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Research letter

Wnt5a and Wnt11 as acute respiratory distress syndrome biomarkers for SARS-CoV-2 patients

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Title: Wnt5a and Wnt11 as acute respiratory distress syndrome biomarkers for SARS-CoV-2 patients

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The coronavirus disease-19 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has spread globally, thereby resulting in declaration of pandemic emergency [1]. COVID-19 patients suffer from various infectious symptoms, 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 also 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 were secreted by immune cells, such as PBMCs, and non-immune cells, including stroma cell, to regulate inflammatory response and immune cell modulation [4-7]. In addition to their roles in inflammation, recent studies have reported that these Wnt ligands play key roles in tissue damage and repair [6]. Interestingly, previous studies have reported significant alterations in Wnt5a and Wnt11 expression compared to other Wnt ligands by analyzing sera of patients with severe sepsis or sepsis mouse model [4, 8]. Wnt5a signaling 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 effect. 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.

Materials and Methods

Whole blood was collected from admitted SARS-CoV-2 patients at Yeungnam University Medical Center 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 at Daegu in Korea.

The concentrations of Wnt5a, Wnt11, or cytokines in SARS-CoV-2 patients' plasma was quantified according to the manufacturer's instructions using a commercially available ELISA kit. Human recombinant WNT11 protein (H00007481-P01, Abnova), anti-Wnt5a antibody (MAB645, R&D system), anti-Wnt11 antibody (ab31962, Abcam), Human Protein Wnt-5a ELISA Kit (MBS2886311, MyBioSource), and Human Protein Wnt-11 ELISA Kit (MBS281148, MyBioSource) were used.

Heparinized blood samples were used fresh within 4 h, and peripheral blood mononuclear cells (PBMCs) were separated from blood using Ficoll–Hypaque or NycoPrep. Following this, more refined PBMCs were obtained via MACSprep™ PBMC Isolation Kit. To verify the effect of Wnt5a neutralizing 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 μg/ml) or recombinant human Wnt11 (10 ng/ml) for 6 hours. The supernatant was used for analysis of cytokines ELISA, and lysate was used for NF-κB activity analysis by an ELISA-based NF-κB family transcription factor assay kit (43296; Active Motif). All experiments were performed independently at least three

times. Statistically significant differences were determined using unpaired t test. Prism software was used for statistical analyses.

Results

In order to establish reliable diagnostic biomarkers, we have conducted a prior study by exploring clinical manifestations and various risk factors on severe SARS-CoV-2 patients admitted to Yeungnam university medical center in Korea [14, 15]. We conducted research to discover new biomarkers in blood based on patient information such as age, BMI, 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 acute respiratory distress syndrome (SARS-CoV-2 ARDS), and discharged individuals after hospitalization by SARS-CoV-2 infection. ELISA analysis showed 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 1Ba). Interestingly, the Wnt5a protein level was rescued in discharged individuals (Figure 1Ba). This was consistent in the survived patients, where the Wnt5a level remained low in the plasma but significant 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 1Bb). On the contrary, 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 1Bc-Bd).

To assess the regulation of Wnt5a and Wnt11 expression by SARS-CoV-2, peripheral blood mononuclear cells (PBMCs) were isolated from SARS-CoV-2, SARS-CoV-2 ARDS, and discharged patients. Based on transcriptional analysis by real-time qPCR (RT-qPCR), it was observed that the secretion in each plasma sample is associated with differential *WNT5a* and *WNT11* mRNA expression level in the PBMCs (Figure 1Be-Bf). 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 high level of Wnt11 were observed in discharged patients (Figure 1Be-Bf). These results suggest that increased level of Wnt5a is associated with the severity of the disease in SARS-CoV-2 ARDS patients, while 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 from the SARS-CoV-2 ARDS patients, while less was detectable for Wnt11 (Figure 1Ca). Indeed, PBMCs isolated from SARS-CoV-2 ARDS showed increase in Wnt5a protein secretion than other groups (Figure 1Cb). Conversely, Wnt11 protein secretion was remained at minimum level in PBMCs isolated from SARS-CoV-2 ARDS, and dramatic increase in Wnt11 protein secretion was observed in PBMCs isolated from discharged individuals (Figure 1Cc). The effects of Wnt5a and Wnt11 on anti-inflammatory responses were further investigated. Patient PBMCs were treated with anti-Wnt5a neutralizing antibody or recombinant Wnt11 (rWnt11). NF-κB activation analysis demonstrated that the treatment with anti-Wnt5a antibody does not significantly reduce the anti-

inflammatory response in the PBMCs of SARS-CoV-2 ARDS (Figure 1Da). Moreover, secretion of various cytokines, including IL-6, IL-1β, IL-4, IFN-γ and TNF-α, was not significantly altered upon anti-Wnt5a antibody treatment (Figure 1Db-Dg). However, treatment of rWnt11 to the PBMCs of SARS-CoV-2 ARDS showed dramatic inhibitory effect on NF-κB activation as well as on cytokine production (Figure 1Db-Dg). 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 up-regulated by TGF-β, which induce pulmonary fibrosis [9], and recent study has reported a significant increase of TGF-β in COVID-19 patent sera [16]. Thus, it is possible that elevation of Wnt5a in patient sera with severe COVID-19 may be due to TGF-mediated lung injury progression, whereby Wnt5a inhibition may not be effective in recovering of inflammatory responses.

In summary, we investigated a single-centre and observational study in South Korea to identify biomarkers that could be used to monitor the progression and severity of the disease in the SARS-CoV-2 patients. By analysing plasma and PBMCs from patients with different pathological severities, our findings reveal that Wnt5a and Wnt11 show opposite expression pattern in SARS-CoV-2 ARDS patients. Based on our results, the measurement of Wnt5a level 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 cytokines production, it could be exploited as a therapeutic target for the treatment of SARS-CoV-2 ARDS patients.

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Author contributions

E.Y.C., H.H.P., W.K., J.-S.B, and W.L. designed and directed the study. H.K., H.N.K, and W.L. carried out ELISA, western blot, immunocytochemistry and cytokine assays. E.Y.C. collected blood samples from patients. E.Y.C., J.-S.B., I.Y.K, S.Y.J and W.L. directed the data analysis. H.H.P., W.K., and W.L. wrote the manuscript. All authors reviewed the manuscript and consented to the description of author contribution.

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Conflicts of Interest

None declared.

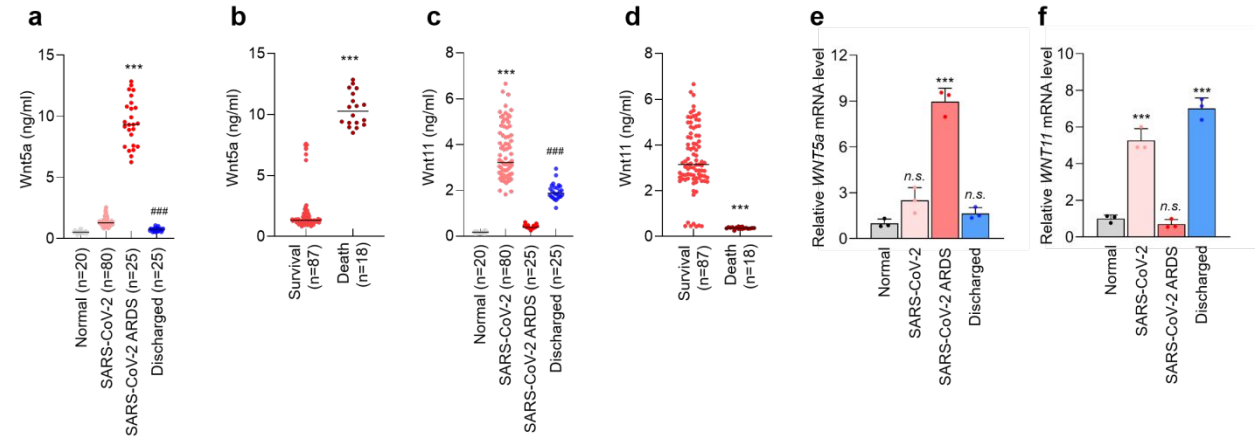
Figure 1 Wnt5a/Wnt11: a promising diagnostic marker for SARS-CoV-2 ARDS. Plasma was secured from 20 healthy volunteers, 80 SARS-CoV-2 patients, 25 patients who progressed to ARDS (SARS-CoV-2 ARDS) and 25 discharged patients (No SARS-CoV-2 detected). (A) Baseline characteristics and clinical outcomes of COVID-19 patients admitted to Yeungnam University Hospital. (Ba-Bd) Analysis of Wnt5a and Wnt11 concentrations in SARS-CoV-2 patients. (Be-Bf) The mRNA expression of Wnt5a and Wnt11 in PBMCs was quantified by qRT-PCR. (Ca) Wnt5a or Wnt11 protein was visualized in PBMCs isolated from normal and SARS-CoV-2 ARDS patients by immunofluorescence staining (x 200). (Cb-Cc) 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) or recombinant human Wnt11 (10 ng/ml) for 6 h (each group n = 18). (Da) Binding activity of NF-κB (p65) in PBMC and (Db-Dg) plasma cytokines levels in SARS-CoV-2 ARDS patients PBMCs treated by anti-Wnt5a antibody- or recombinant human Wnt11. Data are reported as mean ± SEM with significance set at $P< 0.05$; $^{***}P<0.001$ vs. Normal or Survival; $^{###}P<0.001$ vs SARS-CoV-2. $P<0.05$ $^{***}P<0.001$ vs. Normal; $^{*}P<0.05$ vs. SARS-CoV-2; $^{##}P<0.01$ vs. SARS-CoV-2 ARDS; $^{**}P<0.01$ vs. PBS treat.

A

	All patients (n=105)	SARS-CoV-2 ARDS (n=25)	SARS-CoV-2 (n=80)	Discharged (n=25) (From SARS-CoV-2)
Characteristics				
Age, y	45.6 ± 16.2	67.3 ± 15.4	52.4 ± 11.9	37.9 ± 14.7
BMI	23.5 ± 3.9	22.8 ± 4.0	23.4 ± 3.8	23.7 ± 2.6
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)	

Data are presented as mean ± SD (range) or number (percentage).
* Chronic lung disease includes COPD, asthma, bronchiectasis, and interstitial lung disease.
SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

B



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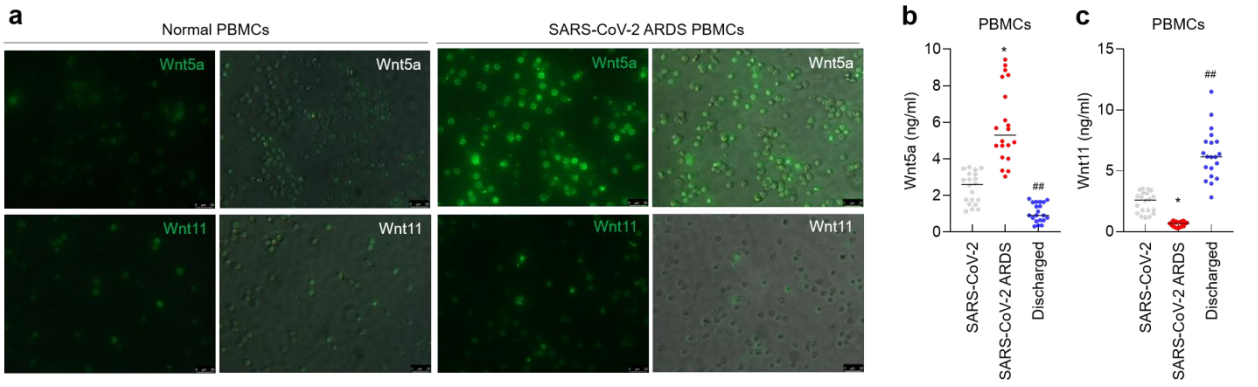

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