COVID-19 Resulting in Potential Hearing Damage of Rodents

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Keywords
► sudden sensorineural hearing loss
► COVID-19
► animal models
► inner ear

Abstract

Objectives To find out the association between the sensorineural hearing loss and coronavirus disease 2019 (COVID-19), the expression of ACE2 and TMPRSS2 in hamsters and mice was detected.

Design Using the public data from the National Center for Biotechnology Information and the Global Initiative on Sharing All Influenza Data, the expression of ACE2 and TMPRSS2 at the transcriptomic, DNA, and protein levels of ACE2 in the brain, inner ear, and muscle from the golden Syrian hamsters (Mesocricetus auratus) and mice (Mus musculus) was assessed.

Results We identified ACE2 and TMPRSS2 expressed at different levels in the inner ear and brain at DNA and transcriptomic levels of both mice and hamsters. The protein expression from the brain and inner ear showed a similar pattern, while the expression of ACE2 from the inner ear was relatively higher than that from the muscle.

Conclusion Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) shows genetic potential to infect the hearing system of rodents and lead to sudden sensorineural hearing loss that can be used as a characteristic to detect asymptomatic patients of COVID-19.

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**Introduction**

Thousands of deaths caused by coronavirus disease 2019 (COVID-19) within a few months highlight the importance of early diagnosis of the disease. Meanwhile, the increasing trend of the confirmed cases draws more attention to the treatment and prognosis of COVID-19 patients. Some recent clinical studies have observed some unspecific symptoms of COVID-19, including headache, diarrhea, and sudden sensorineural hearing loss (SSNHL)\(^1,2\); one case has reported profound sensorineural hearing damage in a 60-year-old COVID-19 patient.\(^3\) To our knowledge, the ACE2 receptor is a key for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entering human cells, while TMPRSS2 is highly involved in virus replication, the co-expression of which can mainly determine the level of damage to the hosts. It has been previously investigated that the expression of ACE2 and TMPRSS2 was high in the intestine and kidneys of humans compared with the brain and lungs. However, several clinical studies have confirmed the most common symptoms of COVID-19, which included SSNHL. They did not draw much attention due to their similarities to the symptoms of the flu or cold. SARS-CoV-2 can infect many organs, including the hearing system, and can invade the cochlear nerve and lead to SSNHL and cause hearing damage after the treatment of the patients with COVID-19. We have already demonstrated that a golden hamster (Mesocricetus auratus)\(^4-6\) can be a better small animal model for COVID-19 compared with a mouse (Mus musculus) for understanding the efficiency and possibility of SARS-CoV-2 attacking human cells with certain or potential consequences.

**Materials and Methods**

**Animals**

The procedures in this study involving animals were reviewed and approved by the Animal Experimentation Ethics Committee of Henan University of Chinese Medicine (DWLL202001301). Syrian hamsters (\(n = 3\)) and C57BL/6 mice (\(n = 6\)) were sacrificed, and tissues of the inner ear, liver, heart, spleen, lungs, brain, and muscle were collected. All tissues were immediately immersed into the TRIzol reagent (TaKaRa, Japan) after dissection and stored at \(-80^\circ C\) before further experiments.

**Transcriptome Analysis**

Raw RNA-seq data of transcriptomes were collected from the National Center for Biotechnology Information (NCBI). The sequences of ACE2 and TMPRSS2 was aligned by using Clustal Omega (V1.2.3) and the phylogeny was reconstructed and calculated by using MEGA v5.2. 1000 maximum number of iterations were applied with Kimura two factor correction method for genetic distance prediction. Cufflinks v2.2.1 with Bowtie2 (Langmead and Salzberg 2012) were used for gene expression analysis.

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**Table 1:** Primers for ACE2 and TMPRSS2 gene expression analysis

<table>
<thead>
<tr>
<th>Species</th>
<th>Genes</th>
<th>Primers</th>
<th>Sequence (5' - 3')</th>
<th>Annealing Temp (°C)</th>
<th>Amplicon size (bp)</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Syrian hamster</td>
<td><strong>ACE2</strong></td>
<td>Primer 1 F: CAATGGTGAATCAGGGCTGG</td>
<td>60</td>
<td>149</td>
<td>XM_005074209.2</td>
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<tr>
<td></td>
<td></td>
<td>R: GTGCGACACACCACCTTCCGAT</td>
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<tr>
<td></td>
<td></td>
<td>Primer 2 F: ACTGACATTTGTTGGACGC</td>
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<td>130</td>
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<tr>
<td></td>
<td></td>
<td>R: ACCAAGATCTCGGCTGTCGTTCA</td>
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<tr>
<td></td>
<td><strong>TMPRSS2</strong></td>
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<td></td>
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<tr>
<td></td>
<td></td>
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<td></td>
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<td>R: TCCAATCATCTCCGTGACAG</td>
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<td><strong>ACTB</strong></td>
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<td></td>
<td></td>
<td>R: GTCTGTTGCCAAATGGTATG</td>
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<td>Mouse</td>
<td><strong>ACE2</strong></td>
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<tr>
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<td>Primer 2 F: TGATGGAATCAGGGCGTGGATG</td>
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<tr>
<td></td>
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<td>R: ATTCGAAAGTCCTCCGGTCCC</td>
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<td><strong>TMPRSS2</strong></td>
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<td>180</td>
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<td><strong>ACTB</strong></td>
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<td>R: AAGGTTGTAATAACCAGCAGCTCA</td>
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</tbody>
</table>

Abbreviations: bp: base pairs; F: forward primer; R: reverse primer; Temp: temperature.
used for expression analysis of transcriptomes from different tissues in humans, mice, and hamsters.

**Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction**

Total RNA was extracted from tissues homogenates and purified with the TRIzol reagent according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed by PrimerScript RT Reagent Kit (TaKaRa, Japan) following manufacturer instructions. Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) reactions were performed using the TB Green Premix TaqII (TaKaRa, Japan) following the manufacturer's instructions. The genes were amplified with specific primers (~*Table 1*). The reference gene, human β-actin gene, and mouse β-actin gene were used for normalization. Samples were run in triplicates, and negative control (no template control) was run concurrently with cDNA to check for primer dimers and contaminants. All reactions were performed twice to ensure the technical reproducibility of the assays. The average standard deviation within duplicates studied was 0.5 cycles.

**Western Blotting**

Tissues protein extracts were isolated from the inner ear, brain, and muscles of Syrian hamsters (n = 3) and C57BL/6 mice (n = 6) with the Tissue Protein Extraction kit (CWBOIO, Beijing, China) following manufacturer's instructions. Bradford Protein Assay (CWBOIO, Beijing, China) was used to estimate the protein concentration. Fifty micrograms of protein supplemented with 5× LSB loading dye was separated in a 10% gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Turbo Midi PVDF by semidry blotting. After blocking for 1 hour at room temperature with a blocking solution of 5% dry milk, the membrane was incubated with the first antibody diluted in blocking solution at 4°C overnight. Incubation with the secondary, horseradish peroxidase (HRP)-conjugated antibodies were kept for 1 hour at room temperature. For the detection of the ACE2 and TMPRSS2 protein, a 1:1,000 dilution of the ACE2 rabbit polyclonal antibody (21115-1-AP, Proteintech, United States) and TMPRSS2 (EPR3861) antibody (ab92323, Abcam, United States) was used as the first antibody. And a 1:12,000 dilution of a goat anti-rabbit IgG (HRP) was used as the secondary antibody (ab205719, Abcam, Cambridge, MA, United States). GAPDH expression was demonstrated with a 1:10,000 dilution of GAPDH mouse monoclonal antibody (60004-1-lg, Proteintech, United States) as the first antibody and a 1:12,000 dilution of the goat anti-mouse antibody (ab205719, Abcam, United States) as the second antibody. All data were analyzed with Graphpad Prism 8.0 software (GraphPad Software, United States).
Statistical Analysis
All data were analyzed with Graphpad Prism 8.0 software (GraphPad Software, United States). Comparison between two groups was performed by Student t-test or unpaired Student t-test. Significance was defined as p < 0.05, data were reported as mean ± SEM, and error bars indicated SEM.

Results
Gene ACE2 and TMPRSS2 of mice and hamsters had more than 80% similarity to humans (Fig. 1A). We found higher expression of ACE2 in the heart of humans and slightly higher expression in the brains of both humans and mice, while TMPRSS2 showed high expression in the lungs of both humans and mice but lower expression in the brain (Fig. 1B). In addition, we found a similarity in patterns between hamsters and humans; both ACE2 and TMPRSS2 were highly expressed in the spleen and were relatively high in the liver, but not in the brain (Fig. 1C). The mRNA expression of ACE2 was high in the inner ear of both mice and hamsters, while TMPRSS2 was much highly expressed in the lungs compared with the inner ear. However, both genes showed lower expression levels in the brains of these two animal models. The protein expression from the brain and inner ear showed a similar pattern, while the expression of ACE2 from the inner ear was relatively higher than that from the muscle (Fig. 1D–F).

Discussion
We hypothesized that SARS-CoV-2 might infect the inner ear by binding to ACE2, which could be a potential reason causing sensorineural hearing loss by pathogens invasion. One study found ACE2, TMPRSS2, and Furin expressed in the middle ear of a mouse, which is consistent with our results. Since data of transcriptome from the inner ear specifically were not available on NCBI, the brain tissue was chosen as an alternative. We found the expression of ACE2 in the inner ear of both mice and hamsters, although the expression level was different compared with ACE2 expression level in the lungs. TMPRSS2 showed a higher expression in the lungs compared to it in the inner ear, which might be the reason that the symptoms of COVID-19 in the respiratory is more severe than it found in the inner ear. More sophisticated work is required to understand the molecular mechanism of SARS-CoV-2 infecting the inner ear and causing sensorineural hearing loss.

Credit Authorship Contribution Statement
Xue Xia, Xu Hongen and Jinxin Miao: Carrying out the experiments. Xue Xia: Analysis and interpretation of data, and writing-original draft. Mingsan Miao and Yaohe Wang: Finishing the qRT-PCR and Western blot. Wenxue Tang, Jinxin Miao and Jianyao Wang: Revising the manuscript.

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Conflict of Interest
The authors declare no conflict of interest.

Reference