Correlation of prechemotherapy urinary megalin ectodomain (A-megalin) levels with the development of cisplatin-induced nephrotoxicity: a prospective observational study

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Abstract

Background: Cisplatin is a potent chemotherapeutic agent used to treat a variety of solid tumors. One of the major side effects of cisplatin is dose-limiting nephrotoxicity. We recently demonstrated that the renal uptake of cisplatin and resultant cisplatin-induced nephrotoxicity are mediated in part by megalin, an endocytic receptor in proximal tubule epithelial cells (PTECs). We also developed sandwich enzyme-linked immunosorbent assays to measure the megalin ectodomain (A-megalin) and full-length megalin (C-megalin) in urine using monoclonal antibodies against the amino- and carboxyl-termini of megalin, respectively. The present study examined the correlation of urinary megalin level with cisplatin-induced nephrotoxicity and its utility as a biomarker in patients with thoracic cancer.

Methods: This prospective observational study involved 45 chemotherapy-naïve patients scheduled to receive chemotherapy with ≥60 mg/m² cisplatin for histologically diagnosed small cell lung cancer, non-small cell lung cancer, or malignant pleural mesothelioma. Before and after the first course of chemotherapy, we measured urinary A- and C-megalin and other markers of PTEC injury, such as N-acetyl-β-D-glucosaminidase, α1-microglobulin, β2-microglobulin, neutrophil gelatinase-associated lipocalin, and liver-type fatty acid-binding protein, and compared the values with the change in the estimated glomerular filtration rate (eGFR) and clinical risk factors for renal impairment.

Results: A negative correlation was found between baseline urinary A-megalin levels and change in eGFR (r = −0.458, P = 0.002). According to Kaplan–Meier survival curves, eGFR decline was associated with the baseline urinary A-megalin quartile (P = 0.038). In addition, according to the hazard ratios (HRs) for eGFR decline > 10 mL/min/1.73 m² calculated using a Cox proportional hazard model, the highest quartile had a significantly higher risk of eGFR decline compared with the lowest quartile (HR 7.243; 95% confidence interval 1.545–33.962). Other baseline urinary markers showed no correlation with eGFR decline.

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Conclusions: This is the first report demonstrating that prechemotherapy urinary A-megalin levels are correlated with the development of cisplatin-induced nephrotoxicity. This finding has clinical implications for the identification of patients at risk for cisplatin-induced nephrotoxicity and the development of possible prophylactic therapies.

Keywords: Chemotherapy, Cisplatin, Nephrotoxicity, Urinary megalin

Background
Cisplatin (cis-dichlorodiammineplatinum [II]) is one of the most potent chemotherapeutic agents used in the treatment of various solid tumors, including bladder cancer, cervical cancer, malignant pleural mesothelioma (MPM), ovarian cancer, squamous cell carcinoma of the head and neck, germ cell cancer, small cell lung cancer (SCLC), and non-small cell lung cancer (NSCLC). Cisplatin chemotherapy in combination with radiotherapy or surgery improves survival in advanced lung cancer and cure rate in locoregional and early stage lung cancer [1, 2].

The dose-limiting toxicity of cisplatin is renal, rather than hematological, typically causing acute kidney injury or even chronic renal impairment [3]. Cisplatin is thus contraindicated for chemotherapy in patients with chronic kidney disease, despite its outstanding efficacy. The kidneys are particularly vulnerable to toxic insult by cisplatin because of its high levels of accumulation in renal tissue [4]. Proximal tubule epithelial cells (PTECs) have been recognized as the primary target of cisplatin-induced toxicity [4]. Candidates for facilitated transport systems associated with cisplatin-induced nephrotoxicity include organic cation transporter 2 and copper transporter 1 in PTECs [5, 6]. However, an organic cation transporter 2 inhibitor, cimetidine, has shown only a partial protective effect against cisplatin-induced nephrotoxicity [5]. Thus, it is important to elucidate the precise mechanism underlying cisplatin-induced nephrotoxicity and develop strategies for its prediction and prevention.

Megalin is a large (~ 600 kDa) glycoprotein member of the low-density lipoprotein receptor family [7] that is expressed at the apical membranes of PTECs [8]. Megalin plays a pivotal role in the tubular reabsorption of glomerular filtrates and mediates intracellular signal transduction [9]. A high-fat-diet–induced mouse model of diabetic kidney disease showed that megalin mediates the proximal tubular uptake of nephrotoxic substances, such as lipoprotein-modified proteins, resulting in tubuloglomerular alterations [10]. Megalin also mediates the uptake of nephrotoxic drugs such as aminoglycosides [11, 12], polymyxin B [11], colistin [12, 13], and vancomycin [12]. We demonstrated that cisplatin is a ligand of megalin using quartz crystal microbalance analysis. Moreover, we showed that PTECs lacking megalin do not [12]. Therefore, megalin likely plays a primary role in the development of cisplatin-induced nephrotoxicity.

We also reported that two forms of megalin are excreted in urine, namely, the ectodomain (A-megalin) and full-length (C-megalin) forms, and that the former is several 100-fold more abundant than the latter [14]. We developed sandwich enzyme-linked immunosorbent assays (ELISAs) to measure urinary A- and C-megalin by using monoclonal antibodies (mAbs) against the amino- and carboxyl-termini of megalin, respectively [14]. Urinary C-megalin levels are increased via exocytosis from residual functioning nephrons that are overloaded by megalin-mediated protein metabolism [15]. In contrast, urinary A-megalin excretion appears to be regulated by the intracellular recycling [16] and intramembrane proteolysis [17–19] of megalin.

In this study, we measured urinary megalin levels, focusing in particular on A-megalin, in patients with advanced thoracic malignancies who were treated with cisplatin (≥60 mg/m²), with the aim of investigating the pathological role of megalin in the development of cisplatin-induced nephrotoxicity and elucidating the potential of urinary megalin measurement as a biomarker to predict nephrotoxicity.

Methods
Patients
Forty-five consecutive chemotherapy-naïve patients with Eastern Cooperative Oncology Group performance status of 0–1 and intact renal function (estimated glomerular filtration rate [eGFR] > 60 mL/min/1.73 m²) who were scheduled to receive chemotherapy with ≥60 mg/m² cisplatin for histologically diagnosed NSCLC, SCLC, or MPM were enrolled at a single institution (Niigata University Medical and Dental Hospital, Niigata, Japan). This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines and was approved by the Niigata University Ethics Committee. The patients were enrolled after providing written informed consent.

Study design
On the day of the first course of chemotherapy (day 0), we obtained baseline serum creatinine (Cr) concentrations and urinary levels of A-megalin, C-megalin, N-acetyl-β-D-
glucosaminidase (NAG), α₁-microglobulin (α₁-MG), β₂-microglobulin (β₂-MG), neutrophil gelatinase-associated lipocalin (NGAL), liver-type fatty acid-binding protein (L-FABP), and Cr. All patients received an intravenous infusion of ≥1500 mL isotonic electrolyte solution, supplemented with magnesium sulfate and diuretics (furosemide or mannitol). After antiemetic therapy consisting of 0.75 mg palonosetron, 9.9 mg dexamethasone, and the oral administration of 125 mg aprepitant, the patients received a 1-h intravenous infusion of cisplatin, followed by 1000 mL isotonic electrolyte solution. Fresh urine samples (20 mL) were scheduled to be collected on day 0 in the morning before cisplatin administration, and on days 1, 2, 4, 6, 7, 12, and 20 after administration. Urine samples were generally collected on the scheduled day ±1 or 2 days. Further urine sampling and measurement of biomarkers were performed at the discretion of the attending physicians. Urine samples were frozen at −80 °C and thawed immediately prior to analysis. Serum Cr concentrations were routinely monitored after cisplatin administration (day 0 in the morning before cisplatin administration, and on days 2, 4, 7, 9, and 12 after administration). Serum Cr was generally

Table 1 Baseline characteristics of all study patients (n = 45)

|                          | All    | Q1     | Q2     | Q3     | Q4     | P     |
|--------------------------|--------|--------|--------|--------|--------|-------|
|                          | n = 45 | n = 11 | n = 12 | n = 11 | n = 11 |       |
| Urinary A-megalin (pmol/g Cr) |        |        |        |        |        |       |
| Mean ± SD or [Median]    | 87.9 ± 46.6 [36.1] | [76.0] | [95.8] | [150.4] |        |       |
| Range                    | 1.6–203.6 | 1.6–52.9 | 68.3–79.8 | 79.9–118.9 | 119.8–203.6 |       |
| Age, years               |        |        |        |        |        |       |
| Mean ± SD                | 64.6 ± 8.2 | 62.1 ± 8.9 | 65.8 ± 4.9 | 65.5 ± 8.4 | 64.8 ± 10.3 | 0.702 |
| Sex, n (%)               |        |        |        |        |        | 0.399 |
| Female                   | 9 (20.0) | 3 (27.3) | 4 (33.3) | 1 (9.1) | 1 (9.1) |       |
| Male                     | 36 (80.0) | 8 (72.7) | 8 (66.7) | 10 (90.9) | 10 (90.9) |       |
| Body height, cm          |        |        |        |        |        | 0.528 |
| Mean ± SD                | 163.9 ± 7.2 | 165.0 ± 6.9 | 161.3 ± 6.0 | 165.2 ± 7.0 | 164.5 ± 8.9 |       |
| Body weight, kg          |        |        |        |        |        | 0.437 |
| Mean ± SD                | 57.7 ± 10.1 | 60.2 ± 10.2 | 59.9 ± 12.8 | 54.3 ± 10.6 | 56.0 ± 4.9 |       |
| Smoking status, n (%)    |        |        |        |        |        | 0.527 |
| Current/former           | 40 (88.9) | 10 (90.9) | 10 (83.3) | 11 (100) | 9 (81.8) |       |
| Never                    | 5 (11.1) | 1 (9.1) | 2 (16.7) | 0 (0.0) | 2 (18.2) |       |
| Baseline therapies, n (%)|        |        |        |        |        |       |
| RAS inhibitors           | 10 (22.2) | 3 (27.3) | 2 (16.7) | 4 (36.3) | 1 (9.1) | 0.457 |
| NSAIDs                   | 22 (48.9) | 5 (45.5) | 5 (45.5) | 4 (36.3) | 8 (72.7) | 0.340 |
| Baseline comorbidity, n (%)|        |        |        |        |        |       |
| Hypertension             | 20 (44.0) | 3 (27.3) | 9 (75.0) | 5 (45.5) | 3 (27.3) | 0.066 |
| Diabetes                 | 9 (20.0) | 1 (9.1) | 2 (16.7) | 3 (27.3) | 3 (27.3) | 0.675 |
| Initial eGFR (mL/min/1.73 m²) |        |        |        |        |        | 0.372 |
| Mean ± SD                | 89.7 ± 15.9 | 83.7 ± 16.2 | 87.9 ± 17.1 | 92.9 ± 17.6 | 94.6 ± 11.9 |       |
| ≥90                      | 24 (53.3) | 4 (36.4) | 8 (66.7) | 6 (54.5) | 6 (54.5) |       |
| 60–89                    | 20 (44.4) | 7 (63.6) | 3 (25.0) | 5 (45.5) | 5 (45.5) |       |
| ≤59                      | 1 (2.2) | 0 (0.0) | 1 (8.3) | 0 (0.0) | 0 (0.0) |       |
| Cisplatin dose (mg/m²)   |        |        |        |        |        | 0.143 |
| Mean ± SD                | 76.0 ± 5.0 | 73.2 ± 6.8 | 77.5 ± 2.6 | 75.9 ± 5.8 | 77.3 ± 2.6 |       |
| Type of malignancy, n (%)|        |        |        |        |        |       |
| NSCLC                    | 33 | | | | |       |
| SCLC                     | 10 | | | | |       |
| MPM                      | 2 | | | | |       |

eGFR, estimate glomerular filtration rate; Cr, creatinine; MPM, malignant pleural mesothelioma; NSAID, non-steroidal anti-inflammatory drug; NSCLC, non-small cell lung cancer; RAS, renin-angiotensin-aldosterone system; SCLC, small cell lung cancer; SD, standard deviation
collected on the scheduled day ±1 or 2 days. An adverse renal event was defined as eGFR decline > 10 mL/min/1.73 m². This definition was based on a report that eGFR levels decrease by approximately 10 mL/min/1.73 m² on average for each cycle of cisplatin treatment in cancer patients [20]. Smoking status and baseline therapies, using either renin-angiotensin system (RAS) inhibitors or non-steroidal anti-inflammatory drugs (NSAIDs), were confirmed from medical records. The presence of hypertension and diabetes was determined by clinical diagnosis.

Measurement of human megalin in urine
Urinary A- and C-megalin were measured by ELISAs in the laboratory of Denka Co., Ltd., as described previously [14]. In brief, the capture mAbs (5 mg/mL) were immobilized on ELISA plates (LumiNunc F16 Maxisorp Surface Plate; Thermo Fisher Scientific, Inc., Waltham, MA) at 4 °C overnight. The Fab’ fragments of the tracer mAbs were conjugated to alkaline phosphatase (Roche Diagnostics, GmbH, Mannheim, Germany). Urine samples (90 μL) were mixed with 10 μL solution A (2 mol/L Tris-HCl, 0.2 mol/L ethylenediaminetetraacetic acid, 10% Triton X-100; pH 8.0) and incubated for 1 min at room temperature for the C-megalin assay, or for 3 h at 50 °C for the A-megalin assay, and reacted with alkaline phosphatase-labeled tracer mAbs in the ELISA plates. Urinary megalin concentrations were standardized by adjustment to urinary Cr concentrations.

Measurement of other markers
Plasma concentrations of Cr were measured by an enzymatic method in the clinical laboratory at Niigata

![Fig. 1](image-url) eGFR levels after cisplatin administration in 24 cases with an adverse renal event. The vertical axis represents the absolute value of eGFR (a) and the change from baseline eGFR (b), respectively.
Table 2  Correlations between renal function and urinary markers

|          | eGFR   |  | ΔeGFR |  |
|----------|--------|---|-------|---|
|          | r      | P |       | P |
| eGFR     | -0.395 | 0.007 | -0.395 | 0.007 |
| ΔeGFR    | 0.299  | 0.016 | -0.458 | 0.002 |
| A-megalin| 0.241  | 0.111 | -0.242 | 0.110 |
| C-megalin| 0.043  | 0.780 | -0.208 | 0.171 |
| ΔC-megalin| 0.075  | 0.623 | -0.179 | 0.239 |
| NAG      | -0.260 | 0.864 | -0.142 | 0.352 |
| ΔNAG     | -0.092 | 0.546 | -0.235 | 0.120 |
| α1-MG    | -0.065 | 0.669 | -0.287 | 0.056 |
| Δα1-MG   | -0.023 | 0.880 | 0.231  | 0.126 |
| β2-MG    | -0.142 | 0.352 | -0.048 | 0.757 |
| Δβ2-MG   | -0.248 | 0.100 | -0.038 | 0.802 |
| NGAL     | -0.071 | 0.642 | 0.124  | 0.417 |
| L-FABP   | 0.123  | 0.421 | -0.074 | 0.629 |

r, Pearson’s correlation coefficient. α1-MG, α1-microglobulin; β2-MG, β2-microglobulin; eGFR, estimated glomerular filtration rate; L-FABP, liver-type fatty acid-binding protein; NAG, N-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin. ΔeGFR, maximum change from the baseline value to the lowest value of eGFR during follow-up. Δurinary marker = (maximum urinary marker after cisplatin administration) – (urinary marker before cisplatin administration).

Statistical analysis

The subjects were classified into 4 groups according to quartile of baseline urinary A-megalin. Numerical data are expressed as the mean ± standard deviation, and categorical data are expressed as n (%). Differences between groups were tested by one-way analysis of variance for numerical variables and the chi-square test for categorical variables. The correlation between two numerical variables was examined using Pearson’s correlation coefficient (r). Event-free survival curves were drawn using the Kaplan-Meier method, and differences between curves were tested by log-rank test. Mean differences in ΔeGFR according to quartile of urinary A-megalin were estimated using crude and adjusted models of linear regression analysis with the lowest quartile (Q1) as the reference group.
The adjusted model was adjusted for baseline eGFR. For these models, P-values for trend were also calculated using the quartile median values. Cox proportional hazards regression analysis was used to calculate crude and adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) for the first adverse renal event according to baseline urinary A-megalin quartiles. All statistical analysis was performed using SPSS v.18.0 (IBM Corp., Armonk, NY). The level of significance was two-tailed P < 0.05.

**Results**

The baseline characteristics of the subjects according to quartile of baseline urinary A-megalin are shown in Table 1. There were no significant differences between the groups in age, sex, smoking history, use of RAS inhibitors or NSAIDs, presence of hypertension or diabetes mellitus, baseline eGFR, and cisplatin dose. Tumor types were NSCLC (n = 33), SCLC (n = 10), and MPM (n = 2). Concomitant chemotherapeutic agents included pemetrexed (n = 18), etoposide (n = 12), gemcitabine (n = 9), CPT-11 (n = 3), docetaxel (n = 2), and vinorelbine (n = 1). Seven patients received the antivascular endothelial growth factor mAb bevacizumab with cisplatin and pemetrexed (Additional file 1: Table S1).

We previously reported that urinary A- and C-megalin levels in healthy control individuals were 73 (35–106) and 0.145 (0.187–0.233) pmol/g Cr, respectively [14]. Mean baseline urinary A-megalin in the present study population (87.9 ± 46.6 pmol/g Cr) was nearly equal to that in our previous report, and mean baseline urinary C-megalin in the present study (0.64 ± 0.76 pmol/g Cr) was slightly higher (Additional file 1: Table S2). Mean NAG, α1-MG, β2-MG, NGAL, and L-FABP are also shown in Additional file 1: Table S2.

During 564 person-days of follow-up, 24 cases (53.3%) experienced adverse renal events (Fig. 1); the incidence rate of the first event was 0.426 per 10 person-days. Of those patients, the mean follow-up period until the first event was 5.8 ± 3.5 days from the start of cisplatin treatment. We found no association between pemetrexed administration and nephrotoxicity (data not shown).

According to Pearson’s correlation coefficients, the baseline values of urinary A-megalin and eGFR showed negative correlations with ΔeGFR (r = −0.458, P = 0.002 and r = −0.395, P = 0.007, respectively): the correlation matrixes are shown in Table 2 and Additional file 1: Table S3, and the scatter diagram is shown in Fig. 2. In addition, A-megalin levels were not correlated with other urinary markers, including C-megalin, at baseline. The other urinary markers did not show any correlation

**Table 3** Difference in ΔeGFR and the risk of adverse renal events according to quartile of urinary A-megalin

| Quartile of urinary A-megalin | Q1 (n = 11) | Q2 (n = 12) | Q3 (n = 11) | Q4 (n = 11) | P          |
|------------------------------|-------------|-------------|-------------|-------------|------------|
| ΔeGFR (mL/min/1.73 m²)       | −3.8 ± 6.7  | −15.2 ± 15.1| −11.9 ± 18.0| −20.8 ± 18.5| 0.085a     |
| Restricted to Q1 (mL/min/1.73 m²) | 0.00 (reference) | −11.39 (−24.30, 1.51)* | −8.05 (−21.23, 5.14) | −16.95 (−30.13, −3.76)* | 0.018b     |
| Adjusted                     | 0.00 (reference) | −10.00 (−22.29, 2.38) | −4.94 (−17.73, 7.85) | −13.27 (−26.17, −0.37)* | 0.064b     |
| Adverse renal events / person-days | 2 / 194 | 7 / 146 | 6 / 129 | 9 / 95 | 0.103 | 0.0479 | 0.465 | 0.947 |
| Event rate (/10 person-days) | 0.103 | 0.479 | 0.465 | 0.947 |
| Hazard ratio (95% CI)         | Crude: 1.00 (reference) | 4.25 (0.88, 20.51)* | 4.04 (0.81, 20.09)* | 7.24 (1.55, 33.96)* | 0.008b     |
| Adjusted                      | 1.00 (reference) | 3.80 (0.78, 18.48)* | 3.03 (0.60, 15.30) | 4.39 (0.91, 21.13)* | 0.093b     |

CI, confidence interval; eGFR, estimated glomerular filtration rate. ΔeGFR, maximum change from the baseline value to the lowest value of eGFR during follow-up.

An adverse renal event was defined as an eGFR decline of > 10 mL/min/1.73 m². Adjusted hazard ratio, adjusted for baseline eGFR. *P < 0.1, *P < 0.05, **P for statistical difference between groups, **P for trend

![Fig. 3](#) Kaplan–Meier curves for adverse-renal-event–free survival. An adverse renal event was defined as eGFR decline > 10 mL/min/1.73 m². Tick marks indicate censored observations. Baseline higher urinary A-megalin levels tended to be associated with poorer adverse-renal-event–free survival (P = 0.038, log-rank test)
Discussion

We examined the ectodomain (A-megalin) and full-length (C-megalin) forms of megalin in urine as markers of cisplatin-induced nephrotoxicity and found that prechemotherapy urinary A-megalin levels were associated with the development of cisplatin-induced nephrotoxicity. This is the first report to describe a relationship between prechemotherapy A-megalin levels and cisplatin-induced nephrotoxicity and to demonstrate that prechemotherapy A-megalin levels may be useful for predicting cisplatin-induced nephrotoxicity.

We previously reported that urinary C-megalin levels are correlated with severity of diabetic kidney disease [14] and IgA nephropathy [22]. The mechanism underlying urinary C-megalin excretion is associated with exocytosis, based on the chronic lysosomal protein metabolic load on PTECs of residual functioning nephrons [15]. However, baseline urinary C-megalin, NAG, α1-MG, β2-MG, NGAL, and L-FABP showed no correlation with the development of cisplatin-induced nephrotoxicity, suggesting that the mechanisms underlying urinary excretion of C-megalin and the other markers are not primarily associated with the pathogenesis of cisplatin-induced nephrotoxicity, at least in the patients enrolled in the present study.

In contrast, urinary A-megalin excretion was not correlated with other urinary markers including C-megalin and appears to be regulated by a distinct mechanism. Megalin undergoes intracellular recycling in PTECs and metalloprotease-mediated ectodomain shedding by regulated intramembrane proteolysis [17, 18]. Thus, urinary A-megalin may be produced as a normal byproduct in the absence of PTECs damage and may be increased by some factors that accelerate intracellular recycling. The efficiency of megalin recycling is also associated with its endocytic function [16]. Hence, it is likely that baseline urinary A-megalin is correlated with the amount and/or endocytic rate of megalin expressed in PTECs. It remains to be determined what factors are involved in the regulation of megalin recycling and/or endocytosis. We recently reported that megalin-mediated cisplatin uptake in PTECs has a primary role in nephrotoxicity [12]. Thus, baseline urinary A-megalin might reflect the efficiency of megalin-mediated cisplatin uptake in PTECs, and thus could be indicative of the development of cisplatin-induced nephrotoxicity. We also demonstrated that cilastatin, a megalin antagonist, blocks the binding of cisplatin to megalin [12], and thereby could protect against cisplatin-induced nephrotoxicity [23]. Hence, it would be worthwhile to investigate whether blockade of cisplatin binding to megalin could prevent cisplatin-induced nephrotoxicity, particularly in patients with elevated urinary A-megalin levels.

Conclusions

This is the first report demonstrating that prechemotherapy urinary A-megalin levels are correlated with the development of cisplatin-induced nephrotoxicity and may be a novel predictor of nephrotoxicity. This has clinical implications for identifying patients at risk for cisplatin-induced nephrotoxicity and developing possible prophylactic therapies.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12885-019-6398-2.

Additional file 1: Table S1. Chemotherapy regimens. Table S2. Baseline urinary markers. Table S3. Correlation matrix for all pairs of tested biomarkers.

Abbreviations

Cl: Confidence interval; Cr: Creatinine; eGFR: Estimate glomerular filtration rate; ELISA: Enzyme-linked immunosorbent assay; HR: Hazard ratio; L-FABP: Liver-type fatty acid-binding protein; mAb: Monoclonal antibody; MEG: Megalin; MPM: Malignant pleural mesothelioma; NAG: N-acetyl-β-D-glucosaminidase; NGAL: Neutrophil gelatinase-associated lipocalin; NSAID: Non-steroidal anti-inflammatory drug; NSCLC: Non-small cell lung cancer; PTECs: Proximal tubule epithelial cells; RAS: Renin-angiotensin-aldosterone system; SCLC: Small cell lung cancer; SD: Standard deviation; α1-MG: α1-microglobulin; β2-MG: β2-microglobulin
Acknowledgements
We thank ThinkSCIENCE, Inc. for English editing of this manuscript.

Authors’ contributions
HK2 and AS conceived and designed the experiments. SS, MH, and AS wrote the manuscript. HK1, RK2, SK, YH, and NT contributed to the analysis and interpretation of the data and assisted in the preparation of the manuscript. RK1, SM, SW, and NA assisted in the care of patients and collection of data. IN and TK provided administrative support. All authors read and approved the final manuscript.

Funding
This work was supported by Grants-in-Aid for Scientific Research to HK2 (no. 26293916) and AS (no. 15H04368) and for Young Scientists (B) to NA (no. 25870245) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant-in-Aid for the Practical Research Project for Renal Diseases from the Japan Agency for Medical Research and Development (18ek0310007h0003). The funding bodies had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request. The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Ethics approval and consent to participate
This study was approved by the Institutional Ethics Committee of Niigata University (approval number: 2015-1789). The study population included 45 patients who had given written informed consent.

Consent for publication
Not applicable.

Competing interests
AS received a research grant from Denka Seiken Co., Ltd. The other authors declare they have no competing interests.

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Received: 12 September 2019 Accepted: 22 November 2019

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