Applied Biosystems), *TMPRSS2* (Hs01122322_m1, Applied Biosystems), *FURIN* (Hs00965485_g1, Applied Biosystems), and *GAPDH* (Hs01122322_m1, Applied Biosystems) expression levels, and it was confirmed that the expression of these target genes could be measured by the ΔΔCT method. Samples with a *GAPDH* CT value less than 28 were included in this analysis. Finally, 245 patients whose normal lung tissues were sampled from 2013 to 2018 were enrolled in this study. The expression level of *ACE2* was not associated with other clinical background factors apart from smoking and COPD/IP. However, the *ACE2* expression of smokers was significantly higher than that of non-smokers (p=0.00272) (Figure 1A), while the *ACE2* expression of current smokers was higher than that of ex-smokers (p=0.00011, Figure 1B), which was consistent with previous results[17]. Moreover, the expression showed a statistically significant decrease with increasing duration of smoking cessation (p<0.0001, Figure 1C and Figure S1). Of the 184 patients with a history of smoking, one patient was excluded because the duration of smoking cessation was unknown. Our results demonstrated that current smokers had higher expression of *ACE2* than ex-smokers and the long period of smoking cessation might decrease *ACE2* expression in normal lung tissue. Meanwhile, *TMPRSS2* and *FURIN* expression was not associated with clinical factors, including smoking status, except for *FURIN* expression differing between females and males (Figure 1D and 1E). These results indicate that ACE2 may be the protein that is most associated with smoking.

3.2. ACE2 expression level is important for the efficiency of SARS-CoV-2 infection

Although ACE2 is a receptor for SARS-CoV-2 infection, it is not yet clear to what extent ACE2 expression levels affect virus propagation. To clarify whether differences of ACE2 expression levels affect the infectivity of SARS-CoV-2, SARS-CoV-2 infectivity to HEK293 cells expressing ACE2 at various levels was examined. First, we established HEK293 cells in which ACE2 expression could be induced with doxycycline (HEK293/tet-ACE2)[15]. Moreover, HA-tagged TMPRSS2 was expressed in HEK293/tet-ACE2 cells (Figure S2). The expression levels of ACE2 depended on the concentration of doxycycline added to the supernatant of HEK293/tet-ACE2. At 1 day after treatment with tetracycline, D614G-bearing B.1.1 isolate (GISAID ID: EPI_ISL_408667), B.1.1.7 variant (GISAID ID: EPI_ISL_804007), B.1.351 variant (GISAID ID: EPI_ISL_1122890), and P.1 variant (GISAID ID: EPI_ISL_833366) were used to infect HEK293 cells expressing ACE2 at various levels. As the expression level of ACE2 increased, N protein levels in the cells were increased (Figure 2A). Moreover, viral RNA in the cells were also increased in a manner dependent on the level of ACE2 expression (Figure 2B). These results suggest that high expression of ACE2 contributes to the efficient propagation of SARS-CoV-2. Interestingly, low expression of ACE2 facilitated replication of B1.1.7, B.1.351, and P.1 variants, compared with that of the B1.1 isolate, suggesting that the spike protein of the variants can bind ACE2 more efficiently than that of B1.1 (Figure 2B). Many reports have shown that N501Y in these variants enhances the efficiency of binding between spike protein and ACE2[18-20]. To confirm that the expression level of ACE2 in normal human lung tissue falls within the range of ACE2 protein levels induced by doxycycline, we quantified the ACE2 protein levels of six human lung tissues. ACE2 expression was normalized using GAPDH. We found that the normalized ACE2 expression level was 0.330–0.769 in human lung tissues (Figure 2C). As shown in Fig. 2A, the expression level of ACE2 protein induced by doxycycline was 0.072–1.759, indicating that the ACE2 expression level in the doxycycline-treated cells included the range of that in human lung tissue. Next, to further clarify the effect of ACE2 expression level on the replication of SARS-CoV-2, we first quantified the maximum level of viral RNA for each strain: P.1 variants showed a higher level of viral RNA (9.2×10^6) than the other variants (B1.1.7: 6.0×10^6, B1.1: 3.7×10^6, B1.1.7: 2.5×10^6). To evaluate the dependence of viral RNA replication on ACE2 expression, we normalized the viral RNA by its maximum level (Figure 2D) and fitted the Hill function [see Eq. (1) in Materials and Methods for details]. Compared with other variants, we found that the B1.1 isolate showed the highest EC_{50} and the lowest m (Figure 2E). This implies that B1.1.7, B.1.351, and P.1 variants replicate their viral RNA more efficiently than the B1.1 isolate at the same ACE2 expression level.

3.3. Identification of pathways involved in ACE2 expression in human lung

To identify representative patterns of gene expression profiles in human normal lung tissues and to investigate the association between *ACE2* expression and clinical background, we performed non-negative matrix factorization on the gene expression matrix of 23 normal lung tissue samples and 8,000 genes. Six representative factors were selected to maximize the evidence lower bound (ELBO), and their contributions to each sample are shown in Figure 3a. Among the six factors, factors 3 and 4 were significantly correlated

with *ACE2* expression (Figure 3B). Pearson's correlation coefficients of factors 3 and 4 for *ACE2* were 0.546 and 0.608, and their significance levels were p=0.0071 and p=0.0021, respectively. We also found that the scores of factors 3 and 4 tended to be higher with a shorter duration of smoking cessation (Figure 3C). Furthermore, we investigated pathways enriched in relation to factors 3 and 4 using Fisher's exact test (Figure 3D). We showed that factor 4 was associated with immune-related pathways such as TNF signaling and IL-17 signaling, and infectious disease-related pathways such as human papillomavirus, Kaposi sarcoma-associated herpesvirus, salmonella, human cytomegalovirus, influenza A, and malaria. Among the six representative patterns of gene expression profiles, the results summarized that two were related to *ACE2* expression and the duration of smoking cessation, and one was associated with immunity- and infection-related molecular pathways.

4. Discussion

Smoking is considered to be one of the risk factors for severe COVID-19. Indeed, smoking has been reported to increase the mortality rate and severity of COVID-19 [21]. The WHO has also indicated that smoking is a factor in the severity of SARS-CoV-2 infections. However, despite the clear involvement of smoking in SARS-CoV-2 infection, many unanswered questions remain regarding how the extent and duration of smoking affect SARS-CoV-2 infection. In this study, we found that the expression level of *ACE2* was significantly increased in smokers (Figure 1A). In addition, the *ACE2* expression of current smokers was higher than that of ex-smokers (Figure 1D). Meanwhile, we found no differences of *TMPRSS2* and *FURIN* expression levels between smokers and non-smokers (Figure 1B and C). These results suggest that smoking enhances the efficiency of SARS-CoV-2 infection through increased expression of ACE2. In fact, the efficiency of SARS-CoV-2 infection increased in a manner dependent on the level of ACE2 expression (Figure 2A and B). Moreover, as a result of mathematical analysis of the dependence of viral RNA replication on ACE2 expression, we found that B1.1.7, B.1.351, and P.1 variants tended to propagate more efficiently than B.1.1 at the same ACE2 expression level (Figure 2D and E). This could indicate one of the reasons why B1.1.7, B.1.351, and P.1 variants spread more rapidly throughout the world. In addition, we identified two groups of genes that correlate with *ACE2* expression using RNA-seq (Figure 3A and B). The duration of smoking cessation as well as *ACE2* expression was associated with the upregulation of Factor 4, which is related to inflammation (Figure 3C). Although the genes comprising Factor 3 have various functions, the genes of Factor 4 are associated with inflammatory signaling (Figure 3D). These results suggest that there is a relationship between *ACE2* expression and inflammatory signaling.

Many papers have reported on the association between smoking and *ACE2* expression [14,17,22-28]. Some papers also reported that nicotine downregulates the expression level of *ACE2* in certain tissues and cell types [23-25,27]. However, in this study, we observed that the *ACE2* expression level was upregulated in current smokers. This discrepancy may have been due to the use of nicotine in previous studies. Tobacco contains many chemical substances, so substances other than nicotine may be involved in inflammation and *ACE2* expression. Smith et al. reported that the *ACE2* expression level is upregulated in lung tissue of COPD patients as well as smokers. Consistent with this report, we showed that not only smoking but also COPD increased *ACE2* expression levels in the lung. In several reports, COPD was identified as a risk factor for severe COVID-19 [29]. Therefore, COPD may also enhance the efficiency of SARS-CoV-2 infection and the severity of COVID-19 via increased expression of *ACE2*.

To understand the mechanism behind the effects of upregulated *ACE2*, we performed transcriptomic analysis to identify the gene expression patterns correlated with *ACE2* expression (Figure 3B). Factor 4 contains multiple pathways related to immune response, suggesting that *ACE2* expression is influenced by immune response. In fact, one study has reported that INF-α, INF-β, and INF-γ treatment increased *ACE2* expression in tracheal cells [17]. Moreover, cigarette smoke is an inflammatory substance and smokers tend to exhibit increases in inflammatory markers [17,30,31]. These findings suggest that inflammatory signaling induced by smoking is associated with the upregulation of *ACE2*. In the current study, we showed that the *ACE2* expression level of current smokers was higher than that of ex-smokers (Figure 1D). Moreover, with increasing duration of smoking cessation, the *ACE2* expression level was downregulated (Figure 1E). These results may indicate that the *ACE2* expression level decreases due to the alleviation of lung inflammation resulting from smoking cessation.

A variety of SARS-CoV-2 variants have been found globally. Among them, the WHO defined B1.1.7, B1.351, and P.1 as variants of concern, and named them alpha, beta, and gamma strains, respectively. The spike proteins of these variants have the N501Y mutation in common. It has been reported that the presence of this mutation may increase the efficiency of infection due to enhanced binding to ACE2 [32]. In this study, the infectivity of SARS-CoV-2 was enhanced in a manner dependent on ACE2 expression, and the infection ratios were efficiently established in these mutations at the same ACE2 expression level compared to the conventional strain (Figure 2A and B). The current data support the finding that N501Y mutation increases the binding of ACE2 to the spike protein of SARS-CoV-2. However, because clinical isolates were used in this study and other mutations may increase the efficiency of infection, detailed analysis using recombinant SARS-CoV-2 with N501Y mutation will be needed in the future.

In summary, we provide evidence that the expression levels of *ACE2* and inflammatory signaling genes are elevated in the lungs of current smokers and ex-smokers with a short period of smoking cessation. Moreover, increased ACE2 expression level is associated with elevated infectivity of SARS-CoV-2. These findings suggest that smoking history may be associated with the infectivity of SARS-CoV-2. An interesting challenge for the future will be to determine the detailed molecular pathway behind the upregulation of *ACE2* induced by smoking and inflammation.

Figure legends

Figure 1.

Expression of *ACE2*, *TMPRSS2*, and *Furin* in human lung tissues

Box plots of gene expression of (A) *ACE2*, (D) *TMPRSS2*, and (E) *FURIN* in normal lung tissue in association with clinical factors. The expression levels of *ACE2* were significantly different between smokers and non-smokers (Mann–Whitney *U*-test, p=0.00272), and between non-COPD/IP and COPD/IP sufferers (Mann–Whitney *U*-test, p=0.00342). No significant difference was found in the expression levels of *TMPRSS2*. The expression level of *FURIN* was significantly different between women and men (Mann–Whitney *U*-test, p=0.00496). (B) A box plot of the expression of *ACE2* among smokers. The expression level was significantly higher in current smokers (cessation period shorter than 6 months) than in former smokers (cessation period longer than 6 months); Mann–Whitney *U*-test, $p = 0.00011$. (C) A box plot of the expression of *ACE2* with the period of smoking cessation. The expression of *ACE2* tended to decrease with increasing duration of smoking cessation. Jonckheere–Terpstra test, $p < 0.0001$.

Figure 2.

ACE2 expression level is important for the efficiency of SARS-CoV-2 infection

(A) Dependence of the infection efficiency of SARS-CoV-2 on the expression level of ACE2. HEK293/tet-ACE2 cells were transfected with HA-tagged TMPRSS2. After 24h of transfection, TMPRSS2 expressing HEK293/tet-ACE2 cells were treated with various concentrations of doxycycline for 24 h. The cells were infected with SARS-CoV-2 (MOI=1). After 18 h of infection, the cell lysate was blotted with anti-ACE2, anti-N, anti-HA, and anti-GAPDH antibodies. (B) Involvement of ACE2 expression in the infection of various SARS-CoV-2 variants. HEK293/tet-ACE2 cells expressing HA-tagged TMPRSS2 with doxycycline treatment were infected with SARS-CoV-2 variants (MOI=1). After 19 h of infection, RNA was isolated from the cells and viral RNA was quantified by RT-PCR. The amount of viral RNA was normalized by dividing by the amount of minimum viral RNA in the doxycycline treatment or non-treatment. (C) The expression level of ACE2 in human lung tissue. The lysates from eight human normal lungs were blotted with anti-ACE2 and anti-GAPDH antibodies. Lower numbers represent the expression level of ACE2 normalized by the expression level of GAPDH. (D) The closed dots and solid line correspond to the observed data and the best-fit solution for Eq. (1). (E) Estimated values of EC_{50} and m are shown.

Figure 3.

Relationships between six factors identified by non-negative matrix factorization and *ACE2* expression, duration of smoking cessation, and pathways

(A) Heatmap of the 23 × 6 contribution matrix $\boldsymbol{H}$ estimated by non-negative matrix factorization and *ACE2* expression. The six factors were selected to maximize the ELBO. Each row represents the contribution of the six factors for each sample. (B) Scatter plots of the third and fourth factor scores (x-axis) and *ACE2* expression (y-axis). The line represents the prediction line for linear model fitting, and the filled colors represent the confidence interval. (C) Boxplots of the third and fourth factor scores for non-smokers and smokers with three durations of smoking cessation (> 6 months, 1 month to < 6 months, and < 1 month). (D) Significantly enriched pathways of the third and fourth factors. The x-axis represents $-\log_{10}(p\text{-value})$ of Fisher's exact test, while the y-axis represents the pathway name.

Author Contributions:

Conceptualization, Y.O., K.N., T.S., S.I., and T.F. Funding acquisition, R.S., K.N., S.I., and T.F. Methodology, R.S., Y.O., K.N., K.K., Y.M., D.O., T.S., S.I., and T.F. Investigation, R.S., Y.O., K.N., K.K., Y.M., H.I., T.T. (Tamura), D.O., T.T. (Tagawa), T.T. (Takenaka), T.Y. T.S., S.I., and T.F. Data curation, R.S., Y.O., K.N., T.S., S.I., and T.F. Writing—original draft preparation, R.S., Y.O., K.N., T.S., S.I., and T.F. Writing—review and editing, R.S., Y.O., K.N., K.K., Y.M., D.O., T.S., S.I., and T.F. Supervision, T.F. All authors have read and agreed to the published version of the manuscript.

References

1. Gorbalenya AE, Baker SC, Baric RS, et al. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol.* 2020;5(4):536-544.
2. Zhu N, Zhang D, Wang W, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med.* 2020;382(8):727-733.
3. Zou L, Ruan F, Huang M, et al. SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. *N Engl J Med.* 2020;382(12):1177-1179.
4. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory disease in China. *Nature.* 2020;579(7798):265-269.
5. Zhou P, Yang XL, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* 2020;579(7798):270-273.
6. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell.* 2020;181(2):271-280.e278.
7. Coutard B, Valle C, de Lamballerie X, Canard B, Seidah NG, Decroly E. The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antiviral Res.* 2020;176:104742.
8. Kragstrup TW, Singh HS, Grundberg I, et al. Plasma ACE2 predicts outcome of COVID-19 in hospitalized patients. *PLoS One.* 2021;16(6):e0252799.
9. Gozdek M, Zieliński K, Pasierski M, et al. Transcatheter Aortic Valve Replacement with Self-Expandable ACURATE neo as compared to Balloon-Expandable SAPIEN 3 in Patients with Severe Aortic Stenosis: Meta-analysis of Randomized and Propensity-Matched Studies. *J Clin Med.* 2020;9(3).
10. MacNee W. Pathophysiology of cor pulmonale in chronic obstructive pulmonary disease. Part One. *Am J Respir Crit Care Med.* 1994;150(3):833-852.
11. Liu W, Tao ZW, Wang L, et al. Analysis of factors associated with disease outcomes in hospitalized patients with 2019 novel coronavirus disease. *Chin Med J (Engl).* 2020;133(9):1032-1038.
12. Adams SH, Park MJ, Schaub JP, Brindis CD, Irwin CE. Medical Vulnerability of Young Adults to Severe COVID-19 Illness-Data From the National Health Interview Survey. *J Adolesc Health.* 2020;67(3):362-368.
13. Patanavanich R, Glantz SA. Smoking Is Associated With COVID-19 Progression: A Meta-analysis. *Nicotine Tob Res.* 2020;22(9):1653-1656.
14. Leung JM, Yang CX, Tam A, et al. ACE-2 expression in the small airway epithelia of smokers and COPD patients: implications for COVID-19. *Eur Respir J.* 2020;55(5).
15. Torii S, Ono C, Suzuki R, et al. Establishment of a reverse genetics system for SARS-CoV-2 using circular polymerase extension reaction. *Cell Rep.* 2021;35(3):109014.
16. Cemgil AT. Bayesian inference for nonnegative matrix factorisation models. *Comput Intell Neurosci.* 2009:785152.
17. Smith JC, Sausville EL, Girish V, et al. Cigarette Smoke Exposure and Inflammatory Signaling Increase the Expression of the SARS-CoV-2 Receptor ACE2 in the Respiratory Tract. *Dev Cell.* 2020;53(5):514-529.e513.

18. Tian F, Tong B, Sun L, et al. N501Y mutation of spike protein in SARS-CoV-2 strengthens its binding to receptor ACE2. *Elife.* 2021;10.
19. Luan B, Wang H, Huynh T. Enhanced binding of the N501Y-mutated SARS-CoV-2 spike protein to the human ACE2 receptor: insights from molecular dynamics simulations. *FEBS Lett.* 2021;595(10):1454-1461.
20. Zhu X, Mannar D, Srivastava SS, et al. Cryo-electron microscopy structures of the N501Y SARS-CoV-2 spike protein in complex with ACE2 and 2 potent neutralizing antibodies. *PLoS Biol.* 2021;19(4):e3001237.
21. Guan WJ, Ni ZY, Hu Y, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med.* 2020;382(18):1708-1720.
22. Brake SJ, Barnsley K, Lu W, McAlinden KD, Eapen MS, Sohal SS. Smoking Upregulates Angiotensin-Converting Enzyme-2 Receptor: A Potential Adhesion Site for Novel Coronavirus SARS-CoV-2 (Covid-19). *J Clin Med.* 2020;9(3).
23. Ferrari MF, Raizada MK, Fior-Chadi DR. Nicotine modulates the renin-angiotensin system of cultured neurons and glial cells from cardiovascular brain areas of Wistar Kyoto and spontaneously hypertensive rats. *J Mol Neurosci.* 2007;33(3):284-293.
24. Ferrari MF, Raizada MK, Fior-Chadi DR. Differential regulation of the renin-angiotensin system by nicotine in WKY and SHR glia. *J Mol Neurosci.* 2008;35(2):151-160.
25. Oakes JM, Fuchs RM, Gardner JD, Lazartigues E, Yue X. Nicotine and the renin-angiotensin system. *Am J Physiol Regul Integr Comp Physiol.* 2018;315(5):R895-R906.
26. Yilin Z, Yandong N, Faguang J. Role of angiotensin-converting enzyme (ACE) and ACE2 in a rat model of smoke inhalation induced acute respiratory distress syndrome. *Burns.* 2015;41(7):1468-1477.
27. Yue X, Basting TM, Flanagan TW, et al. Nicotine Downregulates the Compensatory Angiotensin-Converting Enzyme 2/Angiotensin Type 2 Receptor of the Renin – Angiotensin System. *Annals ATS.* 2018;15:S126-S127.
28. Cai G. Tobacco-use disparity in gene expression of ACE2, the receptor of 2019-nCov. 2020; https://www.preprints.org/manuscript/202002.0051/v1.
29. Lippi G, Henry BM. Chronic obstructive pulmonary disease is associated with severe coronavirus disease 2019 (COVID-19). *Respir Med.* 2020;167:105941.
30. Arnson Y, Shoenfeld Y, Amital H. Effects of tobacco smoke on immunity, inflammation and autoimmunity. *J Autoimmun.* 2010;34(3):J258-265.
31. Gan WQ, Man SF, Sin DD. The interactions between cigarette smoking and reduced lung function on systemic inflammation. *Chest.* 2005;127(2):558-564.
32. Starr TN, Greaney AJ, Hilton SK, et al. Deep Mutational Scanning of SARS-CoV-2 Receptor Binding Domain Reveals Constraints on Folding and ACE2 Binding. *Cell.* 2020;182(5):1295-1310.e1220.

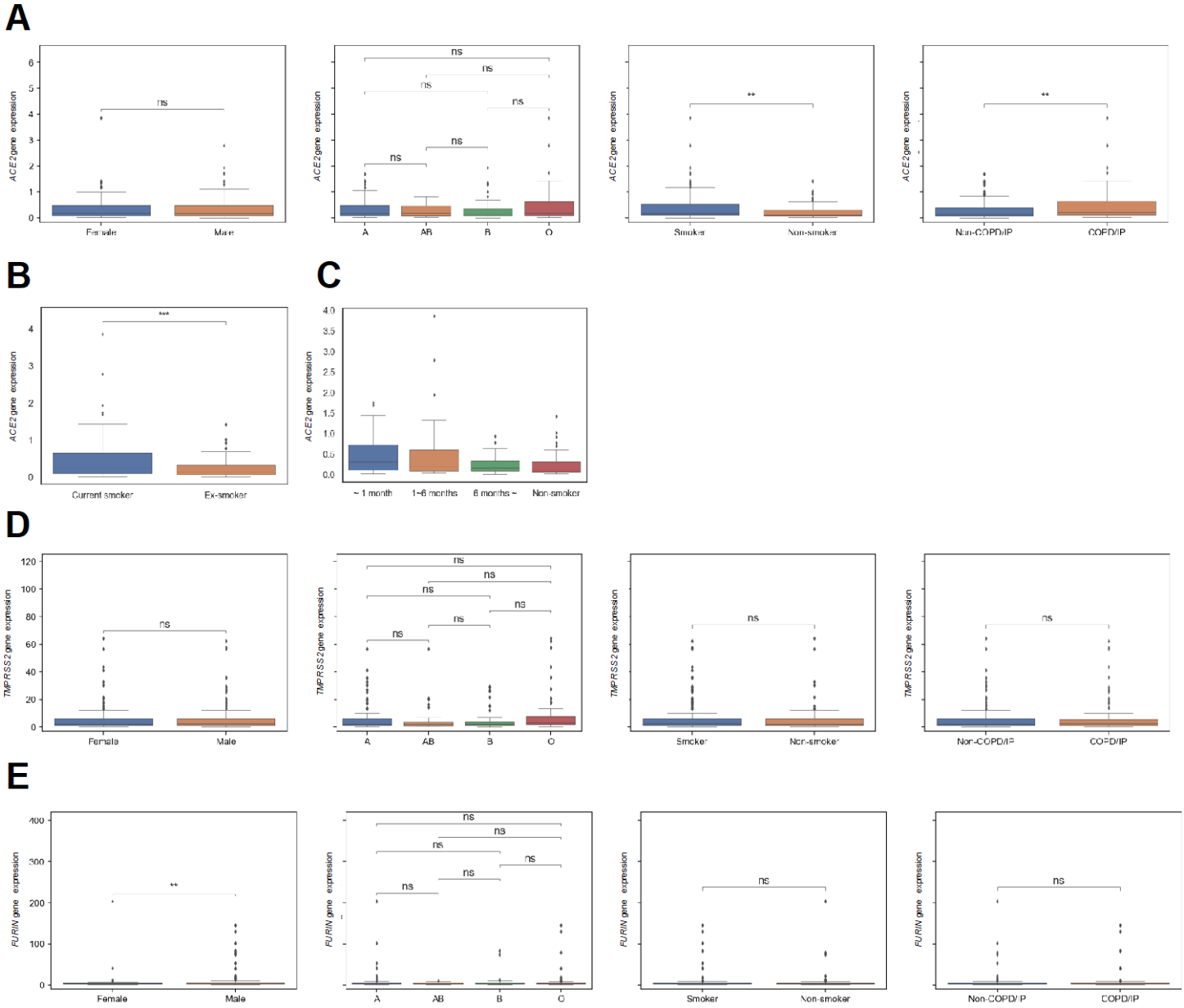

Figure.1 Suzuki *et. al*

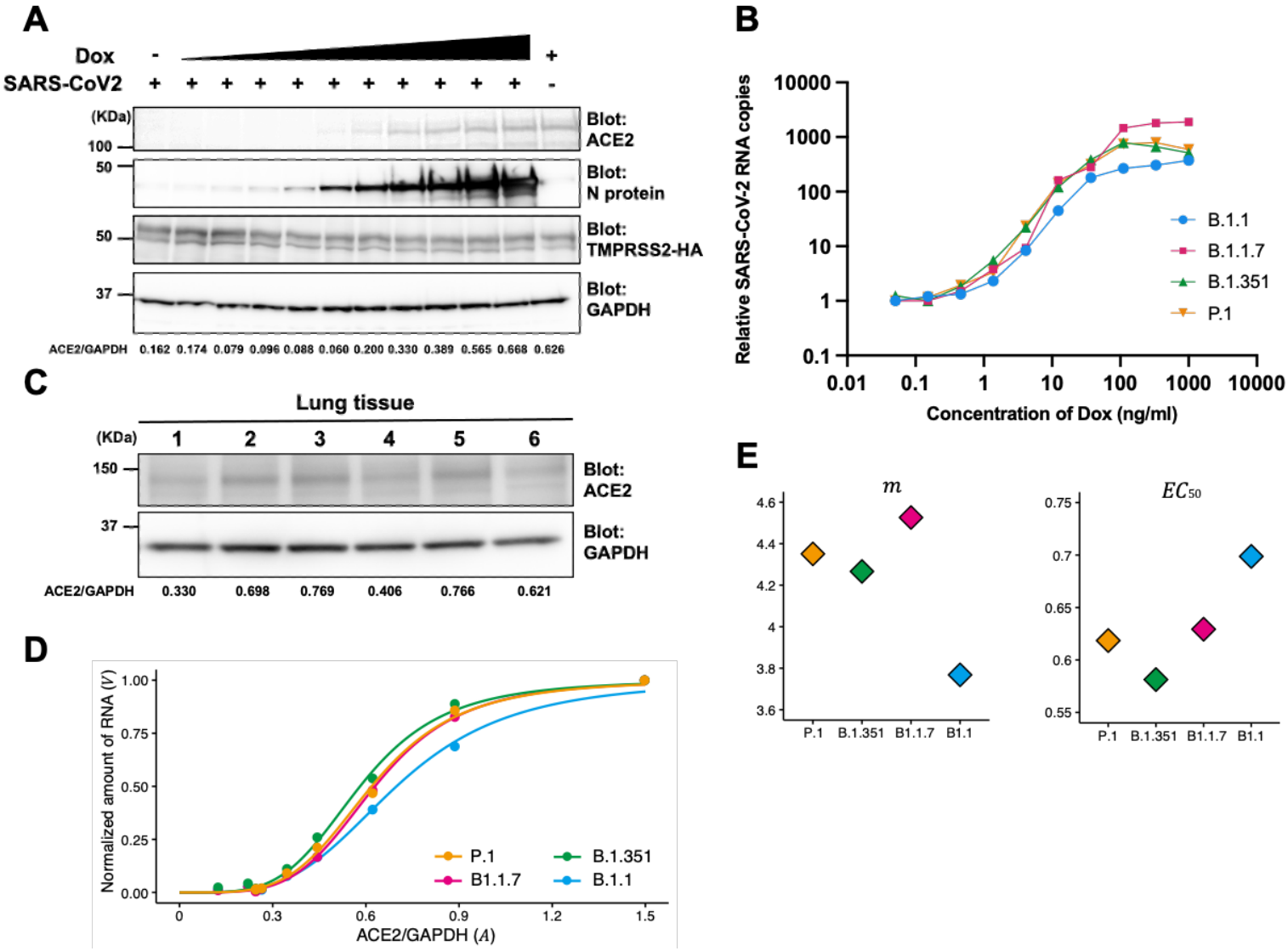

Figure.2 Suzuki *et. al*

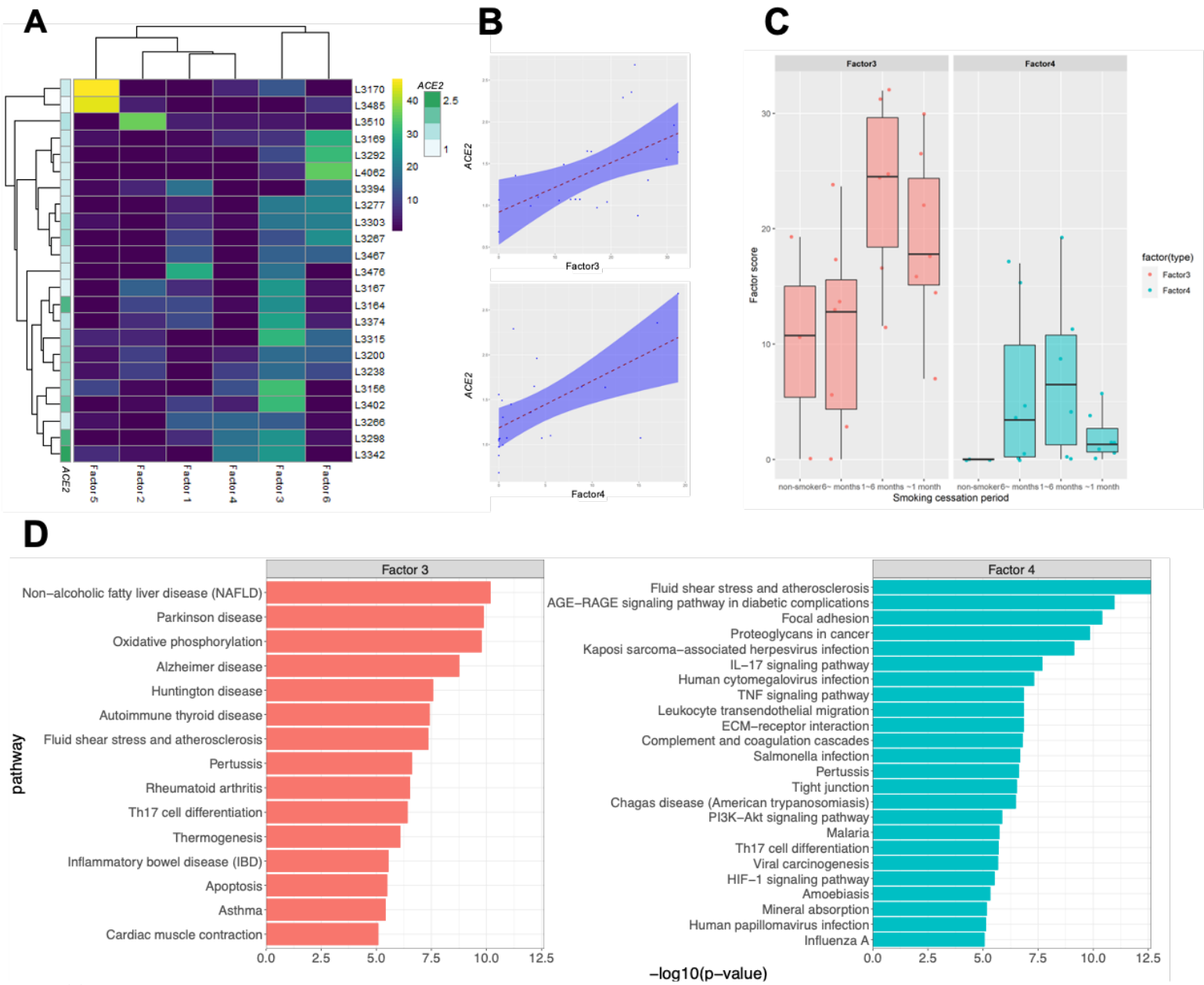

Figure.3 Suzuki *et. al*