Original

Morphological Analysis of Angiotensin-Converting Enzyme 2 Expression in the Salivary Glands and Associated Tissues

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Abstract: We evaluated localization of angiotensin-converting enzyme 2 (ACE2), the receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), in the salivary and associated tissues using immunohistochemistry. Fifty paraffin-embedded blocks from 48 anonymized patients, biopsied or operated on for diseases of the oral and maxillofacial region before 2010, were analyzed. ACE2-expressing cells were observed in the parotid, sublingual and the buccal glands, the conduits, the acinar regions of the serous glands, and sparsely in the mucous glands. Scattered ACE2-positive endothelial cells were also observed in nearby capillaries nourishing the salivary glands, as well as in the juxta-epithelial capillaries of the oral mucosa. ACE-2-positive adipocytes were scattered within the stroma of the parotid gland. These observations suggest the possibility that SARS-CoV-2 may travel through the bloodstream to the capillaries that nourish the salivary glands and oral mucosa, and inducing vasculitis and damage of oral tissues. SARS-CoV-2 infection of salivary glands through the bloodstream implies the main cause of salivary contamination. Similarly, ascending infection from oral fluid to the salivary gland conduit has been shown to be another possible route. Moreover, infection of ACE2-positive parotid adipocytes may lead to parotid glands inflammation and contribute to systemic progression of coronavirus disease 2019.

Key words: ACE-2, Oral mucosa, Endothelial cell, Salivary gland, Immunohistochemistry

Introduction

Near the end of 2019, an outbreak of acute atypical pneumonia with associated respiratory disorder occurred in Wuhan, China. It became apparent that a novel coronavirus was the cause of the outbreak, which rapidly developed into a global pandemic. The causative coronavirus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and was closely related (~80% homology) to SARS-CoV, the causative agent that a novel coronavirus was the cause of the outbreak, which resulted in large numbers of infections and deaths in most countries. The first case was reported in Wuhan, China in December 2019. The causative coronavirus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and was closely related (~80% homology) to SARS-CoV, the cause of the 2003 SARS outbreak1). Global spread of SARS-CoV-2 and its associated syndrome, coronavirus disease 2019 (COVID-19), has resulted in large numbers of infections and deaths in most countries.

Typical pathways of SARS-CoV-2 transmission include (i) direct airborne infection following sneezing, coughing, and verbal communication with inhalation of small droplets, and (ii) contact spread (i.e. contact of the eyes, nasal and oral mucosa with virus-containing material). Saliva is regarded as an obvious source of viral transmission. Thus, it is important to understand the details of SARS-CoV-2 transmission via saliva. Takeuchi et al. demonstrated the usefulness of saliva samples for SARS-CoV-2 screening. Saliva is secreted by the major salivary glands (parotid, sublingual, and submandibular glands) and the minor salivary glands. The first step of SARS-CoV-2 infection is binding of the viral S protein to receptors on host cells, triggering membrane fusion. The receptor for SARS-CoV-2 on human cells is angiotensin-converting enzyme 2 (ACE2).

We hypothesized that there are numerous ACE2-positive cells distributed within the salivary glands. Few previous studies have investigated the regions of salivary glands most susceptible to SARS-CoV-2 infection. Xu et al. analyzed bulk RNA-Seq data from public databases including the cancer genome atlas (TCGA), functional annotation of the mammalian genome (FANTOM5), and cap analysis of gene expression (CAGE) and concluded that ACE2 was expressed in the oral cavity, including on the tongue and the floor of the mouth. This preliminary report supported our hypothesis that there may be numerous ACE2-positive cells in the oral tissues. However, the detailed localization of ACE2 from a morphological standpoint could not be determined from RNA-Seq data alone.

Evaluating the detailed morphological localization of ACE2 in the salivary glands and surrounding tissues is important because it may provide information on viral migration and progression of COVID-19. The aim of our study was to assess localization of ACE2-positive cells in the oral salivary gland and associated tissues from a morphological point of view.

Materials and Methods

Tissue samples

Fifty paraffin-embedded tissue blocks from the maxillofacial regions of 48 patients were used in this study. The tissue blocks were obtained for diagnosis and treatment at the Nippon Dental University Niigata Hospital prior to 2010. Personal information except gender, age, and diagnosis was erased and the blocks were anonymized by randomized order coding using 2-digit numbers (Table 1). Opt-out consent notification
was provided on the webpage of the Nippon Dental University Niigata Hospital (http://www.ngt.ndu.ac.jp/hospital/dental/about/optout/). This study was approved by the Ethics Committee of The Nippon Dental University School of Life Dentistry at Niigata (ECNG-R-409).

**Immunohistochemistry and hematoxylin-eosin staining**

Three-micron thick sections were prepared from the formalin-fixed, paraffin-embedded blocks and placed on slides. The sections were deparaffinized using xylene-substitute dewaxing agent (Clear Plus®, Falma Co., Tokyo, Japan) then immersed in graded series of ethanol followed by tap water. The sections were placed in an antigen retrieval solution (sodium citrate, pH 6.0), heated to 95°C–98°C in a water bath, cooled for 20–30 minutes at room temperature (RT). Then the sections were immersed in 0.3% H2O2 containing methanol for 30 minutes at RT. After rinsing with PBS, the sections were incubated with a goat polyclonal antibody raised against ACE2 (ab15348: Abcam, Cambridge, UK) diluted 1:1500 at RT. The sections were incubated with a rabbit polyclonal antibody (Histofine® Simple Stain™ MAX-PO MUL-TI, Nichirei Bioscience Inc., Tokyo, Japan) for 1 hour at RT. After rinsing with PBS, the sections were incubated with a secondary antibody (Histofine® Simple Stain™ MAX-PO MUL-TI, Nichirei Bioscience Inc., Tokyo, Japan) for 30 minutes at RT. After further rinsing with PBS, the sections were immersed in Tris-buffered...
saline, then visualized by incubation for up to 10 minutes with 3,3′-diaminobenzidine tetrachloride (DAB Substrate Kit, Nichirei Bioscience Inc.). The optimal development time depended on the staining intensity. Nuclear staining was performed using hematoxylin and then the sections were mounted with coverslips. In addition to immunohistochemistry, conventional hematoxylin-eosin staining (Carrarzi’s formula) was carried out for neighboring sections from the tissue samples. Inflammatory changes were confirmed in the observed HE-stained section, and significant cases were excluded. Three types of control tests were carried out for validation of immunohistochemistry.

First, as a negative control, PBS was used instead of primary antibody and incubated using the staining procedure above. Second, we mixed ACE2 polyclonal antibody (ab15348, Abcam) and ACE2 peptide (15352, Abcam) in a 1:20 molar ratio and allowed complexes to form at 4°C overnight. Slides were incubated with the antigen-antibody mixture instead of primary antibody and the staining procedure above was followed. We confirmed negative results for both the first and second types of control slides described above. Third, we confirmed concordance between our ACE2 immunoreactivity results for positive control small intestine slides and immunohistochemical expression of ACE2 in the small intestine from a web-based histological expression database (“Tissue Atlas” in The Human Protein Atlas; https://www.proteinatlas.org/ENSG00000130234-ACE2).

All slides were observed under a light microscope (BX53, Olympus, Tokyo, Japan) and evaluated by two investigators.

Results

The patients comprised 21 males and 27 females, aged from 8 to 82 years (mean 49.5 years). An overview of the patients and samples in this study, including diagnoses, is shown in Table 1. We selected sites for sampling that were unrelated to the pathological findings. Therefore, there were no effects related to disease on the localization of ACE2-positive cells. There were no inflammatory changes or tumors observed in the ACE2-immunopositive tissues.

ACE2-positive cells were found in the parotid and sublingual glands (major salivary glands) (Fig. 1A-J) (Table 2) and in the buccal glands (minor salivary glands) (Fig. 1K, L) (Table 2). In the major salivary glands, parotid glands contained ACE-2 positive cells in the acinus region of the serous gland (9 of 12 cases, 75.0%), as well as in the intercalated and striated ducts (all 12 cases) (Fig. 1B) (Table 2). The sublingual glands contained ACE2-positive cells in the serous demilunes (all 10 cases) and striated conduits (11 of 12 cases, 91.7%) (Fig. 1F) (Table 2). A few ACE2-positive mucous acinar cells were observed (Fig. 1J). In the minor salivary glands, buccal glands contained ACE-2 positive cells in the serous demilunes and the lobular ducts (all 3 cases) (Fig. 1L) (Table 2). ACE2-positive adipocytes were observed in the stroma of the parotid gland (11 of 12 cases, 91.7%), sublingual gland (7 of 9 cases, 77.8%) and buccal gland (all 3 cases), and oral lamina propria (oral floor: 4 of 9 cases, 44.4%, buccal mucosa: all 6 cases, tongue: all 5 cases) (Fig. 1C) (Table 2). Several ACE2-positive endothelial cells were observed that lined nearby capillaries that nourishing the parotid gland (11 of 12 cases, 91.7%), sublingual gland (9 of 11 cases, 81.8%), and

| Table 2. Number of ACE-2 positive cases |
|----------------------------------------|
| Serous gland | Mucous gland | Serous demilune | Conduit | Vascular endothelial cell | Adipocyte | Oral mucosal epithelium |
|-------------|--------------|----------------|---------|--------------------------|-----------|------------------------|
| Parotid gland | 9/12 (75.0%)   | -              | 12/12(100%) | 11/12 (91.7%) | 11/12 (91.7%) | -                      |
| Sublingual gland | 10/10 (100%) | 7/9 (77.8%) | 10/10 (100%) | 11/12 (91.7%) | 9/11 (81.8%) | 3/3 (100%) |
| Buccal gland | 3/3 (100%)* | 0/3 (0%) | 3/3 (100%) | 3/3 (100%) | 3/3 (100%) | -                      |
| Buccal mucosa | -             | -              | -         | -                        | -         | -                      |
| Lingual mucosa | -             | -              | -         | -                        | -         | -                      |
buccal gland (all 3 cases) (Fig. 1D, G, H and L) (Table 2). ACE2 expression were also observed in the oral mucosal epithelium (oral floor: 7 of 11 cases, 63.6%, buccal mucosa: 11 of 12 cases, 91.7%, tongue: all 13 cases) and capillary endothelial cells of lamina propria (oral floor: 7 of 12 cases, 58.3%, buccal mucosa: all 12 cases, tongue: all 13 cases) (Fig. 1N) (Table 2).}

**Discussion**

In this study, ACE2-positive cells were observed in both the major and minor salivary glands; both types of glands had numerous ACE2-positive conduits. Most ACE2-immunopositive acini cells were serous-derived. However, a few mucous acini of the sublingual gland were also ACE2 positive.

Only a few studies have assessed ACE2 expression in the salivary glands using immunohistochemistry. Descamps et al. evaluated ACE2 expression in salivary glands using both a monoclonal antibody (MAB933, R&D Systems) and the same polyclonal antibody (ab15348, Abcam) we used in our study. They reported no staining using the monoclonal antibody but staining of the polyclonal antibody in the sialoducts of the submandibular gland. The authors’ descriptions of ACE2 expression in the minor salivary glands; however, the acini and ducts of seromucous glands were ACE2-positive. There was another report by the Sawa et al. that used the same antibody (ab15348, Abcam) and reported that ACE2-immunopositive cells were observed in human mucous and serous gland tissue in tissues. They also observed equivalent ACE2-immunopositive reaction also in the mouse tissue. At the same time, they detected ACE2 mRNA in the above-mentioned tissue samples. Sakaguchi et al. reported immunohistochemical staining of ACE2 in the submandibular gland using a polyclonal antibody (HPA000288, Sigma) according to their report, serous cells and the ductal cavity were ACE2-positive. Moreover, Usami et al. reported ACE2 immunohistochemistry of the major salivary gland, submandibular gland, and minor salivary glands (lip and palate glands) using a polyclonal antibody (21 115-1-AP, Proteintech). ACE2 expression was observed in the cell membrane, brush borders of the main ducts, interlobular ducts, and interlobular excretory ducts of the submandibular gland. The authors’ descriptions of ACE2 ductal expression concurred with our own observations. In the minor salivary glands (lip and palate), ACE2 expression was observed in the cell membranes of duct components including interlobular ducts and interlobular excretory ducts. However, the authors observed that both the mucinous and serous glands lacked ACE2 expression. In our study, ACE2 expression was observed in the serous acini and a few mucinous acini. We conclude that at some stage of the infection, infected tissue fluid or saliva could be secreted into the oral cavity.

In this study, several ACE2-positive cells were observed in the spinous layer and especially in the basal layers of the epithelium of the oral mucosa. Hamming et al. assessed staining of the oral mucosa using a polyclonal antibody (Millennium Pharmaceuticals) 17). They detected ACE2 expression the basal layer of the non-keratinizing squamous epithelium with staining patterns similar to our own observations. Sakaguchi et al. conducted immunohistochemical staining of the oral mucosa using a polyclonal antibody against ACE2 (HPA000288, Sigma) and found that the lingual mucosa and gingival epithelium, as well as the nuclei and cytoplasm of the spinous-basal layer, were positive. They also detected ACE2-positive cells in the horny layer of the lingual mucosa; however, staining was inconsistent and sporadic. Usami et al. conducted ACE2 immunohistochemistry on the stratified squamous epithelium of the tongue and found that it was ACE2-negative. This result implies that most ACE2-positive mucosal epithelial cells are found in deep and not superficial areas. The oral epithelial barrier is the entryway of the digestive system and physically separates the host from the outside environment. The oral mucosa provides the first line of defense against pathogens, extrinsic substances, and mechanical stress. It comprises a layered of keratinized epithelium with underlying connective tissue and a basement membrane; these cells form a mechanically-resistant surface resulting from terminal differentiation. The mucosa is strengthened by cell-to-cell junctions, such as tight junctions, adherens junctions and gap junctions, as well as by deposition of extracellular matrix. Epithelial cell-to-cell junctions can be altered or destroyed during viral infection. The normal oral mucosa contributes to prevention of virus penetration if the mucosa is made up of thick layered squamous epithelium; however, there is inter-individual variation in the thickness of the oral mucosa. One report suggested that in addition to type II broncho-alveolar cells in the pulmonary parenchyma and intestinal enterocytes, the epithelial cells lining the nasal mucosa, the upper respiratory tract, and the oral cavity express ACE2, making the epithelial mucosa the most likely viral entry point. We speculate that the site of SARS-CoV-2 entry may often be the conduit epithelium of the salivary gland rather than mucosal epithelium because of thickness differences.

In the present study, ACE2-positive cells were observed in the capillary juxta-epithelial region of the oral mucosa and near the salivary glands. These capillaries are likely to nourish the oral mucosal epithelium and the salivary glands. The presence of ACE2 in the vascular endothelium implies that SARS-CoV-2 transported by the blood may be responsible for subsequent invasion of host tissue. During SARS-CoV-2 infection, the epithelium of the oral mucosa can develop blistering lesions and ulcers. The extensive damage to the mucosal epithelium originates from endothelium damage and circulation disorders (e.g., micro-coagulation) of capillaries nourishing the mucosal epithelium. A similar process might occur in the salivary glands nourished by the infected vascular endothelium (vasculitis). Criaudo et al. suggested that the inflammatory response in COVID-19 arises from vasculitis in the skin. Also, several reports have suggested that COVID-19-associated vasculitis results in thrombus formation. If this process also occurs in the oral lesions of SARS-CoV-2-infected individuals, this would reinforce our hypothesis.

In the present study, ACE2 expression was observed in scattered adipoocytes in the parotid stromal area and oral lamina propria. Drummond et al. and Garcia et al. reported increased in the adipose tissue of the parotid stroma associated with aging. Al-Benna reported that high-level ACE2 expression in the adipose tissue was associated with COVID-19 mortality among obese patients. He concluded that adiposity may contribute to COVID-19 severity. A review by Kruglikov et al. suggested that ACE2 expression was upregulated in adipose tissue in obese and diabetic individuals. Adipose tissue is a potential target and viral reservoir because adipoocytes secrete various kinds of adipokines. The amount of adipose tissue in the parotid stroma and oral lamina propria is limited, but may contribute to the pro-inflammatory changes underlying COVID-19 progression. Torabi et al. analyzed COVID-19-induced anoma in the olfactory mucosal epithelium and reported an increase in pro-inflammatory cytokines (interleukin-1 and tumor necrosis factor-α) in the olfactory epithelium following SARS-CoV-2 infection. Similar pro-inflammatory states may also occur in the parotid gland and oral mucosa.

In our study, ACE2-positive cells were found in the major salivary glands (parotid and sublingual glands) and in the minor salivary glands (buccal glands). Notably, ACE-2 positive cells were found in the con-
ducts and acinar regions of serous glands. This implies that salivary tissue could be a source of virus-contaminated saliva after infection with SARS-CoV-2. Wilson and Pandey(52) suggested that the presence of a ductal valve creates a unidirectional flow of saliva out the gland, thus preventing bacteria and viruses from entering. However, this valve may become non-functional, resulting in ascending bacterial or viral infection. Dehydration, drying medications (e.g., atropine, antihistamines, and psychotropic agents), and diseases that decrease salivary output (e.g., Sjögren syndrome), can increase the risk of ascending bacterial or viral infections. These factors may also lead to increased risk of SARS-CoV-2 infection in conduits.

Several reports have assessed the usefulness of PCR from saliva compared with nasopharyngeal swabs for detection of SARS-CoV-2.33,36 To et al. validated the use of nasopharyngeal saliva for PCR diagnosis.37 Recently, a newly-identified tubarial salivary gland was observed by examination using positron emission tomography/computed tomography with prostate-specific membrane antigen ligands.38 We speculate that there are ACE2-positive conduits and gland tissues in the newly-identified oropharyngeal salivary gland. If so, this would imply that this gland could be a source of virus-contaminated saliva around the oropharyngeal mucosa. Current saliva collection methods in the clinic involve the subject spitting saliva into a sample tube. Although a certain level of detection sensitivity has been established, safe and selective collection of parotid saliva, for example, may be effective in further improving detection accuracy. Further studies regarding salivary sample acquisition will be necessary to optimize diagnostic screening of SARS-CoV-2 patients.

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Conflict of Interest

All authors declare that they have no conflict of interest or financial relationship relevant to this article to disclose.

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