Prognostic value of plasminogen activator inhibitor-1 in biomarker exploration using multiplex immunoassay in patients with metastatic renal cell carcinoma treated with axitinib

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Abstract

Background and Aims: Vascular endothelial growth factor-directed therapies play a significant role in patients with metastatic renal cell carcinoma (mRCC). Biomarkers for predicting treatment efficacy and resistance are required to develop personalized medicine. We evaluated multiple serum cytokine levels in patients with mRCC treated with axitinib to explore predictive biomarkers.

Methods: From September 2012 to October 2015, serum samples were collected from 44 patients with mRCC before treatment and 4 weeks after axitinib initiation. Bio-Plex Pro Human Cancer Biomarker Panels 1 and 2 were used to measure levels of 34 serum biomarkers related to angiogenesis and cell proliferation.

Results: Patients with partial response or stable disease had significantly decreased serum plasminogen activator inhibitor-1 (PAI-1) level from pre-treatment to 4 weeks after axitinib initiation compared with those with progressive disease (P = .022). The median progression-free survival (PFS) and median overall survival (OS) in patients with increased serum PAI-1 level from pre-treatment to 4 weeks after axitinib initiation were significantly shorter than those with decreased serum PAI-1 level (P = .027 and P = .026, respectively). Increased serum PAI-1 level from pre-treatment to 4 weeks after axitinib initiation was an independent prognostic marker for shorter PFS and OS in multivariate analyses (P = .015 and P = .032, respectively). The immunohistochemical staining intensity of PAI-1 in tumor specimens was significantly associated with Fuhrman grade and presence of distant metastasis (P = .026 and P = .010, respectively).
Conclusions: The initial change in serum PAI-1 level in the early stage of axitinib treatment could be a useful prognostic biomarker in patients with mRCC.

KEYWORDS
metastatic renal cell carcinoma, molecular-targeted therapy, plasminogen activator inhibitor-1, serum biomarker

1 | INTRODUCTION

In 2017, the age-adjusted incidence and mortality rates of renal cell carcinoma (RCC) in Japanese men were 11.5 and 2.8 per 100,000 person-years, respectively. Distant metastasis is observed in approximately 20% to 30% of patients with RCC at the time of initial diagnosis. Although current first-line treatment for patients with metastatic RCC (mRCC) is either an immune-checkpoint inhibitor (ICI) or vascular endothelial growth factor (VEGF)-directed multitargeted tyrosine kinase inhibitors (TKIs), TKIs improved overall survival (OS) in patients with mRCC with a median value of 8.5 to 14.4 months from 2002 to 2008. Although the treatment paradigm for mRCC is currently shifting from TKIs to ICIs with or without concurrent use of TKIs, personalized biomarker-guided sequential or combination therapies for predicting the efficacy and adverse effects of TKIs are still strongly required for patients with mRCC.

For appropriate use of TKIs in individual patients, useful biomarkers which can be measured during treatment to predict treatment effect, resistance, and prognosis are strongly required. As strategies to predict the treatment effect and prognosis during treatment, serum TKI level can be measured. Pre-treatment evaluation of genetic polymorphisms of drug-metabolizing enzymes and transporters can predict the serum TKI level. In addition, serum VEGF-C, sVEGFR-2, and sVEGFR-3 levels, and the number of endothelial cells in circulating blood have been reported to be biomarkers that correlate with treatment effect and prognosis. However, other potential biomarkers relevant to personalized therapy including TKIs and immunotherapies have not been investigated.

Axitinib is a TKI selective for VEGFR-1, -2, and -3. Patients with mRCC treated with axitinib as second-line therapy had a significantly longer progression-free survival (PFS) than those treated with sorafenib in a randomized, multicenter phase III trial. In this study, we aimed to analyze various potentially prognostic serum cytokines involved in cancer angiogenesis and cell proliferation using the multiplex immunoassay method before treatment and 4 weeks after axitinib initiation in patients with mRCC. We comprehensively explored biomarkers which can predict the clinical effect and prognosis in patients with mRCC treated with axitinib.

2 | MATERIAL AND METHODS

2.1 | Patients

From September 2012 to October 2015, 44 patients with mRCC at the Akita University Hospital were enrolled. An approval (#924) was obtained by Akita University Hospital Institutional Review Board in accordance with the ethical standards based on the Declaration of Helsinki and its later amendments. Written informed consent was obtained by all the patients who participated in this study. Serum samples were obtained before treatment and 4 weeks after axitinib initiation. Patient characteristics are presented in Table 1. The International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk classification at the axitinib initiation treatment was favorable in 11 (25.0%), intermediate in 30 (68.2%), and poor in 7 (15.9%). Twenty-six (59.1%) patients received no other therapies before axitinib. Axitinib treatment was initiated at 10 mg/day twice daily; thereafter, the dosage was increased or decreased according to the discretion of the attending physician based on serum axitinib level, adverse events, and treatment effect. Evaluation of the therapeutic effect was based on the Response Evaluation Criteria in Solid Tumors v1.1.

2.2 | Quantitative analysis of serum biomarkers

Serum samples were centrifuged at 3000 revolutions per min for 10 minutes, and stored at −80°C prior to analysis. Beads array analysis using the Bio-Plex Pro Cancer Biomarker assay kit1 and kit2 (Bio-Rad, Hercules, California) was performed to measure 34 cytokines and tumor growth factors.

Briefly, the capture antibody-coupled beads were first incubated with antigen standards, quality control samples, and serum samples in 96-well plates, followed by incubation with biotinylated detection antibodies. Samples were diluted 1:4 using sample diluent. After washing the unbound biotinylated detection antibodies, the beads were incubated with a reporter streptavidin-phycoerythrin (SA-PE) conjugate. Following the removal of excess SA-PE, the beads were passed through the 2-laser flow cytometer Bio-Plex array reader (Bio-Plex 200 system, Bio-Rad), which measures the fluorescence of the bead and the bound SA-PE. Details of the procedure have been described previously. Assay incubations were performed at room temperature. All washes were performed using the Bio-Plex Pro wash station. Data acquisition was performed using Bio-Plex manager TM 6.0. Using the automatic calibration curve optimization function, the recovery rate was regressed to be in the range of approximately 70% to 130%. All samples were assayed in duplicate. The following biomarkers were determined using the Bio-Plex Pro Human Cancer Biomarker Panel kit1 (#171-AC500M, Bio-
TABLE 1 Patients characteristics of the 44 patients with metastatic renal cell carcinoma treated with axitinib

|                          | No. of patients (%) n = 44 |
|--------------------------|----------------------------|
| **Gender**               |                            |
| Male                     | 31 (70.5)                  |
| Female                   | 13 (29.5)                  |
| **Age**                  |                            |
| Median [range]           | 66.5 [24-83]               |
| **BMI**                  |                            |
| Median [range]           | 22.7 [16.1-31.8]           |
| **IMDC risk group**      |                            |
| Favorable                | 8 (18.2)                   |
| Intermediate             | 24 (54.5)                  |
| Poor                     | 7 (15.9)                   |
| Not available            | 5 (11.4)                   |
| **Histological type**    |                            |
| Clear cell               | 36 (81.8)                  |
| Chromophobe              | 2 (4.5)                    |
| Xp translocation         | 4 (9.1)                    |
| Sarcomatoid              | 2 (4.5)                    |
| **Nephrectomy**          |                            |
| Yes                      | 41 (93.2)                  |
| No                       | 3 (6.8)                    |
| **Target organ**         |                            |
| Lung                     | 29 (65.9)                  |
| Lymph node               | 14 (31.8)                  |
| Bone                     | 11 (25.0)                  |
| Liver                    | 5 (11.4)                   |
| **Previous treatment**   |                            |
| Yes                      | 18 (40.9)                  |
| At least one previous molecular-targeted agent | 12 (66.7) |
| Sunitinib                | 11 (61.1)                  |
| Sorafenib                | 4 (22.2)                   |
| Everolimus               | 7 (38.9)                   |
| Temsirolimus             | 1 (5.6)                    |
| Cytokines only           | 6 (33.3)                   |
| No                       | 26 (59.1)                  |

Rad): soluble epidermal growth factor receptor (sEGFR), fibroblast growth factor basic (FGF-basic), soluble VEGF receptor (sVEGFR)-1, sVEGFR-2, platelet endothelial cell adhesion molecule-1 (PECAM-1), platelet-derived growth factor-AB/BB (PDGF-AB/BB), granulocyte-colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), tyrosine kinase sHER-2/neu (erbB-2), tyrosine kinase sTIE2, sTIE2, sIL-6R, follistatin, prolactin (PRL), lepin, and osteopontin. In addition, the following biomarkers were measured using the Bio-Plex Pro Human Cancer Biomarker Panel kit2 (#171-AC600M, Bio-Rad): VEGF-A, VEGF-C, VEGF-D, epidermal growth factor receptor (EGFR), heparin-binding epidermal growth factor-like growth factor (HB-EGF), placental growth factor (PLGF), transforming growth factor-α (TGF-α), tumor necrosis factor-α (TNF-α), insulin-like growth factor-binding protein 1 (IGFBP-1), soluble Fas ligand (sFASL), IL-6, IL-8, IL-18, plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator (uPA), angiopoietin-2, sCD40L, and endoglin.

2.3 | Immunohistochemistry staining

Tumor specimens obtained by radical nephrectomy or biopsy were fixed in 20% formalin, embedded in paraffin, and evaluated for expression of PAI-1. Specimens were sliced into 3 µm sections and immunohistochemically analyzed using anti-PAI-1 antibody (#66705, Abcam, Cambridge, UK). Peroxidase and 3,3-diaminobenzidine (DAB) were used as labeling enzyme and chromogenic substrate, respectively. Immunohistochemistry (IHC) staining was assessed using an automated quantitative pathology imaging system workstation (Mantra, PerkinElmer, Waltham, Massachusetts). DAB-positive cells were detected, and the staining intensity was scored using inForm ver. 2.3 software (PerkinElmer). Five representative areas were photographed with a 400-fold field of view, and nuclei were automatically recognized. Staining intensity was measured radially from the nucleus, and DAB staining was recognized around the cell membrane (Figure S1). The positive threshold for staining intensity per cell was defined as ≥25% of the maximum staining intensity. The percentage of cells exceeding the threshold was counted, and the average value of the five visualized areas was scored as the final IHC staining intensity.

2.4 | Statistical analysis

The Kolmogorov-Smirnov test was used for nonparametric analysis of the serum biomarkers because of their nonnormal distribution. The relationships between serum biomarker level, treatment response, IHC staining intensity, and pathological parameters were evaluated using the Mann-Whitney U test. Bonferroni’s correction was applied in the multiple comparison. Fisher’s exact test was used to examine the proportion of patients between groups. The Kaplan-Meier method was used to plot time-to-event curves, and statistical significance was estimated using the log-rank test. The Cox proportional hazard model was used to determine independent prognostic factors of PFS and OS. P < .05 was considered as statistically significant. All statistical analyses were performed using SPSS statistics version 23 (IBM, New York).

3 | RESULTS

3.1 | Change in serum biomarker levels from pre-treatment to 4 weeks after axitinib initiation

Among the 34 measured cancer-related biomarkers, the median serum level of sTIE2, sVEGFR-1, sVEGFR-2, and Ang2 significantly decreased from pre-treatment to 4 weeks after axitinib initiation (P < .001, P = .036, P < .001, and P = .006, respectively; Table 2).

In contrast, the median serum level of sEGFR and PRL significantly increased from pre-treatment to 4 weeks after axitinib initiation (P = .032 and P = .010, respectively; Table 2). Using Bonferroni’s correction, only sTIE2, sVEGFR-2, and PRL were significantly decreased or increased. The number of patients for each serum biomarker who exhibited a decrease or increase in level is shown in Table 2.
| Protein name | Abbreviations | Pre-treatment | 4 weeks after initiation of axitinib | Number of patients for change in the serum level |
|--------------|---------------|---------------|--------------------------|-----------------------------------------------|
|              |               | Median (pg/mL) | Range                    | Median (pg/mL) | Range |
|              |               |               |                          | P value     | Increased (n) | Decreased (n) |
| **Bio-Plex Pro Human Cancer Biomarker Panel kit1** | | | | | |
| Soluble epidermal growth factor receptor | sEGFR | 14 779 | 12 669-18 295 | 15 200 | 13 386-20 398 | .032 | 29 | 15 |
| Fibroblast growth factor basic | FGF-basic | 194 | 161-218 | 183 | 160-215 | .090 | 17 | 27 |
| Follistatin | Follistatin | 707 | 506-948 | 629 | 497-1279 | .375 | 23 | 21 |
| Granulocyte-colony stimulating factor | G-CSF | 82 | 60-93 | 76 | 62-90 | .255 | 19 | 25 |
| Tyrosine kinase soluble HER-2/neu | erbB-2 | 2186 | 1705-3348 | 2754 | 1618-3288 | .273 | 24 | 20 |
| Hepatocyte growth factor | HGF | 1246 | 1022-2783 | 1305 | 1050-3174 | .666 | 23 | 21 |
| Soluble IL-6Rα | sIL-6Rα | 10 180 | 8227-11 940 | 10 507 | 8329-12 732 | .161 | 28 | 16 |
| Leptin | Leptin | 1907 | 1016-4364 | 2134 | 924-3545 | .788 | 21 | 23 |
| Osteopontin | OPN | 70 999 | 45 785-90 563 | 69 869 | 47 384-94 053 | .972 | 22 | 22 |
| Platelet-derived growth factor-AB/BB | PDGF-AB/BB | 2732 | 1941-4126 | 2796 | 1939-3815 | .735 | 22 | 22 |
| Platelet endothelial cell adhesion molecule-1 | PECAM-1 | 2981 | 2539-4093 | 3257 | 2662-3849 | .926 | 26 | 18 |
| Prolactin | PRL | 6029 | 4378-11 048 | 8036 | 5323-17 673 | .010 | 33 | 11 |
| Stem cell factor | SCF | 219 | 197-267 | 219 | 193-247 | .076 | 16 | 28 |
| Tyrosine kinase soluble TIE2 | sTIE-2 | 6168 | 5137-8635 | 5510 | 4082-7099 | <.001 | 9 | 35 |
| Soluble vascular endothelial growth factor receptor-1 | sVEGFR-1 | 219 | 138-304 | 188 | 140-257 | .036 | 17 | 27 |
| Soluble vascular endothelial growth factor receptor-1 | sVEGFR-2 | 3558 | 2728-4098 | 2830 | 2209-3217 | <.001 | 7 | 37 |
| **Bio-Plex Pro Human Cancer Biomarker Panel kit2** | | | | | |
| Angiopoietin-2 | Ang2 | 954 | 567-1306 | 751 | 292-1366 | .006 | 13 | 31 |
| Soluble CD40 ligand | sCD40L | 412 | 286-487 | 390 | 308-495 | .797 | 22 | 22 |
| Epidermal growth factor receptor | EGF | 58 | 29-89 | 62 | 33-99 | .161 | 28 | 16 |
| Endoglin | ENG | 906 | 459-1197 | 817 | 413-1186 | .138 | 19 | 25 |
| Soluble Fas ligand | sFASL | 298 | 259-396 | 278 | 226-420 | .118 | 15 | 29 |
| Heparin-binding epidermal growth factor-like growth factor | HB-EGF | 79 | 54-96 | 71 | 46-102 | .197 | 21 | 23 |
| Insulin-like growth factor-binding protein 1 | IGFBP-1 | 12 372 | 4731-18 729 | 11 605 | 3447-28 333 | .135 | 26 | 18 |
| Interleukin-6 | IL-6 | 80 | 33-102 | 80 | 26-108 | .930 | 23 | 21 |
| Interleukin-8 | IL-8 | 24 | 13-29 | 24 | 12-34 | .718 | 23 | 21 |
| Interleukin-18 | IL-18 | 135 | 105-182 | 160 | 91-207 | .243 | 23 | 21 |
| Plasminogen activator inhibitor-1 | PAI-1 | 110 156 | 74 073-165 898 | 107 590 | 76 894-147 861 | .991 | 24 | 20 |
| Placental growth factor | PLGF | 86 | 43-128 | 102 | 52-141 | .067 | 30 | 14 |
3.2 Relationship between serum biomarker levels and treatment response

The treatment responses of 42 patients treated with axitinib were partial remission (PR) in 16 (38.1%) patients, stable disease (SD) in 20 (47.6%), and progressive disease (PD) in 6 (14.3%). Two patients were excluded because of unknown response. The median serum PDGF-AB/BB and sVEGFR-2 levels at baseline were significantly higher in the six patients with PD than in the 36 patients with PR or SD (\( P = .040 \) and \( P = .003 \), respectively); however, the baseline median serum PAI-1 level was significantly lower in the patients with PD than those with PR or SD (\( P = .048 \)) (Table S1). Using Bonferroni’s correction, there was no significant relationship.

The proportion of patients with decreased serum level of PAI-1 and IL-18 from pre-treatment to 4 weeks after axitinib initiation was significantly higher in patients with PR or SD compared to those with PD (\( P = .022 \) and \( P = .022 \), respectively; Table S2). The proportion of patients with decreased serum levels of endoglin, IL-6, and VEGF-A from pre-treatment to 4 weeks after axitinib initiation was significantly higher in patients with PR than those with SD or PD (\( P = .011 \), respectively).

### TABLE 2 (Continued)

| Protein name                                      | Abbreviations | Pre-treatment | 4 weeks after initiation of axitinib | Number of patients for change in the serum level |
|---------------------------------------------------|---------------|--------------|--------------------------------------|-----------------------------------------------|
|                                                   |               | Median (pg/mL) | Range | Median (pg/mL) | Range | \( P \) value | Increased (n) | Decreased (n) |
| Transforming growth factor-α                      | TGF-α         | 60           | 46-81 | 52           | 38-86 | .700          | 21            | 23            |
| Tumor necrosis factor-α                           | TNF-α         | 44           | 16-67 | 39           | 14-61 | .280          | 20            | 24            |
| Urokinase plasminogen activator                   | uPA           | 228          | 74-340| 210          | 69-371| .981          | 21            | 23            |
| Soluble vascular endothelial growth factor A      | VEGF-A        | 580          | 459-754| 610         | 382-862| .401          | 25            | 19            |
| Soluble vascular endothelial growth factor C      | VEGF-C        | 959          | 671-1075| 921         | 580-1167| .815          | 24            | 20            |
| Soluble vascular endothelial growth factor D      | VEGF-D        | 862          | 498-1633| 753         | 466-1600| .155          | 19            | 25            |

FIGURE 1 Kaplan-Meier curves comparing, A, progression-free survival, and B, overall survival in patients with decreased or increased serum plasminogen activator inhibitor-1 (PAI-1) level from pre-treatment to 4 weeks after axitinib initiation.
TABLE 3  Cox proportional hazard model to predict the shorter progression-free survival using baseline clinical parameter and change in the serum biomarker level from pre-treatment to 4 weeks after initiation of axitinib

| Variable | Univariate analysis | Multivariate analysis (stepwise) |
|----------|---------------------|----------------------------------|
|          | HR | 95% CI | P value | HR | 95% CI | P value |
| Age (<median vs >median) | 0.747 | 0.346-1.611 | .456 | | | |
| Gender (male vs female) | 1.048 | 0.441-2.493 | .915 | | | |
| BMI (<25 vs >25) | 0.788 | 0.359-1.730 | .553 | | | |
| Previous treatment (no vs yes) | 0.850 | 0.349-1.831 | .678 | | | |
| pT (≥pT2 vs pT1) | 1.508 | 0.627-3.628 | .359 | | | |
| cN (≥cN1 vs cN0) | 5.476 | 2.039-14.704 | .001 | 10.616 | 3.287-34.280 | <.001 |
| LVI (yes vs no) | 1.226 | 0.409-3.672 | .716 | | | |
| Grade (G2-3 vs G1) | 1.141 | 0.586-2.219 | .699 | | | |
| Number of metastasis (≥3 vs 0-2) | 1.937 | 0.838-4.477 | .122 | | | |
| Lung metastasis (yes vs no) | 1.019 | 0.441-2.353 | .965 | | | |
| Liver metastasis (yes vs no) | 3.236 | 1.180-8.875 | .022 | 2.854 | 0.843-9.662 | .092 |
| Bone metastasis (yes vs no) | 1.890 | 0.823-4.338 | .133 | | | |
| CRP (≥ULN vs <ULN) | 1.114 | 0.486-2.554 | .798 | | | |
| Alb (<LLN vs >LLN) | 2.630 | 0.991-6.981 | .052 | | | |
| Hb (<LLN vs >LLN) | 1.859 | 0.858-4.028 | .112 | | | |
| Thrombocyte (<ULN vs ≥ULN) | 1.802 | 0.674-4.819 | .241 | | | |
| sEGFR (increased vs decreased) | 0.787 | 0.348-1.780 | .565 | | | |
| FGF-basic (increased vs decreased) | 1.217 | 0.686-2.158 | .501 | | | |
| Follistatin (increased vs decreased) | 0.859 | 0.396-1.863 | .700 | | | |
| G-CSF (increased vs decreased) | 1.124 | 0.525-2.406 | .763 | | | |
| erbB-2 (increased vs decreased) | 1.039 | 0.471-2.291 | .925 | | | |
| HGF (increased vs decreased) | 1.492 | 0.689-3.230 | .310 | | | |
| IL-6Ra (increased vs decreased) | 1.573 | 0.687-3.605 | .284 | | | |
| Leptin (increased vs decreased) | 0.953 | 0.446-2.036 | .900 | | | |
| OPN (increased vs decreased) | 1.078 | 0.503-2.313 | .847 | | | |
| PDGF-AB/BB (increased vs decreased) | 0.860 | 0.402-1.837 | .697 | | | |
| PECAM-1 (increased vs decreased) | 1.377 | 0.611-3.104 | .441 | | | |
| PRL (increased vs decreased) | 1.233 | 0.519-2.929 | .635 | | | |
| SCF (increased vs decreased) | 1.002 | 0.458-2.193 | .996 | | | |
| TIE2 (increased vs decreased) | 0.711 | 0.283-1.782 | .466 | | | |
| sVEGFR-1 (increased vs decreased) | 0.764 | 0.378-1.541 | .451 | | | |
| sVEGFR-2 (increased vs decreased) | 0.839 | 0.313-2.245 | .726 | | | |
| Ang2 (increased vs decreased) | 0.809 | 0.341-1.921 | .631 | | | |
| sCD40L (increased vs decreased) | 2.135 | 0.956-4.770 | .064 | | | |
| EGF (increased vs decreased) | 1.809 | 0.763-4.289 | .178 | | | |
| ENG (increased vs decreased) | 1.667 | 0.780-3.563 | .188 | | | |
| sFASL (increased vs decreased) | 1.457 | 0.665-3.193 | .347 | | | |
| HB-EGF (increased vs decreased) | 2.233 | 1.027-4.854 | .043 | 1.937 | 0.208-60.373 | .561 |
| IGFBP-1 (increased vs decreased) | 1.359 | 0.619-2.986 | .444 | | | |
| IL-6 (increased vs decreased) | 2.328 | 1.053-5.143 | .037 | 1.037 | 0.332-3.237 | .949 |
| IL-8 (increased vs decreased) | 1.935 | 0.879-4.258 | .101 | | | |
| IL-18 (increased vs decreased) | 1.675 | 0.759-3.694 | .201 | | | |
| PAI-1 (increased vs decreased) | 2.412 | 1.075-5.412 | .027 | 3.896 | 1.306-11.623 | .015 |
| PLGF (increased vs decreased) | 2.671 | 1.008-7.075 | .048 | 2.018 | 0.547-8.127 | .279 |
### TABLE 3  (Continued)

| Variable | Univariate analysis | Multivariate analysis (stepwise) |
|----------|---------------------|---------------------------------|
|          | HR | 95% CI | P value | HR | 95% CI | P value |
| TGF-α (increased vs decreased) | 2.485 | 1.114-5.546 | .026 | 0.912 | 0.089-9.039 | .938 |
| TNF-α (increased vs decreased) | 1.995 | 0.928-4.291 | .077 |
| uPA (increased vs decreased) | 1.444 | 0.693-3.008 | .327 |
| VEGF-A (increased vs decreased) | 1.435 | 0.656-3.142 | .366 |
| VEGF-C (increased vs decreased) | 1.924 | 0.875-4.233 | .104 |
| VEGF-D (increased vs decreased) | 1.608 | 0.753-3.432 | .220 |

### TABLE 4  Cox proportional hazard model to predict the shorter overall survival using baseline clinical parameter and change in the serum biomarker level from pre-treatment to 4 weeks after initiation of axitinib

| Variable | Univariate | Multivariate |
|----------|------------|--------------|
|          | HR | 95% CI | P value | HR | 95% CI | P value |
| Age (<median vs >median) | 0.480 | 0.174-1.324 | .156 | 2.292 | 0.483-10.883 | .297 |
| Gender (male vs female) | 0.854 | 0.274-2.658 | .785 | 4.104 | 1.487-11.321 | .006 |
| BMI (<25 vs ≥25) | 0.602 | 0.208-1.745 | .350 | 0.912 | 0.311-2.674 | .867 |
| BMI (≥25 vs <25) | 0.534 | 0.182-1.568 | .253 | 2.841 | 0.904-8.924 | .074 |
| cN (≥cN1 vs cN0) | 4.691 | 1.562-14.089 | .006 | 3.255 | 1.198-8.846 | .021 |
| LVI (yes vs no) | 1.494 | 0.326-6.853 | .606 | 3.046 | 0.957-3.473 | .141 |
| Grade (G2-3 vs G1) | 1.439 | 0.597-3.473 | .418 | 3.102 | 0.703-13.684 | .135 |
| Number of metastasis (≥3 vs 0-2) | 4.104 | 1.487-11.321 | .006 | 0.912 | 0.311-2.674 | .867 |
| Lung metastasis (yes vs no) | 2.841 | 0.904-8.924 | .074 | 2.472 | 0.463-13.492 | .35 |
| Liver metastasis (yes vs no) | 3.255 | 1.198-8.846 | .021 | 1.996 | 0.534-7.453 | .304 |
| Bone metastasis (yes vs no) | 4.104 | 1.487-11.321 | .006 | 3.102 | 0.703-13.684 | .135 |
| CRP (≥ULN vs <ULN) | 3.046 | 0.957-9.699 | .059 | 0.753 | 0.273-2.079 | .584 |
| Alb (<LLN vs >LLN) | 3.102 | 0.703-13.684 | .135 | 3.417 | 0.769-15.175 | .106 |
| Hb (<LLN vs >LLN) | 3.102 | 0.703-13.684 | .135 | 3.382 | 1.090-10.496 | .035 |
| Thrombocyte (<ULN vs >ULN) | 3.046 | 0.957-9.699 | .059 | 3.046 | 0.957-9.699 | .059 |
| sEGFR (increased vs decreased) | 1.119 | 0.508-2.464 | .781 | 0.969 | 0.363-2.586 | .949 |
| sVEGFR-1 (increased vs decreased) | 0.753 | 0.273-2.079 | .584 | 0.622 | 0.215-1.799 | .381 |
| sVEGFR-2 (increased vs decreased) | 0.753 | 0.273-2.079 | .584 | 0.701 | 0.261-1.880 | .481 |
| HGF (increased vs decreased) | 1.119 | 0.508-2.464 | .781 | 1.233 | 0.386-3.942 | .724 |
| IL-6Rα (increased vs decreased) | 0.622 | 0.215-1.799 | .381 | 0.912 | 0.311-2.674 | .867 |
| erbB-2 (increased vs decreased) | 0.622 | 0.215-1.799 | .381 | 2.841 | 0.904-8.924 | .074 |
| HGF (increased vs decreased) | 1.233 | 0.386-3.942 | .724 | 3.255 | 1.198-8.846 | .021 |
| IL-6R (increased vs decreased) | 0.701 | 0.261-1.880 | .481 | 3.046 | 0.957-9.699 | .059 |
| Leptin (increased vs decreased) | 0.912 | 0.311-2.674 | .867 | 3.046 | 0.957-9.699 | .059 |
| OPN (increased vs decreased) | 1.233 | 0.386-3.942 | .724 | 1.233 | 0.386-3.942 | .724 |
| PDGF-AB/BB (increased vs decreased) | 1.233 | 0.386-3.942 | .724 | 0.912 | 0.311-2.674 | .867 |
| PECAM-1 (increased vs decreased) | 1.233 | 0.386-3.942 | .724 | 2.841 | 0.904-8.924 | .074 |
| PRL (increased vs decreased) | 1.233 | 0.386-3.942 | .724 | 3.255 | 1.198-8.846 | .021 |
| SCF (increased vs decreased) | 1.233 | 0.386-3.942 | .724 |
| TIE (increased vs decreased) | 1.233 | 0.386-3.942 | .724 | 1.233 | 0.386-3.942 | .724 |
| sVEGFR-1 (increased vs decreased) | 1.233 | 0.386-3.942 | .724 | 0.912 | 0.311-2.674 | .867 |
| sVEGFR-2 (increased vs decreased) | 1.233 | 0.386-3.942 | .724 |
| Ang2 (increased vs decreased) | 1.233 | 0.386-3.942 | .724 | 1.233 | 0.386-3.942 | .724 |
Using Bonferroni’s correction, there was no significant relationship.

### 3.3 Relationship between serum biomarker levels and PFS and OS

The presence of lymph node swelling on initial imaging studies (cN1) and baseline serum leptin level lower than the median were independent factors related to worse PFS in multivariate analysis ($P < .001$ and $P = .026$; Table S3). No independent factor related to OS was found using baseline serum biomarker level (Table S4).

Patients with increased serum PAI-1 level from pre-treatment to 4 weeks after axitinib initiation had significantly shorter PFS and OS than those with decreased serum PAI-1 (15.0 months vs 5.1 months, $P = .027$ and 34.9 months vs 14.2 months, $P = .026$, respectively; Figure 1A,B). The presence of lymph node swelling on initial imaging studies (cN1) and increased serum PAI-1 level from pre-treatment to 4 weeks after axitinib initiation were independent prognostic factors for shorter PFS ($P < .001$ and $P = .015$, respectively; Table 3). Increased serum PAI-1 level from pre-treatment to 4 weeks after axitinib initiation was also an independent prognostic marker for shorter OS ($P = .032$; Table 4).

### 3.4 Relationship between IHC staining intensity and clinical parameters

Of the 44 patients enrolled in this study, 41 (93.2%) underwent radical nephrectomy and 3 (6.8%) underwent tumor biopsy. IHC analysis using PAI-1 antibody was available in 39 specimens from 36 nephrectomies and 3 biopsies. The median IHC staining intensity of PAI-1 was significantly higher in patients with metastatic disease at the time of diagnosis than those with nonmetastatic disease ($P = .010$; Table 5), as well as in patients with Fuhrman grade $\geq 3$ tumors than in those with grade $\leq 2$ ($P = .026$; Table 5). There was no significant relationship between PAI-1 staining intensity and PFS or OS ($r^2 = 0.053$, $\rho = -0.02$, $P = .904$).

### 4 DISCUSSION

The multiplex immunoassay method is a beads array in which various antibodies are loaded on the beads measured by flow cytometry. Previous reports have comprehensively measured angiogenic factors using serum samples from patients with colorectal, ovarian and small cell lung cancer12-14 and urine samples from patients with bladder cancer.15,16 However, few studies have explored biomarkers as predictive factors in patients with metastatic disease using multiplex immunoassay techniques. Although we expected biomarkers other than sVEGFRs to show predictive value in this study, serum PAI-1 level was the only biomarker associated with therapeutic effect, PFS, and OS after axitinib treatment in patients with mRCC.

PAI-1 usually exists in vascular endothelial cells, liver, platelets, and adipocytes, and functions as the principal inhibitor of urokinase-type plasminogen activator (uPA) and its receptor (uPAR) system in fibrinolysis. Furthermore, $\geq90\%$ of PAI-1 is contained in platelets and released into the bloodstream under conditions of vascular endothelial

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**Table 4 (Continued)**

| Variable                  | Univariate HR | 95% CI       | P value |
|---------------------------|---------------|--------------|---------|
| sCD40L (increased vs decreased) | 1.173         | 0.434-3.173  | .753    |
| EGF (increased vs decreased)   | 0.804         | 0.292-2.219  | .674    |
| ENG (increased vs decreased)   | 1.175         | 0.441-3.133  | .747    |
| sFASL (increased vs decreased)  | 1.228         | 0.443-3.399  | .693    |
| HB-EGF (increased vs decreased) | 1.486         | 0.549-4.025  | .436    |
| IGFBP-1 (increased vs decreased) | 1.237         | 0.449-3.408  | .680    |
| IL-6 (increased vs decreased)   | 2.349         | 0.813-6.783  | .115    |
| IL-8 (increased vs decreased)   | 0.916         | 0.331-2.531  | .865    |
| IL-18 (increased vs decreased)  | 1.539         | 0.559-4.240  | .404    |
| PAI-1 (increased vs decreased)  | 3.376         | 1.086-10.497 | .036    |
| PLGF (increased vs decreased)   | 1.424         | 0.453-4.474  | .545    |
| TGF-α (increased vs decreased)  | 1.486         | 0.549-4.025  | .436    |
| TNF-α (increased vs decreased)  | 1.130         | 0.424-3.015  | .807    |
| uPA (increased vs decreased)    | 2.240         | 0.819-6.123  | .116    |
| VEGF-A (increased vs decreased) | 1.057         | 0.383-2.918  | .915    |
| VEGF-C (increased vs decreased) | 1.508         | 0.547-4.152  | .427    |
| VEGF-D (increased vs decreased) | 0.846         | 0.312-2.298  | .743    |

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$P = .025$, and $P = .029$, respectively; Table S2). Using Bonferroni’s correction, there was no significant relationship.
injury. The uPA-uPAR complex activates matrix metalloprotease (MMP) and promotes cancer invasion. Since PAI-1 forms a PAI-1-uPA-uPAR complex and acts repressively on uPA-uPAR, PAI-1 is expected to have a tumor-suppressive effect. However, tumor PAI-1 expression has been reportedly associated with tumor progression. This paradox has been explained by rapid internalization of the PAI-1-uPA-uPAR complex by low-density lipoprotein receptor-related protein.

Regarding the relationship between tumor PAI-1 expression and RCC prognosis, IHC staining intensity of cytoplasmic PAI-1 in paraffin specimens has been previously associated with shorter disease-free survival, OS, and cause-specific survival (CSS) in patients with RCC. In addition, high tissue level of PAI-1 in fresh-frozen RCC specimens measured using enzyme-linked immunosorbent assay has been associated with high grade tumors and shorter CSS. In this study, PAI-1 staining intensity was associated with the presence of metastasis at the time of diagnosis and histologic Fuhrman grade, but not with PFS and OS. However, this study evaluated staining intensity using an automated quantitative imaging system but not using microscopic manual examination as in previous studies. Further IHC studies using an automated quantitative imaging system with larger numbers of patients are required.

In this study, decreased serum PAI-1 level after axitinib treatment was related to improved treatment effect and prognosis. However, the serum PAI-1 level at baseline was not related to the axitinib effect or prognosis. Significant decreases have been observed in both serum PAI-1 and VEGF levels after treatment in a previous study of sunitinib plus interferon in patients with mRCC, whereas no significant decrease in serum PAI-1 level after treatment was observed in our axitinib study. In breast cancer, lower pre-treatment plasma PAI-1 level was an independent prognostic factor for PFS and OS, and plasma PAI-1 level did not correlate with PAI-1 immunostaining intensity. Our results with an inverse correlation between plasma levels and immunostaining intensity were similar to those in the breast cancer results. Since the serum PAI-1 level would reflect PAI-1 released from the tumor, endothelium, and platelets, the successful suppression of both tumor and systemic angiogenesis by axitinib might decrease the serum PAI-1 level. The decrease of the serum PAI-1 level might reflect the change of the tumor microenvironment induced by axitinib which could be associated with the better prognosis. It is assumed that PAI-1 expressed in tumor cells and released into circulation may have a different biological role in patients with mRCC. Although an in vivo murine study using systemic administration of the PAI-1 inhibitor SK-216 for lung cancer and melanoma indicated that PAI-1 generated by host rather than tumor cells plays a determinant role in the anticancer effect, further accumulation of biomarker data in patients with mRCC treated with axitinib is warranted to verify the results.

Additionally, the median serum level of sVEGFR-1 and sVEGFR-2 decreased significantly from pre-treatment to 4 weeks after axitinib initiation, and the decline of serum sVEGFR-2 level was associated with treatment response in this study. However, sVEGFRs were not independent predictive factors for PFS or OS using baseline serum

| TABLE 5 Relationship between IHC staining intensity of PAI-1 and pathological parameters of patients treated with axitinib | n = 39 | Median | Range | Median | Range | Median | Range | Median | Range | Median | Range | Median | Range |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PT ≤ pT2 (n = 19) | ≥ pT3 (n = 20) | P | | | | | | | | | | | |
| ≤ G2 (n = 12) | ≥ G3 (n = 26) | P | | | | | | | | | | | |
| Metastasis | | | | | | | | | | | | | |
| Median | Range | Median | Range | Median | Range | Median | Range |
| 0.686 | 0.268-0.857 | 0.668 | 0.437-0.745 | 0.738 | 0.604-0.788 | 0.126 | 0.083-0.726 |
| 0.955 | 0.730-0.924 | 0.656 | 0.437-0.745 | 0.437 | 0.370-0.574 | 0.726 | 0.604-0.821 |
| 0.286 | 0.143-0.857 | 0.289 | 0.147-0.724 |

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bimarker level or change in level after treatment. These results are partially consistent with previous studies that reported sVEGFR-2 and sVEGFR-3 levels were significant prognostic factors after sunitinib treatment in patients with mRCC.\textsuperscript{6,7} Although serum PAI-1 and sVEGFRs have been identified as markers of tumor hypoxia, and might be affected by systemic VEGF-directed inhibitors,\textsuperscript{28,32} serum PAI-1 level may be a more useful prognostic biomarker than serum sVEGFRs in this axitinib study.

There are several important limitations of this study. First, PAI-1 is ideally measured in plasma, however we used serum samples in this study, which might affect the results. Second, the PAI-1 level measured in this study was not pure PAI-1 but a complex in the blood. The antibody on the beads of the Bio-Plex Pro Human Cancer Biomarker Panel 2 in this study is an anti-total PAI-1 antibody, which measures the sum of the active type, latent type, vitronectin complex, tissue-type plasminogen activator complex, and uPA complex. Third, 40\% of patients received multiple therapies prior to axitinib treatment, which might affect the interpretation of the results. To verify our results, future studies measuring plasma PAI-1 level in larger RCC cohorts should be conducted.

\section*{5 | CONCLUSIONS}

The initial changes in serum PAI-1 level at the early stage of axitinib treatment could be a useful prognostic biomarker in patients with mRCC.

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\section*{CONFLICT OF INTEREST}

The authors declare no conflicts of interest.

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All authors have read and approved the final version of the manuscript.

\section*{TRANSPARENCY STATEMENT}

The corresponding author, Takamitsu Inoue, affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

\section*{DATA AVAILABILITY STATEMENT}

The data that support the findings of this study are openly available in "figshare" at https://figshare.com/s/ea7a0931565d9b361e2, DOI: 10.6084/m9.figshare.12049560.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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