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Early View

Research letter

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Elevated ACE2 expression in the olfactory neuroepithelium: implications for anosmia and upper respiratory SARS-CoV-2 entry and replication

Mengfei Chen*, Wenjuan Shen*, Nicholas R. Rowan, Heather Kulaga, Alexander Hillel, Murugappan Ramanathan Jr, and Andrew P. Lane
Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins University School of Medicine, Baltimore, MD

* Drs. Chen and Shen contributed equally to this manuscript.

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Co-corresponding Authors:
Andrew P. Lane, MD
Department of Otolaryngology – Head and Neck Surgery
Johns Hopkins Outpatient Center, 6th floor
601 N Caroline Street
Baltimore, MD 21287-0910
alane3@jhmi.edu

Mengfei Chen, PhD
Department of Otolaryngology – Head and Neck Surgery
Johns Hopkins University School of Medicine
855 North Wolfe Street, Baltimore, MD 21205
mchen85@jhmi.edu

The ongoing outbreak of coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a major threat to global health [1]. The mechanism of cellular entry by SARS-CoV-2 is through binding to

angiotensin-converting enzyme 2 (ACE2) [2, 3], a metalloproteinase ectoenzyme that primarily functions in the regulation of angiotensin II, but also has non-catalytic roles such as intestinal neutral amino acid transport. The level of ACE2 protein and its subcellular localization in the respiratory tract may be a key determinant of susceptibility to infection, symptoms, and outcomes in COVID-19. In humans, ACE2 protein is broadly expressed in the lung, kidney, and small intestine [4]. Pathological analysis of COVID-19 postmortem samples shows substantial damage in the lung [5], suggesting that the airway is the principal entry and target of SARS-CoV-2. However, analysis of multiple single cell RNA-seq datasets reveal overall low ACE2 RNA transcription in nasal airway epithelium, with further reduced expression in lower airway club cells and rare expression in alveolar epithelial cells [6]. This pattern of ACE2 expression provides evidence that the upper, rather than the lower, airway is the initial site of SARS-CoV-2 infection.

There is growing interest in a presentation of SARS-CoV-2 infection characterized by olfactory loss without concomitant nasal inflammatory symptoms. Disturbances in the sense of smell have been widely reported in COVID-19 patients internationally, with a reported prevalence as high as 85% in a large, multicenter European survey [7]. These reports show, importantly, that some COVID-19 patients manifest olfactory loss as their initial or only symptom. As this presentation is largely not recognized or thought to mandate isolation, this patient group may be a source of continued viral spread and a target population for early intervention and mitigation. The loss of the sense of smell suggests the possibility of direct targeting by SARS-CoV-2 of the olfactory system. However, the cellular location of ACE2 protein in the olfactory epithelium has not been previously demonstrated.

In this study, we performed an immunohistological analysis to determine the location of ACE2 protein in human nasal and tracheal specimens. Nasal tissue included olfactory epithelial or respiratory epithelial samples collected from chronic rhinosinusitis (CRS) patients and control subjects undergoing endonasal surgical approaches for non-CRS disease processes [8]. 4 control (2 females and 2 males ranged from 45 to 63 years old) and 19 CRS biopsies (10 females and 9 males ranged from 37 to 74 years old) were used for detailed immunohistochemistry analysis. 7 tracheal specimens were collected from tracheal stenosis patients who undergoing bronchoscopy. The immunostaining process was carried out after an antigen retrieval step using the following primary antibodies: Goat anti-ACE2 (1:40, AF933; R&D), Rabbit anti-ACE2 (1:100, MA5-32307; Thermo), Goat anti-GFP (1:100, ab6673; Abcam, IgG negative control), Mouse anti-Keratin 18 (1:500, Pierce MA1-39483), Rabbit anti-DCX (1:500, GeneTex, GTX134052), Rabbit anti-PGP9.5 (1:500, Ultraclone RA95101), and Mouse anti-Mucin 5AC (1:200, Abcam ab3649). The research protocol involving human specimens was approved by the Johns Hopkins institutional review board, and all subjects provided signed informed consent.

Within the accessible portion of the olfactory cleft in the nasal cavity, islands of olfactory mucosa are often found surrounded by respiratory epithelium. The specialized olfactory

neuroepithelium has an apical surface consisting mainly of sustentacular cells, which support neuronal dendritic projections containing the odor-sensing cilia. Confocal images demonstrated that the majority of ACE2 staining is localized to the apical surface of $Krt18^{+}$ sustentacular cells in the olfactory neuroepithelium (Figure 1A, B, and G). This distribution of ACE2 protein is similar to that reported in bat nasal epithelium [9]. We further quantified olfactory ACE2 expression and found the number of ACE2 positive cells to be comparable between healthy controls and CRS (Figure 1A, B, and H), a common inflammatory disease of the nasal mucosa that affects the olfactory mucosa [8]. ACE2 is not present in olfactory neurons, demonstrated by co-staining with the immature and mature olfactory neuron marker DCX (Figure 1E) and PGP9.5 (Figure 1F), respectively. We further confirmed the apical location of ACE2 in olfactory epithelium using another ACE2 antibody recognizing a different epitope (Figure 1G). The specificity of ACE2 staining was verified by using a Goat IgG isotype control (Figure 1K).

High intensity ACE2 staining was detected in all 13 olfactory mucosal biopsies. In addition, ACE2 is frequently observed in Bowman's glands and duct cells (Figure 1I). In the adjacent nasal respiratory epithelium, ACE2 is also located on the apical surface (Figure 1J), with a significantly lower level of expression than the olfactory epithelium (Figure 1L). As shown in Figure 1M, in adjacent areas, there is intense ACE2 expression in the $PGP9.5^{+}$ olfactory region, but ACE2 can barely be detected in the $PGP9.5^{-}$ respiratory epithelium. Only 47.4% of nasal respiratory epithelial biopsies (9 in 19) contained ACE2 positive epithelial cells. We quantified the intensity of ACE2 fluorescence and observed striking enrichment (200-700 fold) in the olfactory epithelium (Figure 1N). This cellular tropism of SARS-CoV-2 may be associated with olfactory dysfunction and underlie its high transmissibility. Given the supporting function of $Krt18^{+}$ sustentacular cells for olfactory sensory neurons and sensory cilia, the absence of ACE2 expression in neurons indicates an indirect effect of SARS-CoV-2 infection on COVID-19-associated anosmia.

We further examined ACE2 protein in tracheal epithelium. In 2 of 7 specimens, we detected low expression of ACE2 in $Muc5ac^{+}$ secretory cells (Figure 1O). The expression of ACE2 by secretory cells is reminiscent of the recent findings that increased ACE2 expression in small airway epithelium in COPD patients [10], a disease characterized by secretory cell hyperplasia [11]. Together, the comparatively enhanced human airway expression of ACE2 localized to the olfactory neuroepithelium (Figure 1M and N) suggests a mechanism of olfactory loss and a potential entry point of SARS-CoV-2 into the central nervous system and causes neurological symptoms in COVID-19 patients [12].

Recent studies have suggested a correlation between ACE2 expression level and COVID-19 associated clinical traits. For example, the increased severity of COVID-19 in obese young patients may be linked to increased ACE2 expression in lung epithelial cells [13, 14], and the lower ACE2 gene expression in the nasal epithelium of children relative to adults may help

explain the lower prevalence of COVID-19 children[15]. The cohort presented in the current study are all over 30 years old limited to verify the age factor in ACE2 expression level. However, the relatively increased ACE2 expression in olfactory versus respiratory epithelium is similar across all individuals. Notably, like in the animal [16], the olfactory area in adults may be significantly larger compared to children, with a different geometry and airflow pattern.

Understanding of the pattern of viral load in tissues of COVID-19 patients is critical for diagnosis, management of transmission, and potential treatment strategies. Detection of SARS-CoV-2 in clinical specimens shows that the highest viral copy number is found in nasal swabs (~200 fold), compared to bronchoalveolar lavage or pharyngeal swabs [17, 18]. In the early stages of SARS-CoV-2 infection, viral RNA can readily detected in upper respiratory specimens but not in blood, urine, or stool [19]. These findings, taken together with ACE2 protein cellular localization presented here, suggests that active virus infection and replication occurs in the apical layer of nasal and olfactory mucosa. The differential expression of ACE2 in the olfactory neuroepithelium and respiratory epithelium may help account for the spectrum of nasal-related symptoms, while also raising the intriguing possibility that COVID-19 may be amenable to novel therapeutic approaches. Whether nasal saline irrigation, a common treatment for sinonasal conditions, is beneficial or counter-productive in SARS-CoV-2 infection remains to be determined; however, consideration should be given to the delivery of topical anti-viral additives, such as detergent or povidone-iodine, directed at the nasal and nasopharyngeal viral reservoirs.

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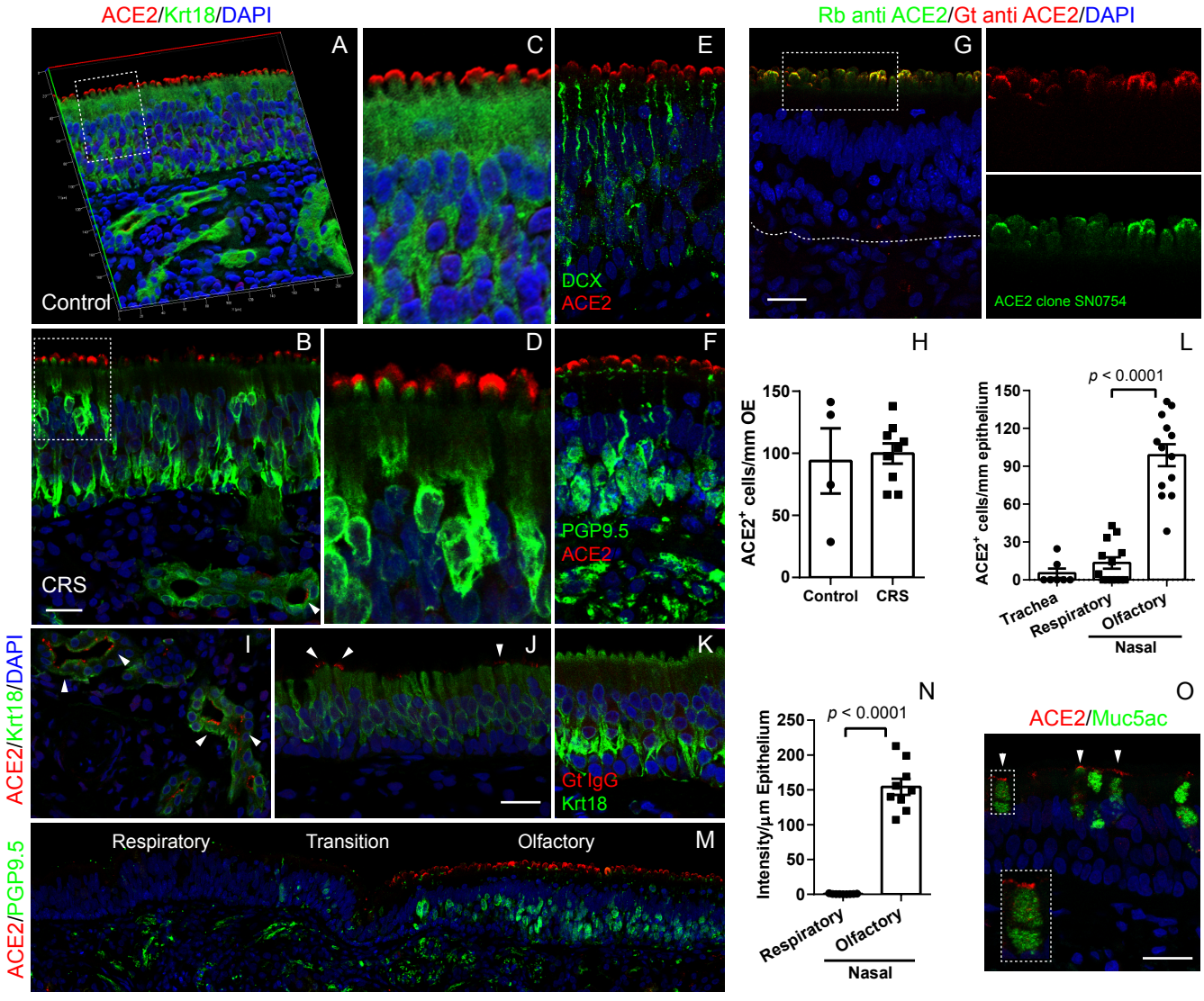
Figure 1. Cellular location of ACE2 in human nasal and tracheal biopsies.

(A-D) Confocal image of ACE2 (red) and Krt18 (green) immunostaining in the olfactory neuroepithelium from healthy control (A and C) and CRS patient (B and D). The 3-dimensional image in A shows that the ACE2 is localized to the apical surface of Krt18 positive sustentacular cells in the olfactory epithelium. Confocal images were obtained under Z stack mode which covered 8 μm in depth. The boxed area in Panel A and B was

highlighted in C and D, respectively. Abbreviations: ACE-2, angiotensin converting enzyme II; CRS, chronic rhinosinusitis.
(E and F) The location of ACE2 and DCX positive immature (E) or PGP9.5 positive mature (F) olfactory sensory neurons in control.
(G) Confocal image verified the apical expression pattern of ACE2 by co-staining of Goat anti ACE2 and another Rabbit anti ACE2 antibody (clone SN0754). The boxed area in G was highlighted in right panels.
(H) Quantification of ACE2 positive cells per mm olfactory epithelium. At least 3 images were collected from each specimen (4 controls and 9 CRS biopsies) using 40 x objectives under the z stack scan mode at same depth.
(I and J) Expression of ACE2 in glands (I) and nasal respiratory epithelium (J).
(K) No detectable signal in Goat IgG isotype control.
(L) Quantification of the $ACE2^{+}$ cell per mm epithelium. The positive cells in 7 tracheal biopsies and 13 nasal specimen that contained both respiratory and olfactory epithelium were counted.
(M) Representative image of respiratory-olfactory mucosa adjacent area. PGP9.5 and ACE2 co-staining image was obtained using confocal microscope under the tile scan mode.
(N) Quantification of the ACE2 fluorescence intensity per μm epithelium. Nasal specimen that contained both respiratory and olfactory epithelium were quantified using Image J.
(O) Co-staining of ACE2 and secretory cell marker Muc5ac in tracheal airway epithelium. The inset represents magnification of the selected area.
Dots in graph represent independent specimens (H, L, and N). Data are represented as mean ± SEM. *p* value was calculated by unpaired two-tailed Student's *t* test. Differences were considered significant when $P < 0.05$. Scale bars, 20 μm.

Figure 1


ACE2/Krt18/DAPI
Rb anti ACE2/Gt anti ACE2/DAPI
A
C
E
G
Control
DCX
ACE2
ACE2 clone SN0754
B
D
F
H
L
CRS
PGP9.5
ACE2
ACE2+ cells/mm OE
Control
CRS
p < 0.0001
ACE2+ cells/mm epithelium
Trachea
Respiratory
Olfactory
Nasal
ACE2/Krt18/DAPI
I
J
K
N
O
Gt IgG
Krt18
Intensity/µm Epithelium
p < 0.0001
Respiratory
Olfactory
Nasal
ACE2/Muc5ac
ACE2/PGP9.5
Respiratory
Transition
Olfactory
M