Original Article

Association of alcohol intake and female gender with high expression of TMPRSS2 in tongue as potential risk for SARS-CoV-2 infection

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COVID-19 is pandemic since 2020 and further information is necessary on the risk factors associated with the infection of SARS-CoV-2. As an entry mechanism, SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) as receptor and transmembrane serine protease 2 (TMPRSS2) to activate fusion with host plasma membrane. Because dysgeusia is an early symptom of COVID-19, we here studied the expression of ACE2 and TMPRSS2 in the tongue and the associated tissues of mice and humans with immunohistochemistry and immunoblot analysis. ACE2 expression was low in the human tongue but was observed in the squamous epithelium, perineurium, arterial wall, salivary glands as well as taste buds. In contrast, mice showed high expression. In sharp contrast, TMPRSS2 expression was high in all the cells mentioned above in humans but relatively low in mice except for salivary glands. We then performed semi-quantitation of immunohistochemistry data of human ACE2 and TMPRSS2 and analyzed for age, sex, alcohol intake, and smoking habit with logistic regression analysis. We found that alcohol intake and female gender were the significant risk factors for increasing TMPRSS2 expression. In conclusion, TMPRSS2 is an important factor to be considered regarding SARS-CoV-2 entry and amplification in the oral cavity, which is promoted through drinking habit.

***Key Words*: coronavirus disease 2019 (COVID-19), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), tongue, angiotensin-converting enzyme 2 (ACE2), transmembrane serine protease 2 (TMPRSS2)**

Coronavirus disease 2019 (COVID-19), an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been pandemic since 2020.[1] Previous studies identified angiotensin-converting enzyme 2 (ACE2) as a receptor for SARS-CoV-2. Spike protein (S protein) organizing the surface of SARS-CoV-2 specifically binds to ACE2 on the plasma membrane of host cells, which is followed by the cleavage of S protein by transmembrane serine protease 2 (TMPRSS2) for the single stranded RNA of SARS-CoV-2 to enter into the cytosol of host cells.[2–4] SARS-CoV-2 can also be internalized to the host cells through the endosomal pathway.[5,6] Thus, two distinct pathways exist for establishing the initiation of SARS-CoV-2 infection (Fig. 1).

Dysgeusia is a complaint in the 41.5% of COVID-19 patients, based on the systematic review of 20 studies.[7] Accordingly, oral mucosa and the associated organs may provide with an important relay base for the SARS-CoV-2 toward lower respiratory infection. Recently, Xu *et al.*[8] reported based on public databases that ACE2 is highly enriched in epithelial cells of tongue. Sakaguchi *et al.*[9] reported that ACE2 is expressed in the stratified squamous epithelium of the dorsal tongue and gingiva and that TMPRSS2 is strongly expressed in stratified squamous epithelium in the keratinized surface layer and detected in the saliva and tongue coating samples via western blot analysis. Usami *et al.*[10] showed ACE2 expression in human salivary gland. However, their results are contradictory each other and none of them explained the molecular mechanism of dysgeusia.

In the present project, we studied the expression of ACE2 and TMPRSS2 in murine and human tongue and the associated tissues with immunohistochemistry and immunoblot analysis, and further analyzed the results with modifying factors in humans. Our results for the first time suggest that TMPRSS2 is an important factor to be considered regarding SARS-CoV-2 entry and amplification in the oral cavity, which is promoted through alcohol drinking habit, and that dysgeusia results from infection of SARS-CoV-2 to taste buds and perineurium.

Materials and Methods

Animal samples. We purchased 5-week old male *BALB/c Cr* mice (SLC, Shizuoka, Japan) and euthanized them with CO_2 inhalation and cervical dislocation. Tongue with associated tissue, submandibular glands and kidneys were dissected. They were divided into two halves; a half was fixed in 10% phosphate-buffered formalin for histology/immunohistochemical analysis and the other half was frozen at −80°C for immunoblot analysis, respectively. All the procedures were approved by the Animal Experimental Committee of Nagoya University Graduate School of Medicine (No. 20279). The animal study was carried out in accordance with the Guidelines for Animal Experiments of the Nagoya University Graduate School of Medicine.

Human tongue samples. This study was carried out in accordance with the principles of the Helsinki Declaration for human research and approved by the Ethics Committee of Nagoya University Graduate School of Medicine (No. 2020-0172). We have enrolled 41 subjects (Table 1), whom included 24 males and 17 females. Among them, we could use 38 samples of tongue, where the patients obtained primary tongue cancer and underwent surgery between January 2016 and April

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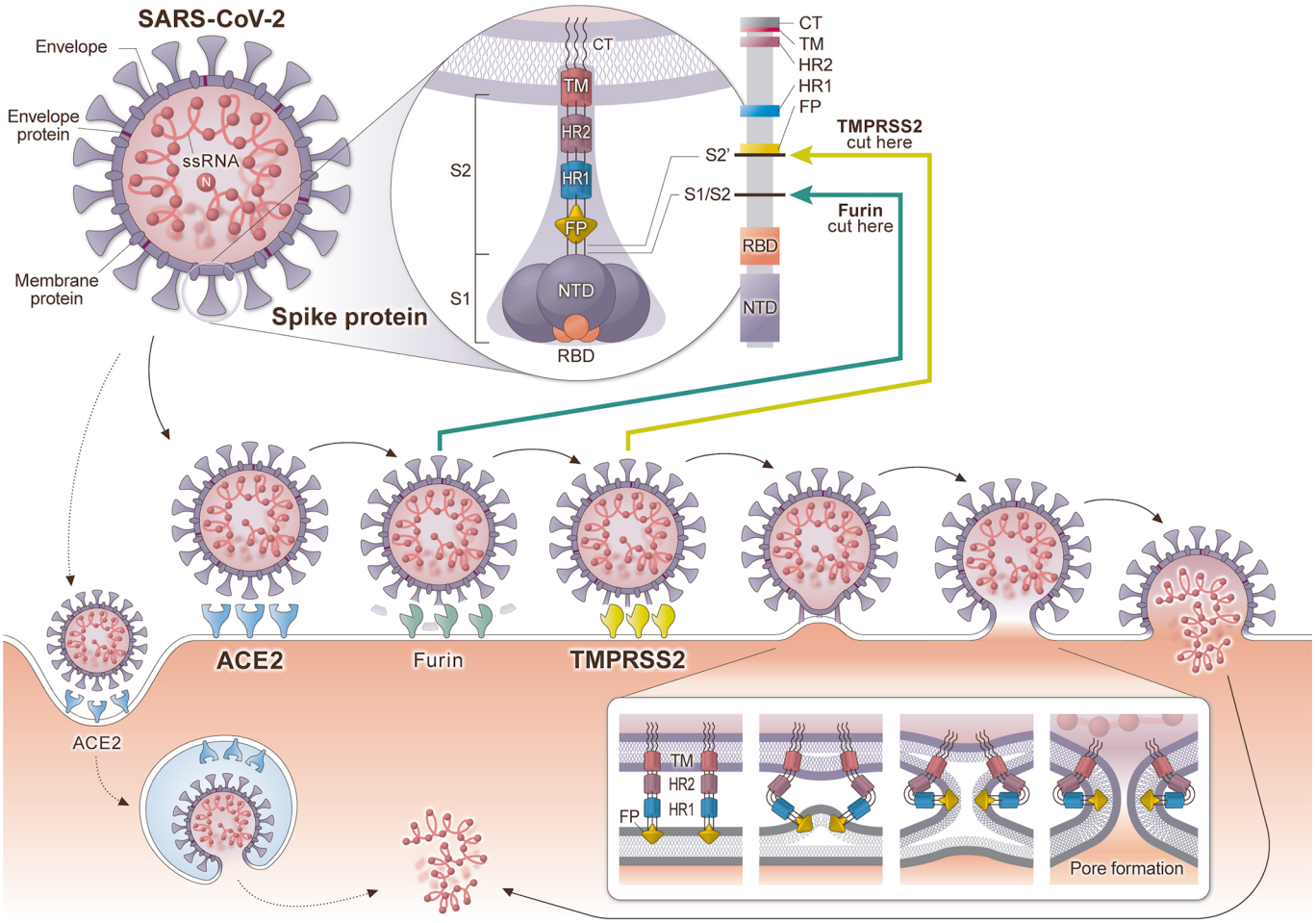

Fig. 1. Schematic illustration of the molecular mechanisms of the entry of SARS-CoV-2 to the host cells. Receptor Binding Domain (RBD) of the spike (S) protein 1 of SARS-CoV-2 attaches to ACE2 and Furin cuts between S protein 1 and 2. Fusion Peptide (FP) of the S protein 2 is exposed thereafter based on the function of TMPRSS2, which can attach to the plasma membrane of host cells. Finally, S protein 2 itself alters its structure for the viral envelope to fuse with the plasma membrane of the host cell, which is followed by the release of viral genome into the host cytoplasm. Alternatively, the virus can enter the host cell through the endosomal pathway. CT, cytoplasmic tail; TM, transmembrane domain; HR, heptad repeat; NTD, N-terminal domain.

2020. Formalin-fixed paraffin-embedded (FFPE) samples were enrolled with an opt-out method. For the immunoblot analysis, we collected 3 fresh samples of tongue from the patients who obtained primary tongue cancer and underwent surgery. All the patients provided with a written informed consent prior to the surgery at Nagoya University Hospital. Non-tumorous area not to be used for pathological diagnosis was selected after the routine surgery and used for the analysis.

Immunohistochemistry (IHC) and its evaluation. Harvested tissues were fixed in 10% phosphate-buffered formalin for 24–48 h. Following fixation, tissues were embedded in paraffin, sectioned at 5 μm, mounted on glass slides and deparaffinized prior to the staining procedures. The sections were immunostained with a BOND-MAX system (Leica Microsystems GmbH, Wetzlar, Germany) using the manufacturer's reagents according to the protocol as described.[11] Anti-ACE2 (1:250, ab108252; Abcam, Cambridge, UK) and anti-TMPRSS2 (1:8000, ab92323; Abcam) antibodies were used after diluting with Primary Antibody Diluent (Leica). Tissue array slides (MNO341; US BIOMAX Inc., Rockville, MD) were used for the analysis of various organs in humans and as positive controls. Regarding the evaluation of ACE2 immunostaining, we classified the samples into three groups depending on the staining intensity in epithelial cells. To secure the reproducibility, two pathologists independently evaluated them, which was assessed with κ coefficient. In terms of TMPRSS2 evaluation, we classified them into three groups: strong (216–256 out of 256-level density), moderate (186–215), weak (156–185) with ImageJ software (National Institutes of Health, Bethesda, MD) (Supplemental Fig. 1*).

Immunoblot analysis (IB). Cells were lysed with a lysis buffer (250 mM NaCl, 50 mM Tris pH 7.4, 50 mM NaF, 0.1 mM $NaVO_4$, 5 mM EDTA, 0.1% Triton-X) including Complete Mini, EDTA-free (Roche, Basel, Switzerland) and PhosStop (Roche). Tissues were homogenized, sonicated, and lysed with the same lysis buffer. Protein concentration was quantified, using Protein Assay Bicinchoninate kit (06385-00; Nacalai Tesque, Kyoto, Japan).[12] SDS-PAGE electrophoresis was carried out by loading 20 μg protein. Blots were incubated with primary antibodies diluted in Can Get Signal® Solution 1 (TOYOBO, Osaka, Japan) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibody (P0448; DAKO, Santa Clara, CA) diluted 1:10,000 in Can Get Signal® Solution 1 with incubation for 1 h at room temperature. Reactions on the membranes were finally visualized by Chemi-Lumi One Super (02230-30; Nacalai) and analyzed with ImageJ software. Anti-ACE2 (1:1,000 dilution),

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Table 1. Immunohistochemical staining grade of ACE2 and TMPRSS2 and Logistic regression analysis with attributes in humans

A

Retrospective (n = 38)		
	Characteristic	n = 38
Age	<50	24
	≧50	14
Sex	Male	22
	Female	16
Alcohol	Yes	28
	No	10
Smoking (Pack-years)	Pack-years <30	30
	Pack-years ≧30	8
ACE2	Weak	20
	Moderate, Strong	18
TMPRSS2	Weak	24
	Moderate, Strong	14

Prospective (n = 3)		
Patient No.	Age (years)	Sex (male/female)
1	74	M
2	63	M
3	27	F

(A) ACE2 and TMPRSS2 grade and clinical features of patients. We enrolled 38 patients as a retrospective study and 3 as a prospective study (total 41 patients).

B

ACE2		
	Characteristic	p value
Age	<50	0.95
	≧50	
Sex	Male	0.301
	Female	
Smoking	Pack-years <30	0.969
	Pack-years ≧30	
Alcohol	Yes	0.929
	No	

TMPRSS2				
	Characteristic	Odds Ratio	95% CI	p value
Age	<50	0.512	0.084–3.103	0.466
	≧50			
Sex	Male	13.878	1.662–115.914	0.015*
	Female			
Smoking	Pack-years <30	8.017	0.820–78.371	0.074
	Pack-years ≧30			
Alcohol	Yes	7.048	1.005–49.426	0.049*
	No			

(B) Logistic regression analysis. Female gender and alcohol intake were the upregulating factors of TMPRSS2 in the tongue (*p<0.05). Smoking habit showed a tendency to increase TMPRSS2 expression in the tongue epithelium (p = 0.07).

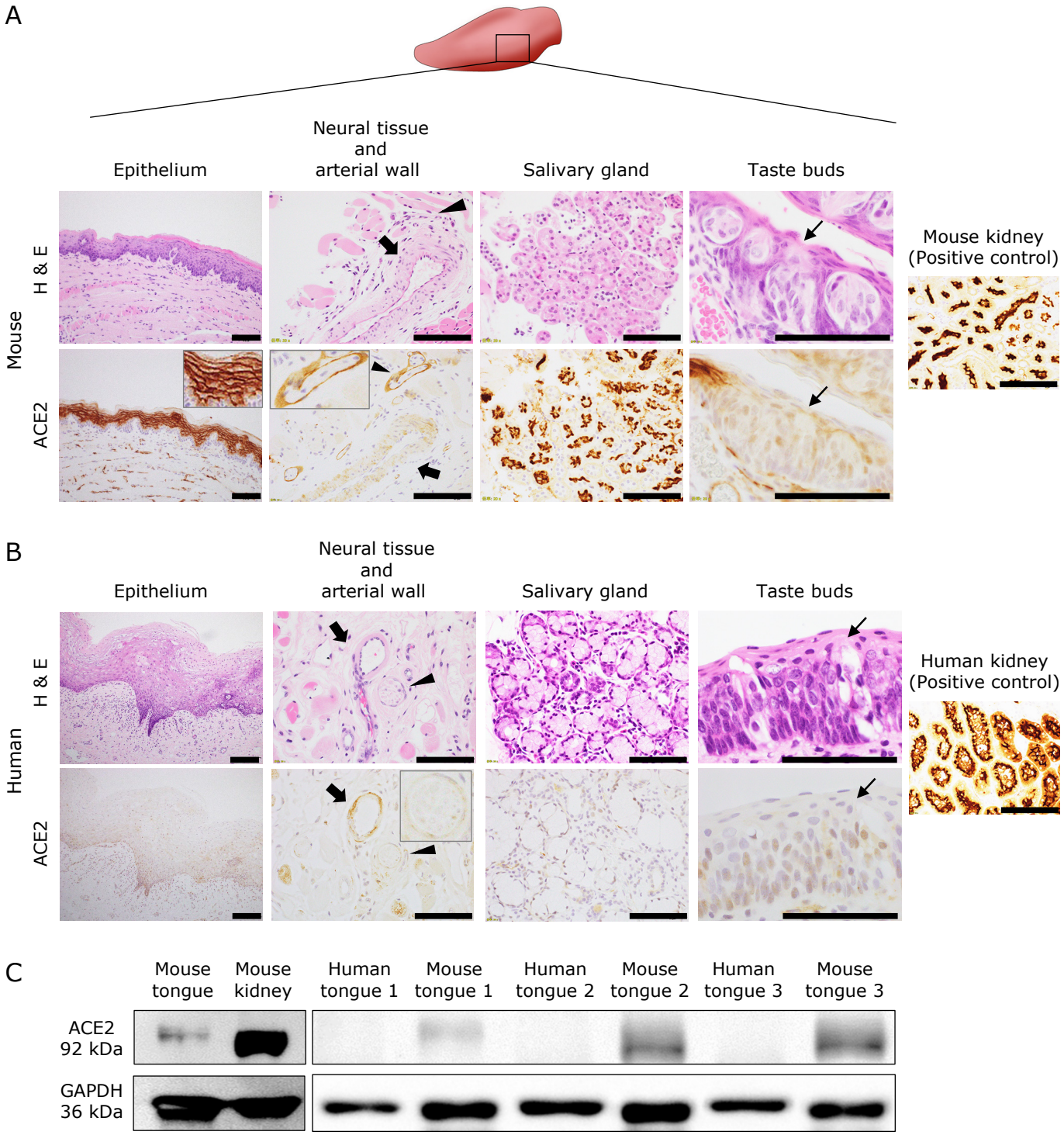

Fig. 2. The ACE2 expression in the human and mouse tongue. (A) Mouse samples. Stratified squamous epithelium of dorsal tongue, perineurium of the peripheral nerve and salivary gland reveal high expression (scale bar = 100 μm; arrow head, peripheral nerve; thin arrow, taste buds; thick arrow, arterial wall). (B) Human samples. Stratified squamous epithelium of dorsal tongue, peripeurium of the peripheral nerve and arterial wall, salivary gland and taste buds are relatively low in expression (scale bar = 100 μm; arrow head, peripheral nerve; thin arrow, taste buds; thick arrow, arterial wall). (C) Immunoblotting. ACE2 expression in the mouse tongue ($n = 3$) was stronger than that of humans ($n = 3$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

anti-TMPRSS2 (1:2,000 dilution) and anti-GAPDH (1:10,000 dilution) were used, respectively.

Statistical analysis. Multiple logistic regression analysis and κ statistics were conducted with IBM SPSS Statistics 28. Significance was defined as $p<0.05$.

Results

ACE2 and TMPRSS2 in tongue are different in expression between mice and humans. We studied the expression of ACE2 and TMPRSS2 in the tongue tissue of mice and humans

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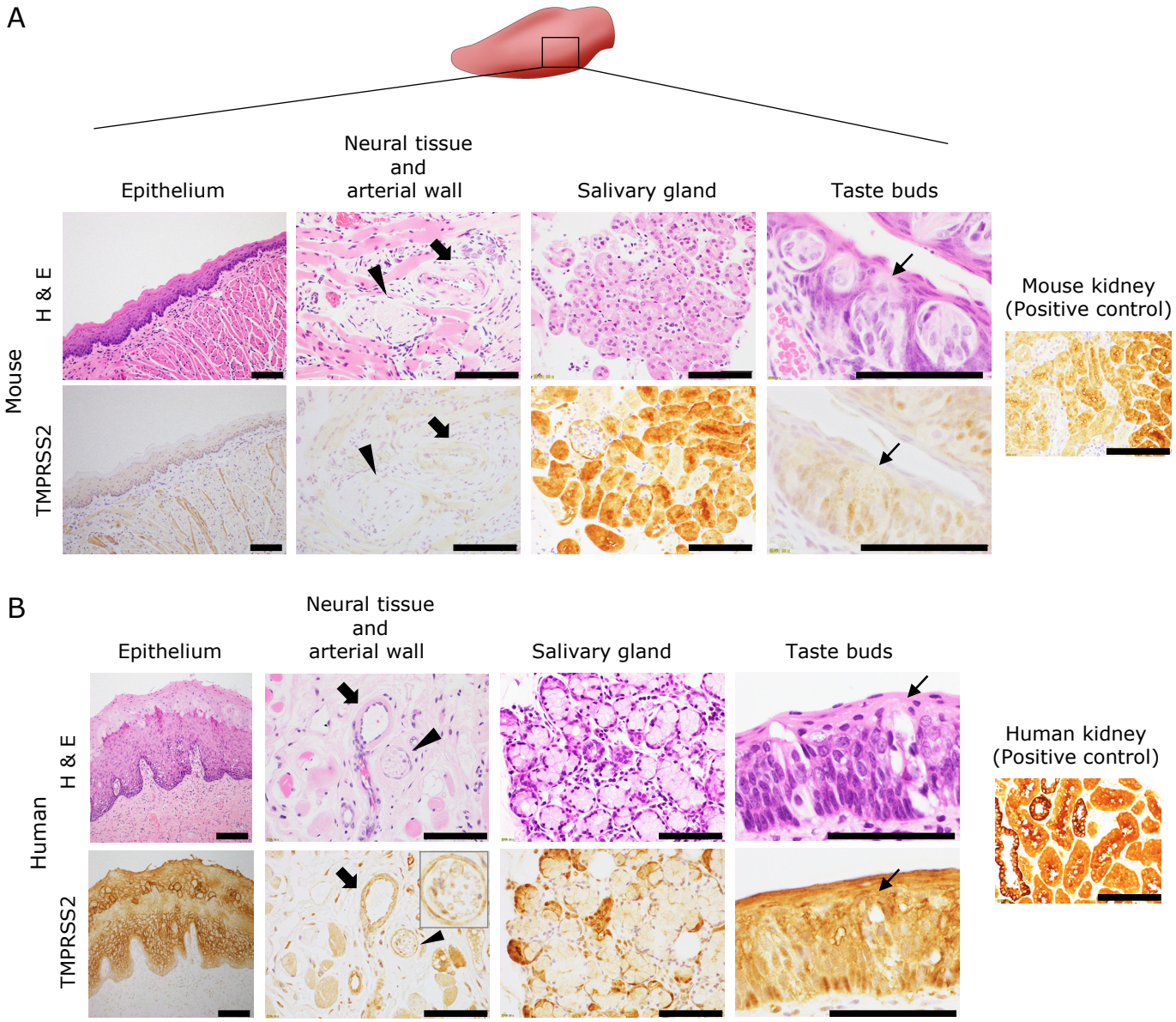

Fig. 3. The TMPRSS2 expression in the human and mouse tongue. (A) Mouse samples. Salivary gland revealed high expression (scale bar = 100 μm; arrow head, peripheral nerve; thin arrow, taste buds; thick arrow, arterial wall). (B) Human samples. Stratified squamous epithelium of dorsal tongue, perineurium of the peripheral nerve and arterial wall, salivary gland and taste buds reveal high expression (scale bar = 100 μm; arrow head, peripheral nerve; thin arrow, taste buds; thick arrow, arterial wall). (C) Immunoblotting. TMPRSS2 expression in the human tongue (n = 3) was stronger than that of mice (n = 3). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

with immunohistochemistry and immunoblot analysis. We found that ACE2 was strongly expressed in the murine tongue, especially plasma membrane of stratified squamous epithelia, perineurium, arterial wall, and luminal side of salivary glands (Fig. 2A). In the human tongue, however, the expression of ACE2 in the squamous epithelium, perineurium, and salivary glands was lower than those of mice (Fig. 2B). As a positive control, luminal brush border membrane of renal tubular cells was intensely immunostained. Immunoblot analysis revealed similar results to those by immunohistochemical analysis and we

could not obtain a specific band with human fresh samples (Fig. 2C). In terms of taste buds in the tongue, the degree of ACE2 immunostaining was almost the same and moderate in mice and humans (Fig. 2A and B).

The expression of TMPRSS2 in the mouse tongue was weak in the stratified squamous epithelium, perineurium, and vessel wall. However, the immunostaining was strong in the cytoplasm and plasma membrane of salivary glands (Fig. 3A). Remarkably, TMPRSS2 immunostaining was strong in humans in the stratified squamous epithelium, perineurium, vascular wall, salivary glands, and taste buds (Fig. 3B). Overall, TMPRSS2 expression was stronger in humans (Fig. 3A and B). As a positive control, cytoplasm, and plasma membrane of renal tubular cells were intensely immunostained. Immunoblot analysis revealed similar results to those by immunohistochemical analysis and we clearly obtained a specific band with human samples (Fig. 3C).

ACE2 and TMPRSS2 expression in the other organs in humans. To evaluate the ACE2 and TMPRSS2 expression in the other organs of human, we used whole organ tissue arrays (Supplemental Fig. 2A*). ACE2 was strongly expressed in the kidney, small intestine and testis (Supplemental Fig. 2B*). In terms of TMPRSS2, liver, kidney, prostate gland, and breast revealed especially high expression in comparison to other organs (Supplemental Fig. 2C*).

Female gender and alcohol intake are the upregulating factors of TMPRSS2 expression in the human tongue epithelium. To identify the upregulating factors of ACE2 and TMPRSS2 expression in the human tongue, we analyzed 38 human samples with the corresponding attributes. In terms of ACE2, the expression level was low, so we scored them as three groups (strong, moderate, or weak) by two pathologists independently (Supplemental Fig. 1*). Reproducibility of the two pathologists was 70.5% as κ coefficient, which was substantial. There were no significant differences for ACE2 expression among age, gender, smoking, and alcohol intake. In contrast, regarding TMPRSS2, we found that female gender and alcohol intake were the significant two factors to upregulate the expression ($p<0.05$) and that smoking habit (pack-years ≥30) showed a tendency to increase TMPRSS2 expression in the tongue squamous epithelia ($p = 0.07$; Table 1B).

Discussion

Here we studied the expression of ACE2 and TMPRSS2, as responsible molecules for infectious entry of SARS-CoV-2, in the tongue of mice and humans. We for the first time identified that female gender and alcohol intake are the upregulating factors of TMPRSS2 expression in the tongue, which may promote SARS-CoV-2 entry through the tongue as an important temporary reservoir to the lower respiratory tract.

ACE2, a 92 kDa protein, is located on plasma membrane and may be secreted. ACE2 controls the blood pressure through renin-angiotensin-aldosterone pathway to decrease the pressure, antagonizing ACE1.[13] TMPRSS2, a 54 kDa protein, is located on plasma membrane and intracellular membrane, but the physiological function has not been elucidated yet. It has been proposed that TMPRSS2 regulates epithelial sodium currents in the lung through proteolytic cleavage of the epithelial sodium channel[14] and is also related with proliferation of prostate cancer cells, leading to extracellular matrix disruption and metastasis.[15] These two proteins have been established to be associated with the entry of SARS-CoV-2.[2,3]

For now, although the fraction of vaccinated population has increased,[16] COVID-19 patients are still increasing in many countries with the appearance of omicron variant.[17] Sakaguchi *et al.*[9] have reported that the expression of ACE2 in the tongue is relatively high in humans, suggesting its relation to dysgeusia in the infected patients. However, the present results of ours revealed that ACE2 expression in the tongue is relatively low in the human tongue (Fig. 2A and B). The immunohistochemistry results were confirmed by immunoblot analysis (Fig. 2C). In sharp contrast, TMPRSS2 was high in expression in the human tongue and low in the mice (Fig. 3). Zhou *et al.*[18] reported that mice do not often get infected with SARS-CoV-2 in comparison to other animal species. Our present results suggest that SARS-CoV-2 susceptibility is more directly associated with the balance between ACE2 and TMPRSS2 expression rather than ACE2 alone. Namely, there is a possibility that SARS-CoV-2 does not infiltrate into the tissue when TMPRSS2 is low even though the ACE2 level is high.

We thus have hypothesized that TMPRSS2 plays a more critical role in initiating infection of SARS-CoV-2 from oral mucosa than ACE2. One study[19] has identified alcohol consumption as a risk factor for severity of COVID-19 epidemiologically in humans but two other studies[20,21] have not so far. Further, male gender has been identified as a risk for death in COVID-19 by global meta-analysis,[22] which is not necessarily inconsistent with the present results. Therefore, our current results of TMPRSS2 expression in tongue need discussion and further investigation to clarify the significance.

The expression levels of ACE2 and TMPRSS2 in the perineurium and taste buds in the human tongue were relatively high in comparison to other portions in the tongue (Fig. 2B and 3B). We believe that our results indicate the relationships between SARS-CoV-2 entry sites and dysgeusia in COVID-19 patients. Additional neuroelectrochemical studies would be necessary to demonstrate the detailed molecular mechanism of SARS-CoV-2 infection and dysgeusia.

In conclusion, the expression of TMPRSS2 is dominant over that of ACE2 in human tongue, which may be important for the initial phase of COVID-19 infection. Alcohol intake and female gender were the risks for higher TMPRSS expression. Expression of both ACE2 and TMPRSS2 in the perineurium and taste buds may be associated with the symptom of dysgeusia in COVID-19 patients.

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Abbreviations

ACE2	angiotensin-converting enzyme 2
COVID-19	coronavirus disease 2019
CT	cytoplasmic tail
FFPE	formalin-fixed paraffin-embedded
FP	fusion peptide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HR	heptad repeat
IB	immunoblotting
IHC	immunohistochemistry
NTD	N-terminal domain
RBD	receptor binding domain
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
S protein	spike protein
TMPRSS2	transmembrane serine protease 2
TM	transmembrane

Conflict of Interest

No potential conflicts of interest were disclosed.

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