

1 Expression of *Ace2*, *Tmprss2*, and *Furin* in mouse ear tissue

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9 **Key words:** SARS-CoV-2, COVID-19, ACE2, TMPRSS2, Furin, Temporal Bone

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16 **Author contributions**

17 T.U developed the concept and performed the experiments, and wrote the draft of the
18 manuscript. K. A., R.U., T.S., H.B., G.Y., and M.K. performed the experiments. K.K
19 reviewed the manuscript. T.Y. developed the concept, designed the experiment, and
20 wrote the draft of the manuscript. All authors contributed to interpretation of the data
21 and writing of the manuscript.

22

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26

27 **Conflict of Interest Statement**

28 We declare no competing interests.

29 **Abstract**

30 Objectives: Intracellular entry of the severe acute respiratory syndrome coronavirus 2
31 (SARS-CoV-2) depends on the interaction between its spike protein to a cellular
32 receptor named angiotensin-converting enzyme 2 (ACE2) and depends on
33 Furin-mediated spike 23 protein cleavage and spike protein priming by host cell
34 proteases including 24 transmembrane protease serine 2 (TMPRSS2). *Tmprss1*,
35 *Tmprss3*, and *Tmprss5* are expressed in the spiral ganglion neurons and the organ of
36 Corti in the inner ear; however, *Ace2*, *Tmprss2*, and *Furin* expression profiles in the
37 middle ear remain unclear. Therefore, this study aimed to analyze *Ace2*, *Tmprss2*, and
38 *Furin* expression in the middle and inner ear of mice.

39 Study Design: Animal research.

40 Setting: Department of Otolaryngology and Head and Neck Surgery, University of
41 Tokyo.

42 Methods: We performed immunohistochemical analysis to examine the distribution of
43 *Ace2*, *Tmprss2*, and *Furin* in the eustachian tube, middle ear space, and cochlea of mice.

44 Results: *Ace2* was expressed in the cytoplasm in the middle ear epithelium, eustachian

45 tube epithelium, stria vascularis, and spiral ganglion. Tmprss2 and Furin were widely
46 expressed in the middle ear spaces and the cochlea.

47 Conclusion: Co-expression of Ace2, Tmprss2, and Furin in the middle ear indicates that
48 the middle ear is susceptible to SARS-CoV-2 infections, thus warranting the use of
49 personal protective equipment during mastoidectomy for coronavirus disease
50 (COVID-19) patients.

51 Key words: SARS-CoV-2, COVID-19, ACE2, TMPRSS2, Furin, Temporal Bone

52

53

54 **Introduction**

55 The recent coronavirus disease (COVID-19) pandemic caused by severe acute
56 respiratory syndrome coronavirus 2 (SARS-CoV-2) poses a serious health concerns.
57 SARS-CoV-2 generally infects the upper respiratory tract and then spreads to various
58 organs. The clinical symptoms of COVID-19 patients include fever, cough, sore throat,
59 fatigue, and loss or decline of smell (anosmia or hyposmia).¹⁻⁴ The Eustachian tube is
60 the conduit between the middle ear space and upper respiratory tract through which,

61 SARS-CoV-2 can spread into the middle ear spaces. Moreover, other human
62 coronaviruses have been identified in middle ear fluid in cases of acute otitis media or
63 otitis media with effusion.⁵⁻⁷ Two studies have reported the occurrence of acute otitis
64 media⁸ and sensorineural hearing loss⁹ among COVID-19 patients. Another study
65 carried out pure tone audiometry and transitory evoked otoacoustic emission (TEOAE)
66 analysis for 20 asymptomatic patients aged 20–50 years positive for COVID-19 on
67 reverse transcription-PCR (RT-PCR) testing and reported that high-frequency pure-tone
68 thresholds and TEOAE amplitudes were significantly worse among the COVID-19
69 patients than among 20 healthy controls.¹⁰

70 Intracellular entry of SARS-CoV-2 depends on the interaction between viral spike
71 proteins and a cellular receptor, angiotensin-converting enzyme 2 (Ace2)¹¹⁻¹³,
72 Furin-mediated Spike cleavage¹³⁻¹⁵, and Spike protein priming by host cell proteases
73 including transmembrane protease serine 2 (Tmprss2).^{13,16} Thus, Ace2, Tmprss2, and
74 Furin upregulation enhances intracellular SARS-CoV-2 entry, thus resulting in clinical
75 symptoms. Previous studies have reported 8 *Tmprss* genes including *Tmprss2* in the
76 inner ear through RT-PCR analysis, although immunohistochemical analysis revealed

77 the expression of only *Tmprss1/3/5* in the inner ear, indicating that *Tmprss1/3/5* are
78 expressed in the spiral ganglion neurons and *Tmprss3* is also expressed in the organ of
79 Corti.¹⁷ To our knowledge, *Ace2*, *Tmprss2*, and *Furin* expression in the middle ear
80 spaces and *Ace2* and *Furin* expression in the inner ear have not been reported.

81 This study aimed to investigate the mechanism underlying middle ear infection and
82 sensorineural hearing loss in COVID-19, by assessing *Ace2*, *Tmprss2*, and *Furin*
83 expression in the middle and inner ear tissues of mice.

84

85 **Materials and Methods**

86

87 *Experimental samples*

88 Tissue samples were obtained from the mice used in our previous studies because
89 the purchase of new animals has been prohibited in our facility owing to the COVID-19
90 pandemic. Tissue samples were obtained from normal 11-week-old male ICR mice, and
91 paraffin-embedded tissue samples including the middle ear spaces, Eustachian tube area,
92 and cochlea were used (Figure 1, 2). Normal tissue morphology in these regions was

93 confirmed through hematoxylin and eosin staining by a qualified pathologist and by
94 otolaryngologists. All experiments were conducted in accordance with institutional
95 guidelines and with the approval of the Animal Care and Use Committee of the
96 University of Tokyo (No. P18-015)

97

98 *Histological analyses*

99 Immunohistochemical staining was performed for Ace2 and Tmprss2.
100 Four-micrometer-thick serial paraffin-embedded sections were deparaffinized in xylene
101 and dehydrated in ethanol and then treated with 3% H₂O₂ to block endogenous
102 peroxidase activity and incubated with Blocking One (Nacalai Tesque, 34 Kyoto, Japan)
103 to block non-specific immunoglobulin binding prior to immunostaining. After antigen
104 activation, the tissue sections were probed with primary anti-Ace2 (1:300 dilution;
105 rabbit monoclonal, Abcam, ab108252; Cambridge, UK), anti-Tmprss2 (1:1000 dilution;
106 rabbit monoclonal, Abcam, ab92323; Cambridge, UK), and anti-Furin (1:100 dilution;
107 rabbit monoclonal, 1 Abcam, ab183495; Cambridge, UK) antibodies, followed by
108 probing with appropriate peroxidase-conjugated secondary antibodies and a

109 diaminobenzidine substrate. Images of all sections were captured using a digital
110 microscope camera (Keyence BZ-X700) with 4×, 10×, and 40× objective lenses.

111

112 **Results**

113 Ace2, Tmprss2, and Furin were detected in the mucosal epithelium of the
114 Eustachian tube and middle ear spaces and in the cochlea, although their expression
115 pattern varied among tissues. Furthermore, Ace2, Tmprss2, and Furin were
116 co-expressed in the mucosal epithelium of the Eustachian tube and the middle ear
117 spaces, the stria vascularis, and spiral ganglion cells.

118 Tmprss2 and Furin were expressed in the mucosal epithelial cells in the
119 middle ear spaces; however, Ace2 was only slightly expressed in the cytoplasm of the
120 epithelial cells (Fig.1A). Furthermore, Ace2 and Tmprss2 were expressed in the nuclei
121 of epithelial cells. Ace2, Tmprss2, and Furin expression profiles in the Eustachian tube
122 were almost identical to those in the middle ear spaces (Fig.1B).

123 In the cochlea, Ace2 was expressed in the nuclei of the hair cells and supporting
124 cells in the organ of Corti, marginal, intermediate, and basal cells in the stria vascularis,

125 fibrocytes of the spiral ligament, and spiral ganglion cells. Furthermore, Ace2 was
126 slightly expressed in the cytoplasm of stria cells and spiral ganglion cells. Tmprss2
127 was diffusely strongly expressed in the nuclei and cytoplasm in the organ of Corti,
128 stria vascularis, spiral ligament, and spiral ganglion cells, being greater in the nucleus
129 than in the cytoplasm. Furin was diffusely expressed in the cytoplasm, but not in the
130 nucleus, in the organ of Corti, stria vascularis, spiral ligament, and spiral ganglion cells
131 (Fig.2).

132

133 **Discussion**

134

135 This immunohistochemical study shows that Ace2, Tmprss2, and Furin are
136 co-expressed in the mucosal epithelium of the Eustachian tube and middle ear spaces
137 and the organ of Corti, lateral wall, and spiral ganglion cells in the cochlea. In both
138 middle ear tissues and the cochlea, Ace2 is primarily expressed in the nucleus, while
139 Tmprss2 is expressed in the nucleus and cytoplasm, and Furin was expressed primarily
140 in the cytoplasm. These results suggest that middle ear spaces are highly susceptible to

141 SARS-CoV-2 infections spreading from the upper respiratory tract through the
142 Eustachian tube, indicating the possibility of severe sensorineural hearing loss upon
143 intracellular entry of SARS-CoV-2 in the cochlea.

144 Herein, we used ICR mice to determine the distribution of Ace2, Tmprss2, and
145 Furin in the ear tissues. We could not obtain human ear tissue samples for
146 immunostaining; hence, the expression patterns of these proteins in human ear tissues
147 might differ from those of the present mouse ear tissues. However, we previously
148 compared the Ace2, Tmprss2, and Furin expression patterns between human and mouse
149 nasal tissues and found that their expression patterns were identical.¹⁸

150 Mastoidectomy is required during major ear surgeries including cochlear
151 implantation and middle ear surgery for extensive cholesteatoma. Mastoidectomy with a
152 high-speed drill generates massive aerosols.¹⁹ Previously, cadaveric simulation of
153 otological procedures during mastoidectomy revealed gross contamination 3 to 6 feet
154 away in all cardinal directions and more significantly on the left side, corresponding to
155 the direction of drill rotation.²⁰ SARS-CoV-2 would substantially infect the middle ear
156 spaces because of the co-expression of Aces, Tmprss2, and Furin. Therefore, high levels

157 of personal protective equipment are strongly recommended during mastoidectomy for
158 COVID-19 patients or all mastoidectomies during the COVID-19 pandemic.

159 The cochlea is isolated and thus is generally resistant to the infection. A recent
160 systemic review on the audio-vestibular symptoms of coronavirus infections found no
161 records of audio-vestibular symptoms reported during previous coronaviral diseases
162 including SARS and Middle East respiratory syndrome.²¹ However, Ace2, Tmprss2, and
163 Furin were co-expressed in the organ of Corti, lateral wall, and spiral ganglion cells in the
164 cochlea. Thus, similar to other viral infections including mumps, when SARS-CoV-2
165 enters the cochlea, it can cause severe labyrinthitis, resulting in marked hearing loss.

166

167 **Conclusions**

168 Ace2, Tmprss2, and Furin are co-expressed in the mucosal epithelium of the
169 Eustachian tube and middle ear spaces and the organ of Corti, lateral wall, and spiral
170 ganglion cells in the cochlea in mice. ACE2 is primarily expressed in the nucleus,
171 Tmprss2 is expressed in the nucleus and cytoplasm, and Furin is primarily expressed in
172 the cytoplasm. These results indicate that middle ear spaces are highly susceptible to the

173 SARS-CoV-2 infection, thus warranting the extensive use of personal protective
174 equipment during mastoidectomy for COVID-19 patients.

175

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178

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238 **Figure Legends**

239 Figure 1. Histological analysis of the middle ear spaces and the Eustachian tube
240 expressing Ace2, Tmprss2, and Furin.

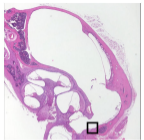
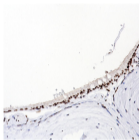
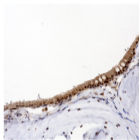
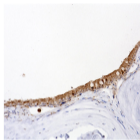
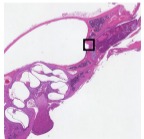
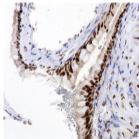
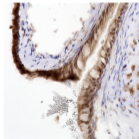
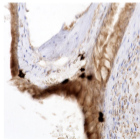
241 **A:** Hematoxylin-eosin staining of the middle ear mucosa (magnification, 40×).
242 Immunohistochemical staining for Ace2 (cytoplasm), Tmprss2 (nucleus and cytoplasm),
243 and Furin (cytoplasm) (400×). **B:** Parts of the Eustachian tube (40×) and Ace2, Tmprss2,
244 and Furin expression in middle ear spaces (400×).

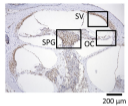
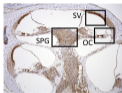
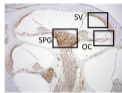
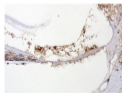
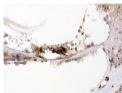
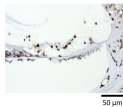
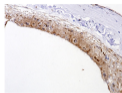
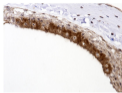
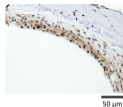
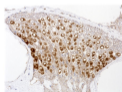
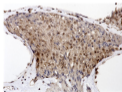
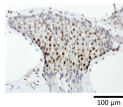
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246 Figure 2: Cochlear Ace2, Tmprss2, and Furin expression.

247 **Top panel:** Parts of the organ of Corti (OC), stria vascularis (SV), and spiral ganglion
248 (SPG) (magnification, 40×). **OC:** Ace2 expressed in the nucleus of the hair cells and OC
249 supporting cells; Tmprss2, diffuse in OC cell nuclei and cytoplasm; Furin, diffuse in
250 the OC cell cytoplasm. **SV:** Ace2, nucleus of marginal, intermediate, and basal cells in
251 the stria vascularis; Tmprss2, nucleus and cytoplasm in the stria vascularis; Furin,
252 cytoplasm in the stria vascularis. **SPG:** Ace2, nucleus of the spiral ganglion cells;
253 Tmprss2, diffuse in the nucleus and cytoplasm in spiral ganglion cells; Furin, diffuse in

254 the cytoplasm in the spiral ganglion cells.

A**ACE2****TMPRSS2****Furin****B****ACE2****TMPRSS2****Furin**

ACE2**TMPRSS2****Furin**200 μm **OC**50 μm **SV**50 μm **SPG**100 μm