Identifying new COVID-19 receptor Neuropilin-1 in severe Alzheimer's diseases patients group brain using genome-wide association study approach

Key-Hwan Lim

Korea Brain Research Institute

Sumin Yang

Korea Brain Research Institute

Sung-Hyun Kim

Korea Brain Research Institute

Jae-Yeol Joo (✉ joojy@kbri.re.kr)

Korea Brain Research Institute <https://orcid.org/0000-0003-2571-3173>

Primary research

Keywords: SARS-Cov-2, Neuropilin-1, Alzheimer's disease, Genome-wide association study

Posted Date: January 25th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-151089/v1>

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Abstract

Background Numerous studies have been conducted on different aspects of the COVID-19 (coronavirus disease 2019) pandemic, which is caused by SARS-CoV-2, since its emergence in late 2019. Mutual relations among SARS-CoV-2 and neuro-pathophysiological phenomena are continuously being demonstrated, and several underlying diseases, such as those in the elderly, are positively correlated with susceptibility to SARS-CoV-2 infection. The expression of angiotensin converting enzyme 2 (ACE2), which is required for SARS-CoV-2 infection, was recently demonstrated to be increased in Alzheimer's disease (AD) patients.

Methods Recent preclinical studies have shown that Neuropilin-1 (NRP1), which is a transmembrane protein with roles in neuronal development, axonal outgrowth, and angiogenesis, also plays a role in the infectivity of SARS-CoV-2. Thus, we hypothesized that NRP1 may be upregulated in AD patients and that a correlation between AD and SARS-CoV-2 NRP1-mediated infectivity may exist. We used an AD mouse model that mimics AD and performed high throughput total RNA-seq with brain tissue and whole blood. For quantification of NRP1 in AD, brain tissues and blood were subjected to western blotting and RT-qPCR analysis. In silico analysis for NRP1 expression in AD patients has been performed on the human hippocampus data sets (GSE4226, GSE1297).

Results Many cases of severe symptom of COVID-19 are concentrated in elderly group who have complications such as diabetes, degenerative disease, and brain disorders. Total RNA-seq analysis showed that *Nrp1* gene was commonly overexpressed in AD model. Similar to ACE2, NRP1 protein also strongly expressed in the AD brain tissues. Interestingly, in silico analysis revealed that the level of expression for NRP1 was distinct at age and AD progression.

Conclusions Given that the NRP1 is highly expressed in AD, it will be important to understand and predict that NRP1 may a risk factor for SARS-CoV-2 infection in AD patients. This will support to development of potential therapeutic drug to reduce SARS-CoV-2 transmission.

Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is being evaluated as a third-high risk contagious infection [1]. People are still highly vulnerable to the ongoing and life-threatening COVID-19 pandemic, as FDA-authorized vaccines or beneficial treatments remain unavailable [2]. The risk of severe complications that are eventually associated with high mortality has been indicated in older people [3, 4]. Moreover, a bidirectional interrelation between neurological complications and COVID-19 has been extensively reported [5].

Age-dependent vulnerability to SARS-CoV-2 has been associated to concomitant symptomatic infections [6, 7]. Alzheimer's disease (AD) is a highly destructive neurodegenerative disorder that mostly affects the elderly and is characterized by a progressive cognitive decline [8]. Although various hypotheses have been proposed to explain its multifactorial properties [9], the exact mechanism and related features of AD

remain obscure. An analysis of 627 patients suggested that AD is a risk factor for SARS-CoV-2 infection [10].

Angiotensin converting enzyme 2 (ACE2) is required for SARS-CoV-2 infection. Recently, it was reported that *Ace2* gene and protein expression is elevated in AD patients compared to that in normal elderly individuals [11–13]. Consistent with these results, an increase in ACE2 expression results in an increased susceptibility to SARS-CoV-2 infection in elderly patients with AD. Furthermore, a recent study suggested that the transmembrane protein Neuropilin-1 (NRP1) also plays a role in SARS-CoV-2 infection [14]. Biochemical experiments and X-ray crystallography showed that NRP1 strongly interacts with a polybasic sequence on the spike protein of SARS-CoV-2, which fits the C-end rule region (CendR) required for NRP1-peptide interaction [14, 15]. NRP1 depletion with RNAi targeting *Nrp1* mRNA inhibits the binding of the SARS-CoV-2 spike protein to NRP1 and consequently decreases the rate of viral infection [14, 15]. In addition, a monoclonal antibody against the b1b2 domain of NRP1 reduces the infectivity of SARS-CoV-2 lentiviral pseudo-particles [16]. NRP1 is a neuronal receptor associated with the regulation of neurite outgrowth through the binding of vascular endothelial growth factor (VEGF) [17]. When NRP1 is activated by CendR, which is a peptide R/KXXR/K motif contained within C-terminal domains, it enables cells to internalize ligands such as viruses containing the motif [18]. Furthermore, NRP1 is expressed in the central nervous system, including the brain olfactory-related regions where SARS-CoV-2 entry may occur, thereby facilitating COVID-19 infection [19].

Thus, we hypothesized that, in addition to ACE2, NRP1 expression might be upregulated in the brains of elderly AD patients. In this study, molecular characterization via high-throughput analysis and biochemical assays revealed that NRP1 is highly expressed in AD, which suggests that NRP1 may be a potential genetic therapy target in AD patients with COVID-19.

Methods

Animals

5×FAD transgenic mice were purchased from the Jackson Laboratory. All animal experiments performed in this study were reviewed and approved by the IACUC committee at Korea Brain Research Institute (IACUC-20-00018).

Total RNA sequencing and human *In silico* analysis

The data analysis of Total RNA-seq from mouse cortex was performed as previously described in [20]. Briefly, brain was extracted from WT and 5×FAD mouse and isolated cortex to prepare the pure RNA and total RNA-seq library. RNA-seq libraries were prepared using TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina Sample Preparation Guide) from isolated mRNA. To profiling the insert length of libraries, we used Agilent 2100 Bioanalyzer and constructed libraries were sequenced from HiSeqTM4000 platform (Illumina, USA). After converted nucleotide sequences using HiSeqTM4000 were sorted and filtered the dirty reads from raw reads. RNA-seq data was accessible using Gene Expression Omnibus (GES)

accession number (GSE147792). In silico data analysis was performed using Affymetrix Human Genome U133 Plus 2.0 Array [11]. The GSE1297 datasets were derived from human hippocampus and GSE4226 datasets were derived from human PBMCs in normal and AD patient.

RNA isolation

Total RNA isolation was performed with mouse cortex according to the TRizol using commercial protocol. Firstly, add phenol based TRizol (Invitrogen) in the cortex tissue contained tube for homogenizing. Then separated into three phases by chloroform for collect only RNA dissolved aqueous phase except DNA and protein precipitated phases. Equal volume of isopropanol was used to precipitate RNA. After centrifugation, discarded supernatant and washed with pre-chilled 75% ethanol once. RNA was dehydrated and crystalized without organic compound contamination and eluted with nuclease free water. RNA was then denatured in the 65°C heat-block for 10 min. The procedure was performed without RNase contamination.

cDNA synthesis

The isolated total RNA was synthesized into complementary DNA (cDNA) following manufacturer's protocol of High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Template RNA (2 ug) was prepared to synthesis of single reaction, and reverse transcription kit components were premixed. The premixture contains 10x RT buffer, 25x dNTP mix (4 mM), 10x RT Random Primers, MultiScribe Reverse Transcriptase (50 U), RNase inhibitor, and nuclease free water for adjusting total volume for reaction. Gently mixed template RNA and equal volume of premixture was placed in the thermal cycler. The condition for reverse transcription was suggested as optimized temperature and time; 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min.

RT-qPCR

Real-time quantitative PCR (RT-qPCR) was performed according to commercial protocol using SYBR Green PCR Master Mix (Applied Biosystems). Primers employed were: *Nrp1* forward, 5' CCTCACATTGGGCGTTATTG 3', reverse, 5' CACTGTAGTTGGCTGAGAAAC 3'; *Gapdh* forward, 5' AGGTCGGTGTGAACGGATTT 3', reverse, 5' TGTAGACCATGTAGTTGAGG 3'. Each reaction contains SYBR Green PCR Master Mix, Template cDNA, forward and reverse primer, and adjusted with nuclease free water.

Western blot

Protein was extracted from mouse cortex and mixed with sample buffer (5% 2-mercaptoethanol) and boiled at 100°C for denaturation. Protein samples were loaded on 4–15% gradient gel (Bio-Rad) to separate on size through vertical SDS-PAGE system. Antibodies used for immune-blot analysis were; anti-Neuropilin-1 (abcam, 1:1000) and anti-β-actin (BETHYL, 1:10000). Images were acquired by ChemiDoc MP imaging system (Bio-Rad).

Results

High-throughput analysis of *Nrp1* expression in AD

Given that the gene expression of ACE2 is upregulated in the brains of patients with AD and may be associated with the mortality rate from COVID-19 in the elderly [11, 21], we hypothesized that NRP1, which codes for a newly recognized Sars-CoV-2 spike receptor, may be also increased in AD patients. To assess *Nrp1* gene expression in AD, we first used a murine model that mimics AD and performed total RNA-seq using mouse brain tissue and whole blood. Total RNA-seq was analyzed by HiSeqTM4000 platform (Illumina, USA) (Fig. 1A). We applied the *Nrp1* gene expression level in the brain and blood from AD and WT, and then mapping the sequencing reads (Fig. 1B). The track of *Nrp1* gene displayed with University of California, Santa Cruz (UCSC) genome browser (Fig. 1B). Interestingly, total RNA-seq analysis revealed upregulation of *Nrp1* gene expression in the brain of AD model compared to wild type (WT) (Fig. 1B), and *Nrp1* fragments per kb per million reads (FPKM) values are increased in AD model brain as well (Fig. 1C). Although *Nrp1* gene expression increased by 319% in AD blood compared to WT blood, the endogenous expression levels of *Nrp1* in the blood were significantly lower than those in the brain (Fig. 1B and 1C). Collectively, our total RNA-seq results showed that *Nrp1* is preferentially expressed in the brain and upregulated in the brains of AD mice.

Nrp1 is upregulated in AD brain

Nrp1 is an abundantly expressed in the neurons and play an important for axon guidance, regeneration, neuronal plasticity or various human disease such as epilepsy and seizure [22].

We confirmed *Nrp1* gene expression both WT and AD model mouse brain through the total RNA-seq (Fig. 1). To further analyze *Nrp1* expression during AD progression, we measured the *Nrp1* mRNA levels in 3-month-old to 9-month-old AD brains. RT-qPCR revealed an approximately 145% increase in *Nrp1* mRNA expression in 9-month-old AD brains compared to that in WT brains (Fig. 2A). In addition, NRP1 protein expression was also significantly increased in 9-month-old AD brains compared to that in the WT (Fig. 2B and 2C). Taken together, these findings indicate that *NRP1* gene and protein expression levels are significantly increased in the brains of aged AD mice.

Severe AD patients are highly expressed with *Nrp1*

Having found increased *Nrp1* gene expression in the brains of AD mice, we next performed *Nrp1* gene expression profiling of brains and peripheral blood mononuclear cells (PBMCs) from human patients with different stages of AD. To identify the fold change of ratio for *Nrp1* gene from AD patients, we performed the in silico analysis using GSE1297 and GSE4296 microarray dataset. Patients with severe AD showed significantly upregulated *Nrp1* gene expression (179%) compared to the control group (individuals without AD), whereas incipient and moderate AD patients did not show increases in brain *Nrp1* gene expression (Fig. 3A). Interestingly, we did not find differences in PBMC *Nrp1* gene expression between any of the groups (Fig. 3B). These data correlated with the results from the AD murine model. Together, the

results demonstrate that NRP1 mRNA and protein expression is significantly elevated in the brains of late-stage AD patients.

Discussion

Since the beginning of the COVID-19 pandemic, there have been significant efforts on identifying unique SARS-CoV-2-associated proteins that could serve as targets for novel vaccines or therapeutic agents. Despite notable studies suggesting the possibility of developing other COVID-19-targeted drugs, the first-generation drugs have mostly focused on the viral spike protein receptor ACE2 [23]. As high-throughput genomic studies begin to define the abnormal expression of individual DNAs in particular diseases, it may become possible to rationally determine disease-specific gene expression and thus, establish biomarkers for risk prediction in older people with complications such as AD. Recently, we showed the increase of ACE2 expression in an elderly group with AD; therefore, our *in silico* analysis accurately predicts high risk for SARS-CoV-2 infection in elderly patients with AD [11]. In addition, our research scheme may be useful for predicting the risk of AD in patients with SARS-CoV-2 infection.

Our findings have implications for the prevention and treatment of SARS-CoV-2 infection in elderly patients with AD. First, both *Ace2* and *Nrp1* are preferentially expressed in the brain and their expression level may determine the sensitivity to SARS-CoV-2 infection (Fig. 4). Interestingly, it was recently suggested that differences in cytokines such as IL-1 β and TNF- α are less pronounced in peripheral blood in SARS-CoV-2 infection [24, 25]. Second, in addition to *Ace2*, *Nrp1* expression was also upregulated in patients with severe AD. Although predictive immune biomarkers have been suggested for the clinical treatment of COVID-19 [26], our high-throughput analysis-based approach would probably provide an accurate prediction of SARS-CoV-2 risk in elderly AD patients. Notably, *Ace2* gene expression gradually increased with the severity of AD symptoms (from incipient to severe stage) [11], whereas elevated *Nrp1* gene expression was only present in the severe AD patient group (Figs. 1B and 1D). This result indicates that ACE2 may be a more fundamental gene for SARS-CoV-2 infection compared to NRP1.

Conclusions

Recently, the spread of SARS-CoV-2 infection has accelerated worldwide. Efforts on the clinical treatment of SARS-CoV-2 infection are concentrated on the development of vaccines and drugs, including gene therapy [27]. To our knowledge, this is the first study examining NRP1 expression in AD patients and reporting its higher expression in these individuals. Moreover, it reveals the importance of determining SARS-CoV-2 spike protein receptor gene expression. Our gene profiling could potentially be used to predict the risk for SARS-CoV-2 infection in elderly AD patients.

Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing financial interests.

Availability of supporting data

Not applicable

Funding

This work was supported by Korea Brain Research Institute (KBRI) basic research program through KBRI funded by the Ministry of Science and ICT (20-BR-02-13), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2019R1F1A1059595).

Author Contributions

J.-Y.J. and K.-H.L. designed research; K.-H.L., S.Y., S.-H.K., and J.-Y.J. wrote the paper; K.-H.L., S.Y., S.-H.K., and J.-Y.J., performed research; J.-Y.J. performed total RNA-seq and RNA preparation; K.-H.L., S.Y., S.-H.K., performed western blot; and K.-H.L., S.Y., S.-H.K., and J.-Y.J. discussed the results and commented on the manuscript.

Acknowledgements

We would like to thank Dr. Sun-Woo Lee for assistance of bioinformatics analysis. 5xFAD (9-month) mice were kindly provided by Professor Sungkun Chun at Jeonbuk National University Medicine School. A graphic figure was made with biorender.com. We would like to thank Editage for English language editing.

Authors' information

Affiliation: Neurodegenerative Disease Research Group, Korea Brain Research Institute, Rep. of Korea; Key-Hwan Lim, Sumin Yang, Sung-Hyun Kim, and Jae-Yeol Joo

Corresponding author: Correspondence to Jae-Yeol Joo.

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Figures

Figure 1

Correlation between *Nrp1* gene and protein expression in AD brain. (A) Graphic representation of *Nrp1* gene expression in the cortex of 5 familial AD mutations (5×FAD) mice, which are used as a murine model of AD. The representation is shown on the UCSC genome browser following total RNA-seq. *Nrp1* gene expression levels are increased by 129% in 5×FAD cortex compared to control WT cortex.

Figure 2

The expression of NRP1 in mouse AD brain. (A) RT-qPCR analysis showing the *Nrp1* mRNA expression levels in the cortex of WT and 5x*FAD* mice. *Nrp1* mRNA expression is significantly increased in 9-month-old 5x*FAD* cortex compared to that in WT cortex. No significant differences are observed in the early disease stages of 5x*FAD* mice (3- and 6-month). The data are shown as the mean $\pm$ standard error of the mean (s.e.m) from $n = 3$ mice per group; statistical differences were assessed using unpaired t-test. (B) Representative western blot analyzing the NRP1 protein levels in 5x*FAD* brains. Endogenous NRP1 is highly expressed in 9-month-old 5x*FAD* brains compared to that in WT brain. β -actin was used as a loading control. The arrowhead indicates the NRP1 protein, and the asterisk indicates a non-specific band ($n = 5$ mice per group).

Figure 3

In-silico analysis of Nrp1 gene expression in human hippocampus and PBMCs from AD patients. (A) Nrp1 expression is significantly increased in the human hippocampus of severe AD patients compared to that in the control group (179%). No statistical difference is observed when WT is compared to incipient and moderate AD patients. Normal control group $n = 6$, incipient group $n = 7$, moderate group $n = 8$, and severe group $n = 6$. Statistical differences were assessed using one-way ANOVA. (B) Nrp1 expression in PBMCs

from AD patients is not statistically different from that in the control group. Normal elderly control, female n = 7 and male n = 7; AD patient group, female n = 7 and male n = 7.

Figure 4

Schematic model of NRP1- and ACE2-mediated SARS-CoV-2 infection in AD. NRP1 and ACE2 mediate SARS-CoV-2 binding to the cell membrane and consequently, infection. Because these two receptors are highly expressed in AD patients, these individuals may be more sensitive to SARS-CoV-2 infection.