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BRIEF COMMUNICATION

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Brief communication: Immunohistochemical detection of ACE2 in human salivary gland

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Abstract

The novel, severe acute respiratory syndrome coronavirus (SARS-CoV-2) was firstly reported in late December of 2019 and subsequently caused a global outbreak. It has been shown that SARS-CoV-2 uses ACE2 (Angiotensin Converting Enzyme 2) as a cellular receptor for host cell entry through the surface unit of SARS-CoV spike glycoprotein. In this brief report, we analyze ACE2 protein expression and localization in human salivary gland, and propose a possible role of saliva in the pathogenesis of Coronavirus disease 2019 (COVID-19).

KEYWORDS

aspiration pneumonia, COVID-19, salivary gland, SARS-CoV-2

1 | INTRODUCTION

Coronaviruses are enveloped RNA virions, which belong to the family of Coronaviridae, and cause mild respiratory disease in humans; however, the SARS-CoV (Severe acute respiratory syndrome coronavirus) and the MERS-CoV (Middle East respiratory syndrome coronavirus) explored in 2002-2003 and in 2012, respectively, caused fatal severe respiratory diseases. The novel coronavirus SARS-CoV-2, the causative agent of coronavirus disease 19 (COVID-19), was firstly reported late in December 2019 and subsequently caused a global outbreak. SARS-CoV-2 shares ~80% amino acid identity with SARS-CoV, and it has been shown in the involvement of ACE2 (angiotensin converting enzyme 2) as a cellular receptor of a surface unit of SARS-CoV spike glycoprotein.[1]

Several transmission routes of SARS-CoV-2 have been proposed including both direct and contact transmission.[2] Importantly, recent studies have proposed saliva as a potential reservoir for COVID-19 asymptomatic infection.[3,4] Some studies have shown ACE2 expression in human salivary gland by analyzing the mRNA expression,[1,3] however, evidence of immunohistochemical ACE2 expression and its localization in human salivary gland is still unknown. Herein, we evaluate ACE2 protein expression and localization in human salivary gland, and propose a possible role of saliva in the pathogenesis of COVID-19.

2 | MATERIALS AND METHODS

2.1 | Ethics Statement

This study was approved by the Institutional Ethical Review Board of Osaka University Graduate School of Dentistry (no. R1 E-46) and performed in accordance with the Committee guidelines and regulations.

2.2 | Tissue materials

Four salivary gland tissues, two tongue tissues (Table 1) and C57BL/6 mouse tissues (for positive control) were fixed with 10% neutral-buffered formalin and embedded in paraffin wax. All the tissues were confirmed to be normal by two pathologists. The 4 μm-thick sections were used for further studies.

2.3 | Immunohistochemistry

Immunohistochemical staining was performed using the streptavidin–biotin complex peroxidase method. Briefly, the sections were deparaffinized and pretreated for antigen retrieval as follows; heat-induced antigen retrieval method (HIAR, 121°C for 10 minutes, 95°C for 10 minutes, using Dako Pascal, Dako Cytomation, Glostrup, Denmark, with 10 mM sodium citrate buffer pH 6.0), protease-induced antigen retrieval method (Pro-K, Protease K 5 minutes in room temperature (RT), Ready-to-use, Dako), and without any pretreatment (Untreated). The sections were then incubated with blocking reagent (5% Bovine serum albumin in Phospate buffered saline, 30 minutes, RT) before being incubated over night with primary anti-rabbit polyclonal antibody against ACE2 (1:2000, 21 115-1-AP, Proteintech) at 4°C. After washing with Tris-buffered saline buffer, the sections were incubated with the anti-rabbit biotinylated secondary antibodies (1:200, Dako, 60 minutes RT) and then incubated with Streptavidin-Horseradish peroxidase antibody (1:200, Dako, 30 minutes RT). Chromogenic fixation was performed in a solution of 3,3'-diaminobenzidine (DAB, Dako) according to manufacturer's instructions. Negative controls for immunostaining were obtained by substituting the primary antibody with normal mouse or rabbit IgG (Dako).

TABLE 1 Detail of human samples

Sample no.	Tissue	Age	Gender
1	Minor salivary gland of the lip	30s	Female
2	Minor salivary gland of the palate	40s	Male
3	Submandibular gland	50s	Female
4	Submandibular gland	70s	Female
5	Tongue	6-year-old	Male
6	Tongue	80s	Female

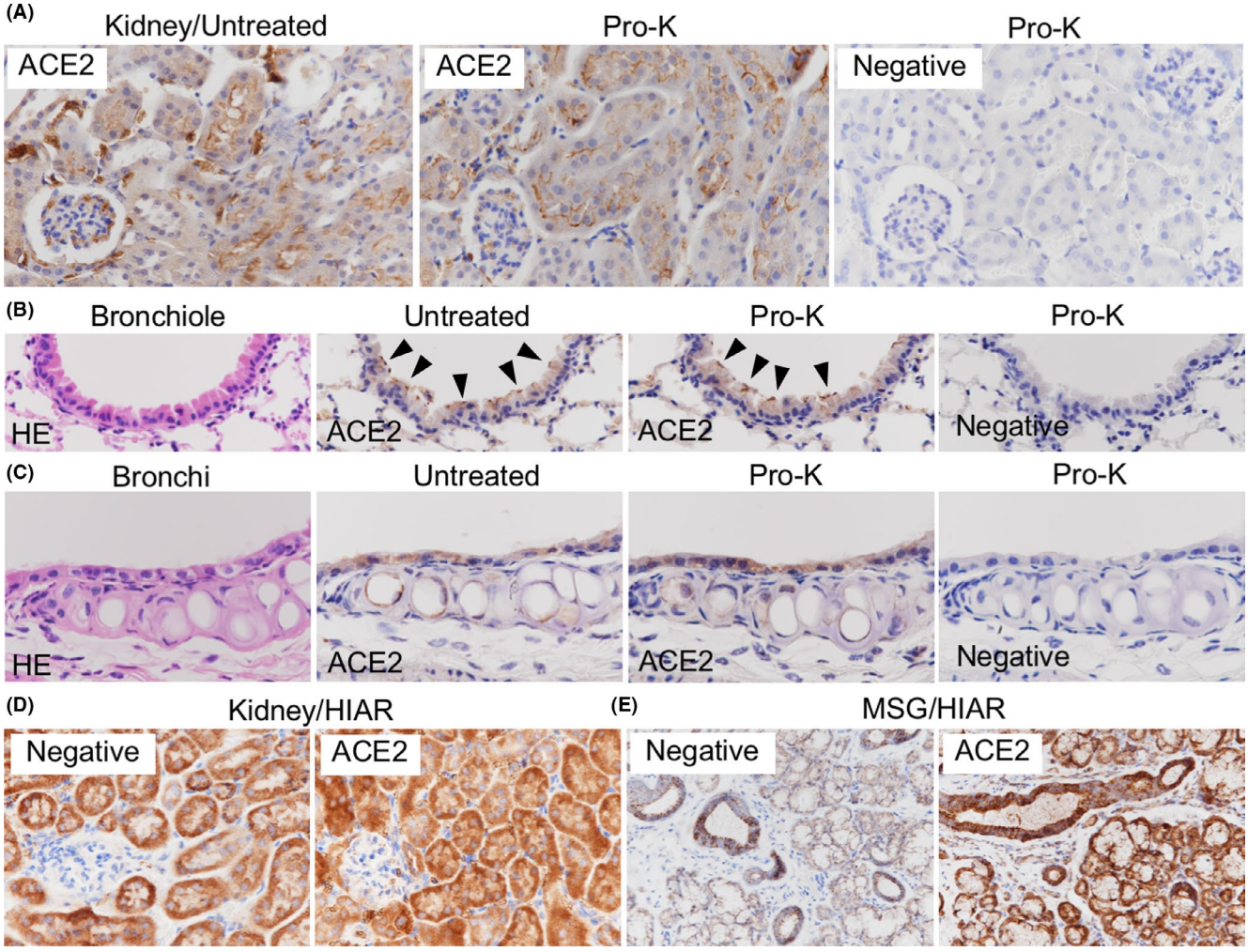

FIGURE 1 Pretreatment for immunohistochemistry using anti-ACE2 antibody. (A) Renal tubules, (B) bronchiole and (C) bronchi of C57Bl/6 mouse were used as positive control. Untreated: without any pretreatment before applying the primary antibody, Pro-K: protease K-induced antigen retrieval method, Negative: negative control. Heat-induced antigen retrieval before applying the primary antibody induced strong endogenous biotin in both (D) mouse kidney and (E) human minor salivary gland tissue of the lower lip. HIAR, heat-induced antigen retrieval method; MSG, minor salivary gland

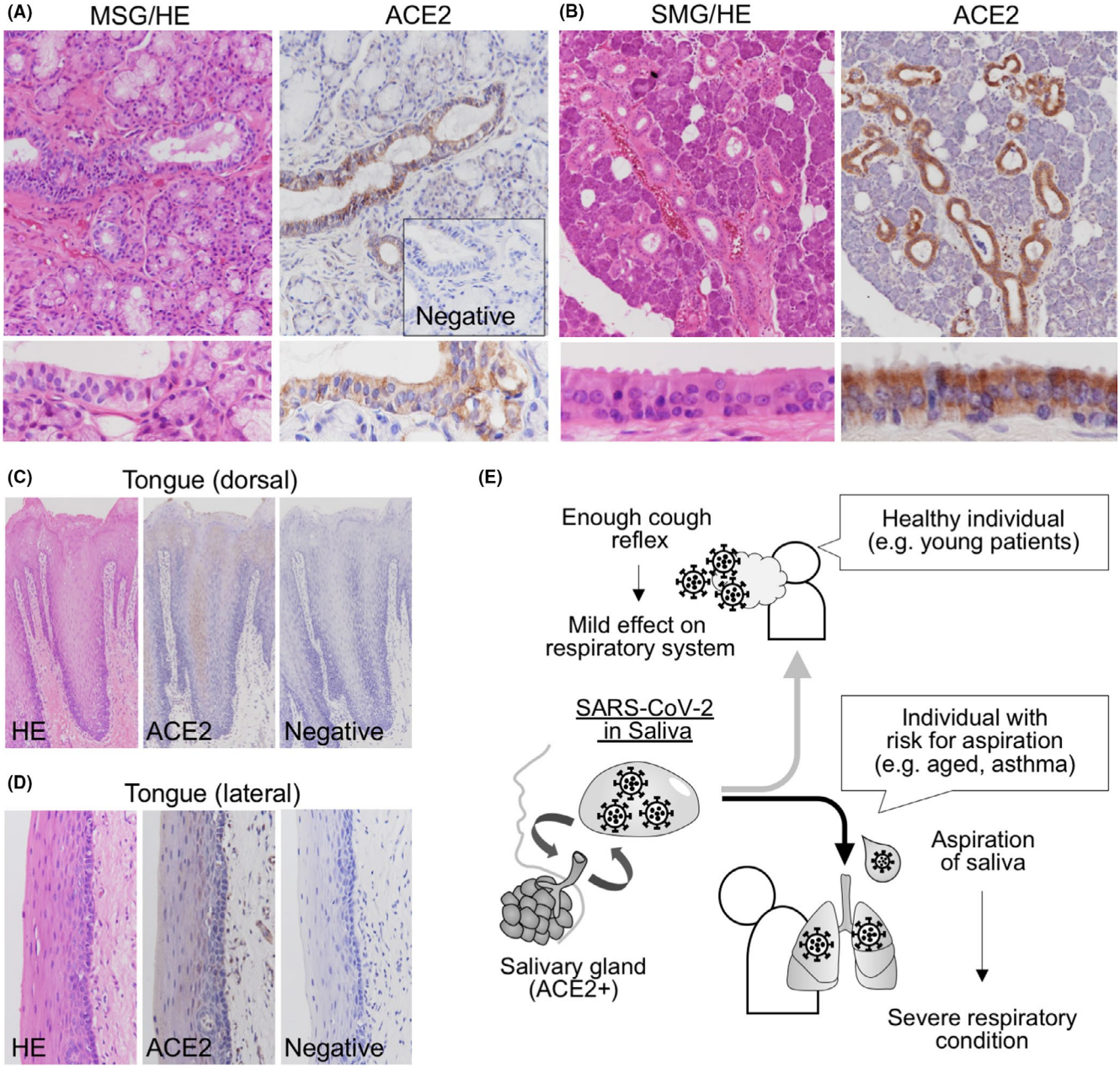

FIGURE 2 ACE2 expression in salivary gland. ACE2 expression in ductal epithelium of (A) minor salivary gland of lower lip and (B) submandibular gland. Lower panel: membranous expression of ACE2 in ductal cells. Inset: negative control. (C and D) ACE2 expression was undetectable in squamous epithelium of the tongue. (C) Dorsal epithelium. (D) Lateral epithelium. (E) Possible role of saliva in COVID-19. SARS-CoV-2 infected saliva will be a reservoir of the virus. In individuals with risk of aspiration, SARS-CoV-2 in saliva will induce severe respiratory condition of COVID-19

3 | RESULTS AND DISCUSSION

3.1 | Immunohistochemical expression and localization of ACE2

ACE2 is a carboxypeptidase, negatively regulating the renin angiotensin system, which is well known to be involved in vasodilation by cleaving angiotensin II. Because the ACE2 protein shares its role as cellular receptor of SARS-CoV-2, several researchers have been focusing on ACE2 expression in various human organs, especially in respiratory systems which can be the entrance for the SARS-CoV-2.[1] ACE2 expression in the salivary gland has been analyzed by using a tissue-specific gene expression database, however, it will be difficult to conclude its expression without the direct evidence of ACE2 protein expression and localization.

Before evaluating the expression and localization of ACE2 protein in the salivary gland, we first established the protocol to obtain the appropriate expression of ACE2 protein using a mouse kidney and lung as a positive control. Correct ACE2 expression and localization was successfully observed employing both Pro-K and

without any pretreatment (untreated); ACE2 protein expression was observed in brush border of renal tubules (Figure 1A), bronchiole (Figure 1B) and bronchi (Figure 1C). Because the results of immunostaining were slightly sharp in the Pro-K-group when compared to the untreated group, we decided to employ the Pro-K pretreatment for further analysis. Notably, using the HIAR method, the most widely used method for antigen retrieval, both mouse kidney and human minor salivary gland (MSG) tissue showed strong cytoplasmic DAB staining, due to strong endogenous biotin (retrieved endogenous biotin) of both tissues (Figure 1D,E).

In human MSG, ACE2 expression was observed in the cell membrane of duct components including interlobular excretory ducts and interlobular ducts (Figure 2A). In human submandibular gland, ACE2 expression was observed in the cell membrane/brush border of the main ducts (not shown), interlobular excretory ducts and interlobular ducts (Figure 2B). Both mucinous and serous acini lack ACE2 expression (Figure 2A,B). Squamous epithelium of the tongue was negative for ACE2 expression (Figure 2C,D). Endogenous biotin was not seen in the negative control (inset in Figure 2A). Our results clearly show that ACE2 protein is expressed in the duct component of salivary glands, and not expressed in squamous epithelium.

Recent epidemiological studies have shown that transmission of SARS-CoV-2 can occur from the pre-symptomatic patient (before the first onset of symptoms), therefore, it will be important to capture potential transmission events for effective control of the outbreak.[5] Since the early diagnosis of COVID-19 is still difficult, diagnostic value of saliva for SARS-CoV-2 nucleic acid examination will be promising. In fact, studies of both saliva swab and saliva directly from salivary gland ducts have successfully shown the evidence of SARS-CoV-2 RNA in COVID-19 patients.[4] Moreover, it has been shown that SARS-CoV RNA can be detected in saliva before lung lesions appear,[4] suggesting the role of saliva as an early diagnostic tool as well as a potential transmission root of asymptomatic infections.

3.2 | Possible role of saliva in COVID-19

Because the ductal elements of salivary gland express ACE2, and SARS-CoV-2 can be found in the saliva of COVID-19 patients, we want to emphasize the role of saliva as reservoir of SARS-CoV-2 (Figure 2E). When SARS-CoV-2 enters through the respiratory route, some may reach the deep respiratory organs and some may not. In contrast, ducts of salivary glands are open to the oral cavity, and will be easier for the virus to reach, when compared to the deep respiratory organs. In individuals with risk of aspiration (eg aged individuals, individuals with asthma, etc), SARS-CoV-2-positive saliva will easily reach the deep respiratory organs, and lead to a severe respiratory condition and severe viremia (Figure 2E). To confirm this hypothesis, large cohort studies and well-designed animal studies will be necessary.

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