Wnt5a and Wnt11 as acute respiratory distress syndrome biomarkers for severe acute respiratory syndrome coronavirus 2 patients

To the Editor:

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has spread globally, resulting in declaration of pandemic emergency [1]. COVID-19 patients suffer from various symptoms of infection, including pneumonia, acute respiratory distress syndrome (ARDS) and sepsis. Some known antiviral drugs, including remdesivir, have been proposed as effective agents for the treatment of SARS-CoV-2 infection [2, 3]. Along with the development of potential therapeutics, there is urgency to mitigate the transmission and economic crisis of SARS-CoV-2 *via* identification of biomarkers that can rapidly indicate the severity of the disease in infected patients. Wnt ligands are secreted glycoproteins and their downstream signalling plays a pivotal role in embryonic development and tissue homeostasis. With remarkable progress in the immunology field, Wnt signalling has gained much attention as a critical regulator in various inflammatory diseases. A large body of evidence has suggested that Wnt ligands are secreted by immune cells, such as peripheral blood mononuclear cells (PBMCs) and non-immune cells, including stroma cells, to regulate inflammatory response and immune cell modulation [4–7]. In addition to their roles in inflammation, studies have reported that these Wnt ligands play key roles in tissue damage and repair [6]. Interestingly, prior studies have reported significant alterations in Wnt5a and Wnt11 expression compared to other Wnt ligands by analysing sera of patients with severe sepsis or sepsis mouse models [4, 8]. Wnt5a signalling has been known to activate in sepsis or ARDS and play a pivotal role in lung inflammation and fibrosis [5, 9], whereas Wnt11 protein has been reported to suppress induction of inflammatory cytokines by regulating NF-κB activity [10, 11]. Previous reports have demonstrated that Wnt5a and Wnt11 have opposite functions to one another in response to inflammation [12, 13]; hence it is thought that Wnt5a has pro-inflammatory effect and Wnt11 may be anti-inflammatory. Therefore, we focused on Wnt5a and Wnt11 to explore their potential relevance to COVID-19-related diseases. In this study, we report Wnt5a and Wnt11 as reliable biomarkers for monitoring of pathological progression in SARS-CoV-2 patients.

Whole blood was collected from admitted SARS-CoV-2 patients at Yeungnam University Medical Center (Daegu, Republic of Korea) when these patients were diagnosed with the SARS-CoV-2 infection at the public health centre in Daegu. None of the patients had taken any medications nor used any mechanical devices upon admission to the hospital. The study protocol (YUH 2020-03-057, 2020-05-031-001) was approved by the institutional review board of Yeungnam University Hospital.

The concentrations of Wnt5a, Wnt11 or cytokines in SARS-CoV-2 patient plasma was quantified according to the manufacturer's instructions using a commercially available ELISA kit. Human recombinant WNT11 protein (H00007481-P01; Abnova, Aachen, Germany), anti-Wnt5a antibody (MAB645; R&D Systems, Minneapolis, MN, USA), anti-Wnt11 antibody (ab31962; Abcam, Cambridge, MA, USA), Human Protein Wnt-5a ELISA Kit (MBS2886311; MyBioSource, San Diego, CA, USA), and Human Protein Wnt-11 ELISA Kit (MBS281148; MyBioSource) were used.

Heparinised blood samples were used fresh within 4 h, and PBMCs were separated from blood using Ficoll–paque^{TM} PLUS (17-1440-02, GE Healthcare, Uppsala, Sweden) or NycoPrep (PROGEN

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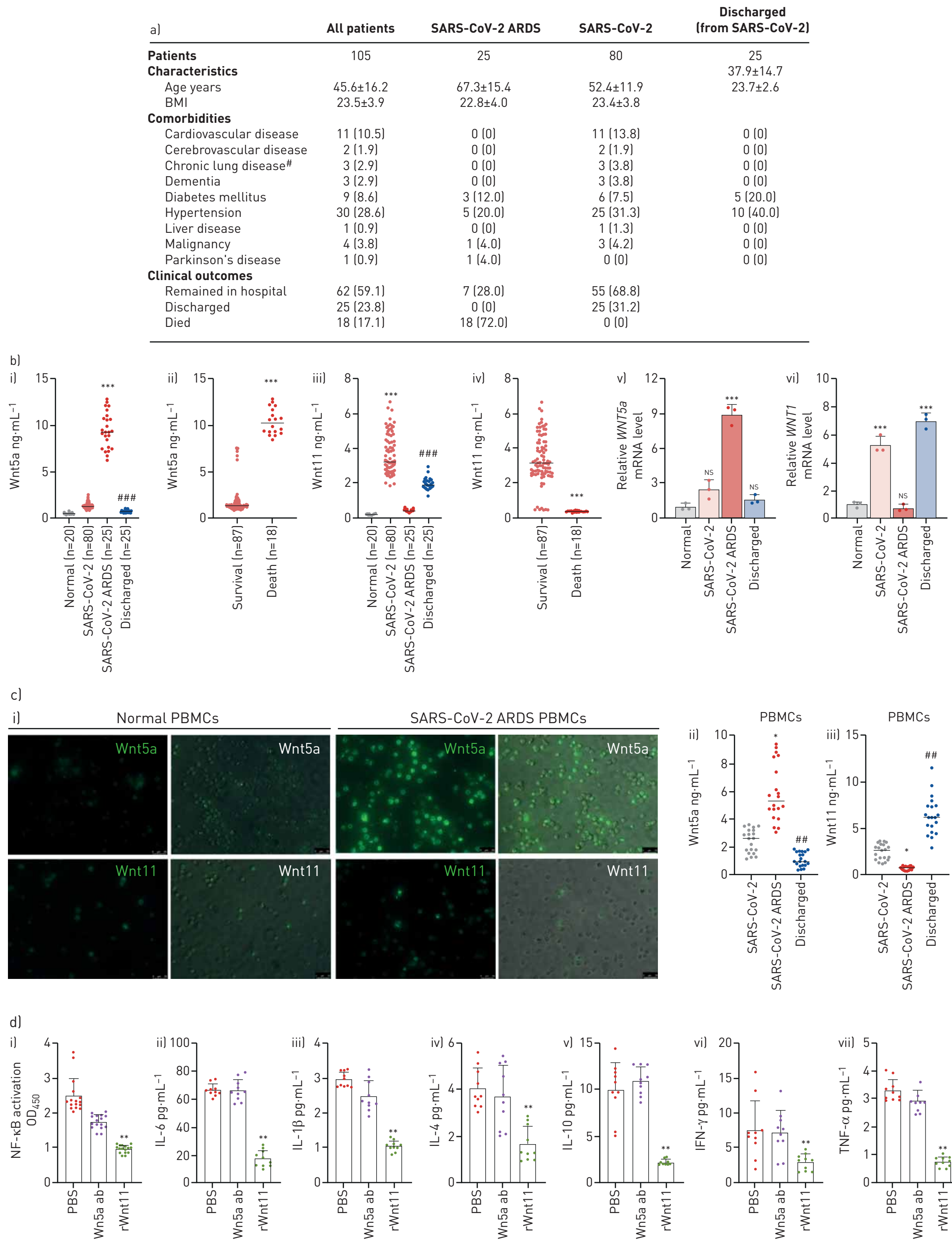
Biotechnik, Oslo, Norway). Following this, more refined PBMCs were obtained *via* MACSprep™ Human PBMC Isolation Kit (130-115-169, Miltenyi Biotec, Bergisch Gladbach, Germany). To verify the effect of Wnt5a-neutralising antibodies or recombinant human Wnt11 on the suppression of cytokine secretion and NF-κB activation, PBMCs isolated from SARS-CoV-2 patients were incubated with the Wnt5a antibody (20 $\mu g \cdot mL^{-1}$) or recombinant human Wnt11 (10 $ng \cdot mL^{-1}$) for 6 h. The supernatant was used for analysis of cytokines ELISA, and the lysate was used for NF-κB activity analysis using an ELISA-based NF-κB family transcription factor assay kit (43 296; Active Motif, Carlsbad, CA, USA). All experiments were performed independently at least three times. Statistically significant differences were determined using unpaired t-tests. Prism software (GraphPad, La Jolla, CA, USA) was used for statistical analyses.

In order to establish reliable diagnostic biomarkers, we conducted a prior study exploring clinical manifestations and various risk factors on severe SARS-CoV-2 patients admitted to Yeungnam University Medical Center [14, 15]. We conducted research to discover new biomarkers in blood based on patient information such as age, body mass index and comorbidities (figure 1a). The SARS-CoV-2 patient blood plasma samples were divided according to the severity of the disease: normal individuals (control group, tested for SARS-CoV-2 infection, but negative); SARS-CoV-2 patients; SARS-CoV-2 patients with ARDS (SARS-CoV-2 ARDS); and individuals discharged after hospitalisation for SARS-CoV-2 infection. ELISA analysis showed a marginal difference in the Wnt5a secretion level between the SARS-CoV2 infection and the control group. Irrespectively, the Wnt5a protein level was dramatically increased in the blood of SARS-CoV-2 ARDS (figure 1b(i)). Interestingly, the Wnt5a protein level was rescued in discharged individuals (figure 1b(i)). This was consistent in the patients who survived, where the Wnt5a level remained low in the plasma, but significant a high level of Wnt5a was still observed in the dead patients, thus demonstrating correlation of the Wnt5 level with the severity of the disease (figure 1b(ii)). In contrast, Wnt11 protein level was robustly induced in the plasma of SARS-CoV2 patients and discharged individuals, but remained at normal levels in SARS-CoV-2 ARDS, where the Wnt5a level was detected at its highest (figure 1b(iii) and (iv)).

To assess the regulation of Wnt5a and Wnt11 expression by SARS-CoV-2, PBMCs were isolated from SARS-CoV-2, SARS-CoV-2 ARDS and discharged patients. Based on transcriptional analysis using real-time quantitative PCR, it was observed that the secretion in each plasma sample is associated with differential *WNT5a* and *WNT11* mRNA expression level in the PBMCs (figure 1b(v) and (vi)). Likewise, Wnt5 level was significantly higher and Wnt11 level was significantly lower in the SARS-CoV-2 ARDS patients. Similar restoration in the Wnt5 level and a high level of Wnt11 were observed in discharged patients (figure 1b(v) and (vi)). These results suggest that an increased level of Wnt5a is associated with the severity of the disease in SARS-CoV-2 ARDS patients, while a low level of Wnt11 is related with insufficient capability to suppress and alleviate the inflammatory cytokine-induced SARS-CoV-2. To further confirm our findings and expand to clinical significance, PBMCs isolated from normal, SARS-CoV-2, SARS-CoV-2 ARDS and discharged patients were cultured for immunocytochemical analysis. The patient PBMCs were immunostained with specific antibodies and a high level of Wnt5a expression was observed in the SARS-CoV-2 ARDS patients, while less was detectable for Wnt11 (figure 1c(i)). Indeed, PBMCs isolated from SARS-CoV-2 ARDS showed a greater increase in Wnt5a protein secretion than other groups (figure 1c(ii)). Conversely, Wnt11 protein secretion remained at minimum level in PBMCs isolated from SARS-CoV-2 ARDS, and a dramatic increase in Wnt11 protein secretion was observed in PBMCs isolated from discharged individuals (figure 1c(iii)). The effects of Wnt5a and Wnt11 on anti-inflammatory responses were further investigated. Patient PBMCs were treated with anti-Wnt5a neutralising antibody or recombinant (r)Wnt11. NF-κB activation analysis demonstrated that treatment with anti-Wnt5a antibody does not significantly reduce the anti-inflammatory response in the PBMCs of SARS-CoV-2 ARDS (figure 1d(i)). Moreover, secretion of various cytokines, including interleukin (IL)-6, IL-1β, IL-4, interferon-γ and tumour necrosis factor-α was not significantly altered upon anti-Wnt5a antibody treatment (figure 1d(ii–vii)). However, treatment of SARS-CoV-2 ARDS PBMCs with rWnt11 showed a dramatic inhibitory effect on NF-κB activation as well as on cytokine production (figure 1d(ii–vii)). These results suggest that Wnt11 protein has great efficacy in reducing inflammatory responses caused by SARS-CoV-2 infection, but Wnt5a is unlikely to be the potential therapeutic target. Previously, Wnt5a expression was upregulated by transforming growth factor (TGF)-β, which induces pulmonary fibrosis [9], and a recent study has reported a significant increase in TGF-β in sera of COVID-19 patients [16]. Thus, it is possible that elevation of Wnt5a in sera of patients with severe COVID-19 may be due to progression of TGF-mediated lung injury, whereby Wnt5a inhibition may not be effective in recovery from inflammatory responses.

In summary, we conducted a single-centre observational study in South Korea to identify biomarkers that could be used to monitor the progression and severity of disease in SARS-CoV-2 patients. By analysing



a)

	All patients	SARS-CoV-2 ARDS	SARS-CoV-2	Discharged (from SARS-CoV-2)
Patients	105	25	80	25
Characteristics				37.9±14.7
Age years	45.6±16.2	67.3±15.4	52.4±11.9	23.7±2.6
BMI	23.5±3.9	22.8±4.0	23.4±3.8	
Comorbidities				
Cardiovascular disease	11 (10.5)	0 (0)	11 (13.8)	0 (0)
Cerebrovascular disease	2 (1.9)	0 (0)	2 (1.9)	0 (0)
Chronic lung disease#	3 (2.9)	0 (0)	3 (3.8)	0 (0)
Dementia	3 (2.9)	0 (0)	3 (3.8)	0 (0)
Diabetes mellitus	9 (8.6)	3 (12.0)	6 (7.5)	5 (20.0)
Hypertension	30 (28.6)	5 (20.0)	25 (31.3)	10 (40.0)
Liver disease	1 (0.9)	0 (0)	1 (1.3)	0 (0)
Malignancy	4 (3.8)	1 (4.0)	3 (4.2)	0 (0)
Parkinson's disease	1 (0.9)	1 (4.0)	0 (0)	0 (0)
Clinical outcomes				
Remained in hospital	62 (59.1)	7 (28.0)	55 (68.8)	
Discharged	25 (23.8)	0 (0)	25 (31.2)	
Died	18 (17.1)	18 (72.0)	0 (0)	

FIGURE 1 Wnt5a/Wnt11: a promising diagnostic marker for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) acute respiratory distress syndrome (ARDS). Plasma was secured from 20 healthy volunteers, 80 SARS-CoV-2 patients, 25 patients who progressed to ARDS (SARS-CoV-2 ARDS) and 25 discharged patients (no SARS-CoV-2 detected). a) Baseline characteristics and clinical outcomes of COVID-19 patients admitted to Yeungnam University Hospital (Daegu, Republic of Korea). Data are presented as n, mean±SD or n (%). BMI: body mass index. #: includes COPD, asthma, bronchiectasis and interstitial lung disease. b) i–iv) Analysis of Wnt5a and Wnt11 concentrations in SARS-CoV-2 patients; b) v, vi) the mRNA expression of Wnt5a and Wnt11 in peripheral blood mononuclear cells (PBMCs) was quantified using quantitative reverse transcriptase-PCR. ***: $p<0.001$ *versus* normal or survival; ###: $p<0.001$ *versus* SARS-CoV-2. c) i) Wnt5a or Wnt11 protein was visualised in PBMCs isolated from normal and SARS-CoV-2 ARDS patients by immunofluorescence staining (×200); c) ii, iii) Wnt5a or Wnt11 secreted from SARS-CoV-2, SARS-CoV-2 ARDS and discharged patients were detected by ELISA. SARS-CoV-2 sepsis patient plasma was incubated with Wnt5a antibody (20 μg·mL^{-1}) or recombinant human Wnt11 (10 ng·mL^{-1}) for 6 h (each group n=18). *: $p<0.05$ *versus* SARS-CoV-2; ##: $p<0.01$ *versus* SARS-CoV-2 ARDS. d) i) Binding activity of NF-κB (p65) in PBMCs and d) ii–vii) plasma cytokine levels in SARS-CoV-2 ARDS patient PBMCs treated with anti-Wnt5a antibody or recombinant human Wnt11. Data are presented as mean±SEM. IL: interleukin; IFN: interferon; TNF: tumour necrosis factor. Significance was set at $p<0.05$. **: $p<0.01$ *versus* PBS.

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plasma and PBMCs from patients with different pathological severities, our findings reveal that Wnt5a and Wnt11 show opposite expression patterns in SARS-CoV-2 ARDS patients. Based on our results, the measurement of Wnt5a levels in SARS-CoV-2 ARDS patients may be a good indicator for poor prognosis, whereas Wnt11 levels may be a good indicator for ability to survive the disease. Given that Wnt11, not Wnt5a, efficiently inhibits inflammatory responses and cytokine production, it could be exploited as a therapeutic target for the treatment of SARS-CoV-2 ARDS patients.

Eun Young Choi[1,9], Hee Ho Park[2,9], Hyelim Kim[3,9], Hong Nam Kim[4,5], Inyoung Kim[6], Soyoung Jeon[6], Wantae Kim[6,10], Jong-Sup Bae[7,10] and Wonhwa Lee[8,10]
[1]Division of Pulmonary and Allergy, Dept of Internal Medicine, College of Medicine, Yeungnam University and Respiratory Center, Yeungnam University Medical Center, Daegu, Republic of Korea. [2]Dept of Biotechnology and Bioengineering, Kangwon National University, Chuncheon, Republic of Korea. [3]College of Pharmacy, Chungnam National University, Daejeon, Republic of Korea. [4]Center for BioMicrosystems, Brain Science Institute, Korea Institute of Science and Technology (KIST), Seoul, Republic of Korea. [5]Division of Bio-Medical Science and Technology, KIST School, Korea University of Science and Technology, Seoul, Republic of Korea. [6]Dept of Biochemistry, College of Natural Sciences, Chungnam National University, Daejeon, Korea. [7]College of Pharmacy, CMRI, Research Institute of Pharmaceutical Sciences, BK21 Plus KNU Multi-Omics based Creative Drug Research Team, Kyungpook National University, Daegu, Republic of Korea. [8]Aging Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea. [9]These authors contributed equally to this work. [10]These authors contributed equally to this article as lead authors and supervised the work.

Correspondence: Wonhwa Lee, Aging Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon, 34141, Korea. E-mail: bywonhwalee@gmail.com

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