Endostatin and Vascular Endothelial Growth Factor-A165b May Contribute to Classification of Pulmonary Hypertension

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Background: Pulmonary hypertension (PH) is characterized by dysregulation of small pulmonary arteries. In addition to endostatin (ES), placenta growth factor (PIGF), vascular endothelial growth factor-A (VEGF-A), and the anti-angiogenesis isoform of VEGF-A (VEGF-A165b) are associated with PH. However, the usefulness of these biomarkers in PH is unknown. We investigated whether these 4 biomarkers are related to PH classification.

Methods and Results: Between July 2015 and August 2017, 33 control patients and 107 PH patients were enrolled in the study. Among the PH patients, 48 had pulmonary arterial hypertension (PAH), 5 had left heart disease-associated PH (LHD-PH), 4 had lung disease-associated PH (LD-PH), and 50 had chronic thromboembolic PH (CTEPH). Among the PAH patients, 16 had idiopathic PAH (IPAH) and 17 had connective tissue disease-associated PAH (CTD-PAH). PIGF, total VEGF-A, and VEGF-A165b levels were measured in the control and PH groups. ES was only measured in the PH group. VEGF-A165b levels were significantly higher in the LD-PH group than in the PAH, LHD-PH, and CTEPH groups (all P<0.001). PIGF levels were significantly higher in the CTD-PAH group than in the IPAH and control groups. ES levels were significantly correlated with the 6-min walk distance (P<0.001), B-type natriuretic peptide (P<0.001), and pulmonary vascular resistance (P=0.008).

Conclusions: ES could detect CTD-PAH in PAH and may be an indicator of PH severity. VEGF-A165b was useful in detecting LD-PH.

Key Words: Endostatin; Placenta growth factor (PIGF); Pulmonary hypertension; Vascular endothelial growth factor (VEGF)-A; VEGF-A165b

Pulmonary hypertension (PH) is a pathophysiological disorder that may involve multiple clinical conditions and can complicate the majority of cardiovascular and respiratory diseases. PH is generally divided into 5 groups according to the cause of PH, as follows: Group 1, pulmonary arterial hypertension (PAH); Group 2, PH due to left heart disease (LHD-PH); Group 3, PH due to chronic lung disease and/or hypoxia (LD-PH); Group 4, chronic thromboembolic PH (CTEPH); and Group 5, PH due to unclear multifactorial mechanisms. Group 1 PH includes idiopathic PAH (IPAH) and hereditary PAH (HPAH), as well as PAH associated with conditions such as connective tissue disease (CTD-PAH), congenital heart disease (CHD-PAH), and cirrhosis and portal hypertension (PoPH). At present, B-type natriuretic peptide (BNP) and N-terminal pro BNP (NT-proBNP) are considered useful biomarkers for the diagnosis of PH. However, BNP and NT-proBNP reflect cardiac wall stress due to high pulmonary arterial pressure of both PAH and LHD-PH. Therefore, other biomarkers that are specific for PH and do not depend on cardiac wall stress are needed.

PAH progression depends on the remodeling of the endothelium and smooth muscle cells of the pulmonary artery, which is known to be closely related to angiogenesis. Therefore, angiogenic and angiostatic factors, such as endostatin (ES), placenta growth factor (PIGF), vascular endothelial growth factor-A (VEGF-A), and the anti-angiogenesis isoform of VEGF-A (VEGF-A165b), which are thought to be associated with the development and progression of PH, may be potential biomarkers.

The aim of the present study was to verify the usefulness of serum ES (sES), PIGF, VEGF-A and VEGF-A165b concentrations in classifying the etiology of PH, as well as...
those with PAWP >15 mmHg were classified as Group 2; those with a percent predicted forced expiratory volume in 1 second (FEV1.0%) <70% or total lung volume (TLC) <60% in pulmonary function tests, and lung disease (e.g., chronic obstructive pulmonary disease [COPD] or idiopathic pulmonary fibrosis [IPF]) that was identified by computed tomography (CT) were classified as Group 3; and patients who developed a mismatch in ventilation-perfusion scintigraphy and who had a thrombus identified by contrast CT were classified as Group 4. Patients who were allocated to Group 5 were excluded from the study. The remaining patients who had not been allocated to Groups 2–5 were classified as Group 1.

Based on physical findings, the results of blood tests, echocardiography, and radiographic findings, patients in Group 1 were subdivided into CTD-PAH, CHD-PAH, and PoPH. IPAH was defined as any patient in whom the cause of PH could not be determined. Patients who had a

### Methods

#### Study Subjects

In all, 107 consecutive patients with PH diagnosed by right heart catheterization (RHC) were enrolled in this study from July 2013 to June 2017. The exclusion criteria were infection, cancer, use of VEGF-targeted drugs, and hemodialysis. PH was divided into 5 groups according to the guidelines, namely PAH (Group 1), LHD-PH (Group 2), LD-PAH (Group 3), CTEPH (Group 4), and PH caused by unknown mechanisms (Group 5). Group 1 was further divided into 4 subgroups: IPAH, CTD-PAH, CHD-PAH, and PoPH. PH was defined as mean pulmonary arterial pressure (mPAP) ≥25 mmHg at rest, and PAH was defined as both mPAP ≥25 mmHg and pulmonary artery wedge pressure (PAWP) ≤15 mmHg. Among the PH patients, those with PAWP >15 mmHg were classified as Group 2; those with a percent predicted forced expiratory volume in 1 second (FEV1.0%) <70% or total lung volume (TLC) <60% in pulmonary function tests, and lung disease (e.g., chronic obstructive pulmonary disease [COPD] or idiopathic pulmonary fibrosis [IPF]) that was identified by computed tomography (CT) were classified as Group 3; and patients who developed a mismatch in ventilation-perfusion scintigraphy and who had a thrombus identified by contrast CT were classified as Group 4. Patients who were allocated to Group 5 were excluded from the study. The remaining patients who had not been allocated to Groups 2–5 were classified as Group 1.

### Table 1. Characteristics and Hemodynamics of Patients With PH

| Variables                      | Group 1 (n=48) | Group 2 (n=5) | Group 3 (n=4) | Group 4 (n=50) | P value |
|-------------------------------|----------------|---------------|---------------|----------------|---------|
| Age (years)                   | 49.4±17.8      | 62.0±9.4      | 69.0±5.1      | 62.1±14.5      | 0.001   |
| Female sex (%)                | 85             | 0             | 0             | 58             | <0.001  |
| BMI (kg/m²)                   | 23.5±5.7       | 24.2±3.7      | 23.0±1.9      | 23.8±5.0       | 0.978   |
| SBP (mmHg)                    | 124.6±24.1     | 146.4±25.0    | 129.3±19.1    | 127.3±19.8     | 0.219   |
| DBP (mmHg)                    | 63.5±11.2      | 67.4±3.2      | 63.5±7.7      | 63.8±9.6       | 0.883   |
| Heart rate (beats/min)        | 74.3±12.9      | 73.0±17.6     | 57.5±7.3      | 73.9±13.6      | 0.120   |
| WHO functional class          |                |               |               |                |         |
| I                             | 1 (2.1)        | 0 (0)         | 0 (0)         | 3 (6.0)        |         |
| II                            | 22 (45.8)      | 3 (60.0)      | 0 (0)         | 19 (38.0)      |         |
| III                           | 23 (47.9)      | 2 (40.0)      | 4 (100.0)     | 24 (48.0)      |         |
| IV                            | 2 (4.2)        | 0 (0)         | 0 (0)         | 4 (8.0)        |         |
| Laboratory test               |                |               |               |                |         |
| Hemoglobin (g/dL)             | 12.3±1.9       | 12.0±1.5      | 15.7±1.7      | 13.2±2.2       | 0.005   |
| Platelets (×10³/μL)           | 204.0±76.1     | 184.2±95.8    | 180.5±35.4    | 231.7±64.3     | 0.127   |
| Uric acid (mg/dL)             | 6.0±2.1        | 6.1±1.2       | 5.6±1.2       | 6.0±1.7        | 0.977   |
| eGFR (mL/min/1.73m²)          | 81.8±29.7      | 67.7±25.3     | 68.1±21.9     | 74.2±26.2      | 0.060   |
| BNP (pg/mL)                   | 111.1±355.3    | 144.9±90.8    | 62.2±5.9      | 124.5±230.0    | 0.970   |
| 6MWD (m)                      | 410.6±141.0    | 344.5±96.1    | 426.9±121.9   | 0.655          |
| Peak VO₂ (mL/kg/min)          | 15.8±5.9       | 19.5          | 14.5±3.9      | 0.664          |
| Hemodynamics                  |                |               |               |                |         |
| sPAP (mmHg)                   | 60.7±20.6      | 60.6±15.8     | 55.0±14.5     | 61.5±18.3      | 0.934   |
| dPAP (mmHg)                   | 23.4±9.4       | 20.4±3.8      | 21.3±8.2      | 18.6±6.0       | 0.033   |
| mPAP (mmHg)                   | 37.5±12.4      | 34.2±7.5      | 33.8±7.8      | 33.8±7.8       | 0.616   |
| Cardiac index (L·min⁻¹·m⁻²)   | 2.70±0.79      | 1.99±0.48     | 1.96±0.24     | 2.33±0.59      | 0.010   |
| RAP (mmHg)                    | 5.90±3.00      | 12.40±5.81    | 6.00±4.08     | 5.87±3.89      | 0.002   |
| PVR (mmHg)                    | 7.73±5.51      | 4.74±3.35     | 5.74±1.75     | 7.57±4.33      | 0.523   |
| PAWP (mmHg)                   | 8.73±3.33      | 20.40±5.08    | 13.80±5.19    | 9.18±3.62      | <0.001  |
| Echocardiogram parameters     |                |               |               |                |         |
| TRPG (mmHg)                   | 56.2±20.4      | 63.7±18.7     | 56.5±11.9     | 60.4±23.8      | 0.779   |
| TAPSE (mm)                    | 19.0±3.8       | 14.0          | 17.7±5.4      | 18.5±4.4       | 0.612   |
| s’ (mm)                       | 11.4±2.4       | 10.5±3.2      | 11.7±2.8      | 0.764          |
| RVFAC (%)                     | 36.1±10.3      | 27.9±9.8      | 36.2±12.6     | 0.606          |

Data are given as the mean±SD, median (45–75%), or n (%). 6MWD, 6-min walk distance; BMI, body mass index; BNP, B-type natriuretic peptide; DBP, diastolic blood pressure; dPAP, diastolic pulmonary artery pressure; eGFR, estimated glomerular filtration rate; Group 1, pulmonary arterial hypertension; Group 2, pulmonary hypertension (PH) due to left heart disease; Group 3, PH due to chronic lung disease and/or hypoxia; Group 4, chronic thromboembolic pulmonary hypertension; mPAP, mean pulmonary artery pressure; PAWP, pulmonary artery wedge pressure; PVR, pulmonary vascular resistance; RAP, right atrial pressure; RVFAC, tricuspid annular plane systolic excursion; TRPG, tricuspid regurgitation pressure gradient; WHO, World Health Organization.
Angiogenic and Angiostatic Biomarkers in PH

Determined using an ELISA kit (Human VEGF Quantikine ELISA Kit, DVE00; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. The limit of detection of the assay was 9 pg/mL, with intra- and interassay coefficients of variation (CVs) of 4.5% and 7.0%, respectively. Serum VEGF-A 165 b (sVEGF-A 165 b) concentrations were also determined using an ELISA kit (Human Vascular Endothelial Growth Factor-165b ELISA Kit, MBS720132; MyBiosource, San Diego, CA, USA). The limit of detection of this assay was 1 pg/mL, with intra- and interassay CVs both being <10%.

Measurement of ES and PlGF

sES concentrations were determined using a human Solid Phase Sandwich ELISA (R&D System, Wiesbaden, Germany). The analytical sensitivity of this kit is 0.063 ng/mL (assay range 0.031–10 ng/mL), with and intra-assay CV of 5.5%. Recombinant human PlGF was obtained from the Cobas system.

Statistical Analysis

All PAH patients were diagnosed by RHC. The signifi-
ences among the groups in terms of age, sex, and plasma hemoglobin concentrations; 85% and 58% of patients in Groups 1 and 4, respectively, were female. Hemoglobin levels were significantly higher in Group 3 than in the other groups. With regard to hemodynamics, there were significant differences among the groups in diastolic pulmonary pressure (dPAP), cardiac index (CI), mean right pressure (mRAP), and PAWP. dPAP and CI were significantly higher in Group 1, and mRAP was significantly lower in Group 3. There were no significant differences in the other indicators among the groups.

The clinical characteristics and hemodynamics of the patient population and each group are presented in Table 1. Of the 107 PH patients, 48 (44.9%) were in Group 1, 5 (4.7%) were in Group 2, 4 (3.7%) were in Group 3, and 50 (46.7%) were in Group 4. There were significant differences among the groups in terms of age, sex, and plasma hemoglobin concentrations; 85% and 58% of patients in Groups 1 and 4, respectively, were female. Hemoglobin levels were significantly higher in Group 3 than in the other groups. With regard to hemodynamics, there were significant differences among the groups in diastolic pulmonary pressure (dPAP), cardiac index (CI), mean right pressure (mRAP), and PAWP, dPAP and CI were significantly higher in Group 1, and mRAP was significantly lower in Group 3. There were no significant differences in the other indicators among the groups.

The clinical characteristics and hemodynamics of the Group 1 patient population and subgroups are presented in Table 2. In Group 1, 16 patients had IPAH, 17 had CTD-PAH, 6 had CHD-PAH, and 5 had PoPH. There were significant differences in age and plasma platelet levels, and in the 6-min walk distance (6MWD) between the subgroups. Patients with CTD-PAH were older and had a significantly shorter 6MWD. With regard to hemodynamics, systolic pulmonary arterial pressure and mean pulmonary arterial pressure (mPAP) differed significantly among the subgroups. mPAP was significantly higher in the IPAH subgroup and pulmonary vascular resistance (PVR) tended
to be higher in the IPAH than in the other subgroups. There were no significant differences in the other indicators among the subgroups.

Comparisons of Serum Biomarker Levels Among PH Groups
Comparisons of sES, sPlGF, sVEGF-A, and sVEGF-A_{165b} among the groups are shown in Figure 1. There were no significant differences in sVEGF-A, sPlGF, and sES among Groups 1–4. However, sVEGF-A_{165b} was significantly higher in Group 3 than in the other groups (P<0.001 for all).

Comparisons of Serum Biomarker Levels Among Group 1 Subgroups
Comparisons of sES, sPlGF, sVEGF-A, and sVEGF-A_{165b} among the Group 1 subgroups are shown in Figure 2. There were no significant differences in sVEGF-A among the subgroups. However, sVEGF-A_{165b} was significantly higher in the CTD-PAH and CHD-PAH groups compared with the control cohort (CTD-PAH, P=0.015; CHD-PAH, P=0.009). sPlGF was significantly higher in patients with CTD-PAH than IPAH (P<0.001). Compared with the control cohort, there were significant increases in PI GF in both the IPAH or HPAH and CTD-PAH groups. sES was significantly higher in patients with CTD-PAH than PoPH.

Relationship Between Biomarkers and Hemodynamics or Exercise Tolerance
Correlation analysis was used to determine the relationship between each biomarker and hemodynamics or exercise tolerance. As shown in Figure 3, sVEGF-A was correlated with PVR (r=0.339; P=0.001), sVEGF-A_{165b} was not correlated with any other hemodynamic parameters or with exercise tolerance. sPlGF was correlated with 6MWD (r=−0.542; P<0.001) and BNP levels (r=0.531; P<0.001), and there was a significant difference in WHO-FC (P<0.001; Figure 4). sES was also significantly correlated with 6MWD (r=−0.542; P<0.001) and BNP levels (r=0.531; P<0.001), and there was a significant difference in WHO-FC (P<0.001). As shown in Figure 5, there was a weak but statistically significant correlation between sES
and both PVR \((r=0.254; P=0.008)\) and mixed venous oxygen saturation \((\text{SvO}_2; r=-0.295; P=0.003)\).

**Discussion**

PAH is a refractory disease that, at present, has no cure. Proper treatment needs to estimate risk stratification and find the right causes leading to the development of PH.\(^8\) However, it is often difficult to clearly distinguish the cause of PH because of overlapping etiology of PH in the clinical setting, specifically overlapping lung disease with connective tissue disease, left heart disease with IPAH, and CTD-PAH with IPAH. Biomarkers to evaluate PH may help obtain a proper diagnosis and treatment. BNP/NT-proBNP levels are useful biomarkers that reflect cardiac wall stress in PH.\(^6\) However, there are few biomarkers to classify causes of PH. In the present study we focused on serum concentrations of angiogenic and angiostatic markers, because the development of PH, as well as lung and connective tissue diseases (which can be causes of PH), is partly attributed to pathological angiogenesis of the pulmonary vessels.\(^6,12,13\)

The main findings of the present study were that: (1) sES was significantly correlated with hemodynamics as well as symptoms and exercise tolerance; (2) sPIGF was significantly higher in the CTD-PAH group than in the control cohort and the IPAH subgroup of Group 1 PAH; and (3) sVEGF-A\(_{165b}\) was significantly higher in the LD-PH (Group 3) group than in the other groups, including the control cohort.

In the present study, sES was significantly correlated not only with WHO-FC and 6MWD, but also PVR and SvO\(_2\). A previous study showed that there were significant differences in WHO-FC, NT-proBNP levels, and 6MWD, but not in resting hemodynamics (e.g., mPAP, PVR, RAP, and CI), between high and low sES.\(^7\) In the present study, 37.8% of patients were treatment naive, compared with only 15.8% in the previous study.\(^7\) Thus, the present study reflected patients’ severity more than the previous report. Recently, risk stratification at diagnosis was recommended by the European Society of Cardiology (ESC) guidelines to determine the treatment strategy for PAH.\(^11\) Specifically, a single index is inadequate to estimate the severity and prognosis of PAH. The ESC risk table contains symptoms, exercise tolerance, biomarkers (BNP or NT-proBNP levels), right heart failure (right atrial area or pericardial effu-
Angiogenic and Angiostatic Biomarkers in PH

PIGF, a member of the VEGF family, binds VEGF receptor 1 and is a main effector of proangiogenic signaling, which stimulates the proliferation of endothelial cell and results in PH. Abnormalities in the regulators of angiogenesis in systemic sclerosis (SSc) play a role in scleroderma vascular disease. It was reported that 7–12% of patients with SSc develop PAH during the course of their disease. In addition, PIGF has been recognized as a marker of the development of PH in SSc. In the present study, 7 of 17 Group 1 patients (41%) had SSc-PAH, and this proportion was higher than reported in a previous Japanese epidemiological study. These results may reflect the significant increases in sPIGF levels in CTD-PAH. In the subanalysis, the lack of correlations between sPlGF and hemodynamics such as PVR and SvO₂ is in agreement with a previous report. In contrast, we found significant correlations between sPIGF and both 6MWD and WHO-FC. Thus, sPIGF may partially mirror severity. In cases in which CTD-PAH is complicated by lung disease, such as pulmonary fibrosis and interstitial pulmonary pneumonia, it is difficult to find the main cause of PH. Furthermore, primary Sjogren’s syndrome is known to be underdiagnosed with routine evaluation as a primary cause of PH.

sVEGF-A₁₆₅b was significantly higher in the LD-PAH (Group 3) group than in the other groups. In particular, the increase in sVEGF-A₁₆₅b in the LD-PH but not CTEPH (Group 4) group indicates that the cause of PH is not simply hypoxia. Remodeling of lung tissue may have contributed to the increase in sVEGF-A₁₆₅b. Previous studies reported increases in sVEGF-A₁₆₅b in IPAH and SSc-associated PAH. The findings of the present study are consistent with these previous reports. In the present study, VEGF-A₁₆₅b was measured using samples eluting from serum. Conversely, previous studies used samples from plasma. Platelet aggregation is much less in serum than in plasma, which may have affected the results. Overexpression of VRGF-A₁₆₅b leads to insufficient angiogen-
estimate the severity of PH. Because sES was correlated with the main items of the ESC risk table, sES is thought to reflect the severity of PH in recent treatment strategies. Further studies are needed in a treatment-naive cohort to verify the usefulness of sES.

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IRB Information
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