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Urinary afamin levels are associated with the progression of diabetic nephropathy

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ABSTRACT

Aims: In this study, we applied quantitative proteomic analysis to identify urinary proteins associated with diabetic nephropathy (DN).

Methods: Two-dimensional image-converted analysis of liquid chromatography and mass spectrometry detected the proteins differentially excreted between normoalbuminuric and macroalbuminuric patients with type 2 diabetes mellitus (T2DM) ($n = 6$ each). Urinary levels of excreted proteins were measured by multiple reaction monitoring (MRM) analysis

Abbreviations: 2DICAL, 2-dimensional image-converted analysis of liquid chromatography and MS; A1AG, α-1-acid glycoprotein 1; A1AT, α-1-antitrypsin; A1BG, α-1B-glycoprotein; AACT, α-1-antichymotrypsin; ACN, acetonitrile; ACR, urinary albumin to creatinine ratio; Afa/Cre, urinary afamin to creatinine ratio; CD44, CD44 antigen; DN, diabetic nephropathy; FA, formic acid; GO, gene ontology; LAMP2, lysosome-associated membrane glycoprotein 2; LC, liquid chromatography; MRM, multiple reaction monitoring; T2DM, type 2 diabetes mellitus

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using an independent sample set ($n = 77$). Urinary afamin levels were measured by ELISA in T2DM and DN patients enrolled in this cohort study ($n = 203$).

Results: One-hundred-four proteins displayed significant alterations in excretion. Nine of these candidates were validated by MRM analysis. Among them, the levels of afamin, CD44 antigen, and lysosome-associated membrane glycoprotein 2, which have not previously been implicated in DN, were significantly associated with both the urinary albumin to creatinine ratio (ACR) and eGFR. We further measured afamin levels in urine collected from T2DM patients who did not yet have significant kidney disease (ACR < 300 mg/g or eGFR change rate $\leq$ 3.3%/year). The urinary afamin to creatinine ratio (Afa/Cre) was significantly higher in patients who progressed to a more severe DN stage or had early renal decline than in patients who did not.

Conclusions: Afa/Cre was significantly increased in T2DM patients who subsequently developed DN. Afa/Cre may be useful to predict patients with T2DM at high risk of nephropathy before the development of macroalbuminuria or reduced kidney function, although further validation studies in a larger population are needed.

1. Introduction

Diabetes mellitus is a growing concern worldwide. The International Diabetic Foundation has predicted that the total number of diabetic people worldwide will rise to 642 million by 2040 [1]. Diabetic nephropathy (DN), one of the major microvascular complications of diabetes, has been a major cause of incident dialysis in Japan since 1998 [2]. DN is also a risk factor for cardiovascular disease, with a mortality rate more than 10-fold higher in type 2 diabetes mellitus (T2DM) patients with end-stage renal disease than in T2DM patients without DN [3]. Recently, in a retrospective cohort study, Wada et al. [4] reported that an elevated urinary albumin to creatinine ratio (ACR) and an eGFR decline increase the risk of cardiovascular events.

Microalbuminuria is the most dependable diagnostic marker of DN [5,6]. However, Krolewski et al. [7] recently reported that early progressive renal function decline precedes the onset of microalbuminuria. Other reports have also suggested that microalbuminuria alone may be insufficient as a predictive factor for DN [8], indicating the need for new factors predicting DN that work alone or as a complement to urinary albumin and eGFR.

Body fluids such as blood and urine contain numerous proteins and peptides and may reflect health conditions. Progress in MS technology has enabled the identification of thousands of proteins and peptides in body fluids at a time. Ono et al. [9,10] recently developed an integrated platform for label-free quantitative analysis, named 2-dimensional image-converted analysis of liquid chromatography and MS (2DICAL). This software detects the MS peaks that display differential intensities compared with controls and then identifies the corresponding peaks using a protein sequence database. 2DICAL is useful for large-scale proteomics due to its simple process, high throughput, and quantification accuracy and shows great potential in clinical proteomics [11–16]. Accordingly, we performed quantitative urinary proteomic analysis using 2DICAL to identify novel urinary proteins associated with DN.

2. Materials and Methods

2.1. Clinical samples

Urine samples were acquired from T2DM patients recruited as outpatients or inpatients at the Center Hospital of National Center for Global Health and Medicine (NCGM), JR Tokyo General Hospital, Toyama University Hospital, and Chiba East Hospital. In total, 1268 participants were recruited for this cross-sectional study between 2010 and 2014; 65 of them were selected for the proteome analysis. Urine samples were also collected from 651 healthy subjects without T2DM who were enrolled from an annual health checkup conducted at the Department of Complete Medical Checkup of NCGM between 2010 and 2013, and 24 of these participants were randomly selected for this study as controls. In the discovery phase proteome analysis, urine samples from T2DM patients ($n = 6$) and stage III to IV DN patients (DN3–4; $n = 6$) were analyzed. In the validation phase proteome analysis, urine samples were analyzed using the independent samples obtained from healthy subjects without T2DM ($n = 24$), T2DM patients without albuminuria ($n = 19$), stage II DN patients (DN2; $n = 19$), and DN3–4 patients ($n = 15$). The clinical characteristics of the participants used in the discovery phase proteome analysis and validation phase proteome analysis are shown in electronic Supplementary Tables 1 and 2, respectively.

Participants at the Center Hospital of NCGM and JR Tokyo General Hospital ($n = 510$) were also followed in the cohort study. DN progression was determined by reference to medical records, and the mean follow-up period was 2.4 years. Of these 510 participants, T2DM patients without DN ($n = 122$) and DN2 patients ($n = 81$) were retrospectively selected by reference to the medical records deposited during the follow-up period. Their baseline clinical characteristics are shown in Supplementary Tables 3 and 4, respectively.

T2DM was diagnosed according to World Health Organization criteria as described previously [17]. DN was diagnosed as follows: T2DM (normoalbuminuria), urinary albumin < 30 mg/g creatinine, and eGFR $\geq$ 30 mL min^{-1} 1.73 m^{-2}; DN2, urinary

albumin 30–299 mg/g creatinine, and eGFR $\geq$ 30 mL min^{-1} 1.73 m^{-2}; stage III DN, urinary albumin $\geq$ 300 mg/g creatinine, and eGFR $\geq$ 30 mL min^{-1} 1.73 m^{-2}; and stage IV DN, eGFR < 30 mL min^{-1} 1.73 m^{-2}. This study was approved by the local ethics committees of NCGM, JR Tokyo General Hospital, Chiba East Hospital, and Toyama University Hospital, and written informed consent was obtained from all participants.

2.2. Sample preparation

Urine samples were obtained by centrifugation at 3000*g* for 10 min and stored at −80 °C until analysis. Two hundred microliters of concentrated urine samples were applied to an Albumin & IgG Depletion SpinTrap (GE Healthcare, Little Chalfont, UK) to remove albumin and IgG. The depleted urine samples were then dried and dissolved in a solution containing 12 mM sodium deoxycholate, 12 mM sodium *N*-lauroyl sarcosinate, and 100 mM Tris-HCl. The urine samples were digested using MS-grade lysyl endopeptidase (Wako, Osaka, Japan) as described previously [18] and resolved in a solution containing 0.1% trifluoroacetic acid and 2% acetonitrile (ACN) (Kantokagaku, Tokyo, Japan).

2.3. Label-free quantitative proteomics

The peptide samples were analyzed using nano-liquid chromatography (LC) coupled with QSTAR Elite quadruple time-of-flight MS (Sciex, Framingham, MA). The peptide samples were concentrated and desalted with a trap column (0.3 × 5 mm, L-Column ODS; Chemicals Evaluation and Research Institute, Tokyo, Japan) and separated using a C18 column (L-Column Micro, L-Column ODS; Chemicals Evaluation and Research Institute) at a flow rate of 300 nL/min by a binary gradient composed of solvent A (2% ACN, 0.1% formic acid [FA]) and solvent B (90% ACN, 0.1% FA). The gradient started with 5% of B and linearly increased to 35% of B at 120 min, 95% of B at 120.1–140 min, 59% of B at 140.1–155 min, and 5% of B at 155.1 min (total run time, 175 min). Mass spectra were acquired for 0.5 s in the 400–1800 *m/z* range. MS peaks were detected, normalized, and quantified by 2DICAL software (Mitsui Knowledge Industry, Tokyo, Japan). A peak identification number (ID) was applied to each MS peak (1–2602). MS and tandem MS (MS/MS) data were searched using Mascot software version 2.4.1 (Matrix Science, London, UK). Then, the UniprotKB/Swiss-Prot database was searched with *Homo sapiens* as taxonomy (release-2013_12). The search parameters used were as follows: enzyme: trypsin/P, allowing 1 missed cleavage; fixed modification: carbamidomethyl (C); variable modifications: oxidation (M); precursor mass tolerance: ±0.1 Da; fragment ion mass tolerance: ±0.1 Da. The peptides identified with an expected value of $P < 0.05$ were considered to be reliable. If a MS peak was assigned to multiple peptides, the peptide with the highest Mascot score was selected. Duplicate analyses were performed for each sample.

2.4. MRM analysis

MRM analysis was performed largely as described previously [19]. Briefly, the MRM run was performed using a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Sciex) coupled with an LC800 HPLC system (GL Science, Tokyo, Japan) in the MRM mode. The peptide samples were separated using an ACQUITY UPLC BEH C18 column at a flow rate of 100 μl/min by a binary gradient composed of solvent A (2% ACN, 0.1% FA) and solvent B (90% ACN, 0.1% FA). The gradient started with 0% of B and linearly increased to 5% of B at 5 min, 30% of B at 75 min, 45% of B at 80 min, and 95% of B at 83–90 min. MRM transition peptides that had modifications, such as partially oxidized methionine, were avoided. Ten femtomoles of stable isotopically labeled human angiogenin peptide (DINTFL*IHGNK: *Leucine was labeled with the stable isotopes ^{13}C and ^{15}N) was also added to each sample as an internal standard. Samples were analyzed by LC-MRM on the 4000 QTRAP using the predetermined MRM method. Data were processed using the MultiQuant program (version 1.2; Sciex). The most intense peak of the transition was used for quantitation. The area under the most intense peak was calculated and normalized to the input internal standard. The peak of the transition for the input internal standard was also used for the quality control measure. Duplicate analyses were performed for each sample.

2.5. Measurement of urinary afamin levels

Urinary levels of human afamin were measured using a Human Afamin DuoSet ELISA (R&D Systems, Minneapolis, MN) according to the protocols provided by the manufacturer.

2.6. Statistical analysis

Data are expressed as the mean ± SD or the median (interquartile range). Differences between the two groups were analyzed using the Mann–Whitney *U* test or two-tailed Student *t*-test. One-way ANOVA was used for more than two group means, and multiple testing corrections were performed using the Tukey's honest significant difference test or Games-Howell test. Sex differences between the two groups were tested using the chi-square test. Correlations were calculated using the Pearson correlation coefficient. Using variables showing a correlation with afamin ($P \leq 0.1$) as potential independent variables, multivariable stepwise linear regression analysis was conducted with significance set at $P < 0.05$ for entry in the model and >0.10 for removal from using afamin as the dependent variable. Receiver operating characteristic (ROC) analysis was conducted to assess the predictive performance of a single predictor (ACR or urinary afamin to creatinine ratio [Afa/Cre]) in the DN2 patients enrolled in the cohort study. In the ROC analysis, patients who progressed from DN2 to DN3–4 (DN2 → DN3–4; $n = 11$) were defined as progressors and patients who did not show a change in DN severity (DN2 → DN2; $n = 52$) or who regressed from DN2 to T2DM (DN2 → T2DM; $n = 18$) were defined as non-progressors. We computed the area under the curve (AUC), sensitivity, specificity, positive predictive value, and negative predictive value of each predictor using various cut-off points to predict DN progression. Logistic regression analysis was also conducted in DN2 patients enrolled in the cohort study, using the progression of DN as the binary response variable and the clinical parameters (including

urinary afamin level) as the independent variables. ORs and corresponding significance levels (*P* value) were calculated from the logistic regression. All skewed variables were logarithmically transformed before analyses. Statistical analyses were performed using 2DICAL, Microsoft Excel 2011, or SPSS software version 20 (IBM Corporation, New York, NY). A *P* value <0.05 was considered significant.

3. Results

3.1. *Identification of differentially excreted proteins in the urine of DN patients by 2DICAL*

We compared the profiles of urinary proteins derived from DN3–4 and T2DM patients without nephropathy using 2DICAL software. We detected 2602 MS peaks within 400–1800 *m/z* and 0–175 min, with 1198 peaks showing significant differences between the DN3–4 and T2DM groups (fold change $\geq$ 2, Mann–Whitney *U* test and ratio *P* value <0.05). Fig. 1a shows a representative result of a 2-dimensional peak map derived from the analysis of the T2DM and DN3–4 patients. A representative peptide peak intensity map and box plot of the corresponding peak (peak ID 168) are also shown in Fig. 1b, c. MS/MS spectra acquired from the 286 peaks matched 220 peptide sequences derived from 104 proteins. In total, 99 peptides derived from 62 proteins were decreased, and 121 peptides derived from 42 proteins were increased in the DN patients compared with T2DM patients (Fig. 2). Eight proteins – apolipoprotein D, basement membrane-specific heparin sulfate proteoglycan core protein, Ig alpha-1 chain C region, phosphoinositide-3-kinase-interacting protein 1, polymeric immunoglobulin receptor, protein shisa-5, prothrombin, and thyroxine-binding globulin – were identified in both increased and decreased peptide peaks (Supplementary Table 5). Theoretically, the peptide mixture obtained from an individual protein is equimolar; however, many factors can violate the assumption of proportionality [20]. One possible explanation for these results is that the eight proteins are present in different isoforms or are otherwise post-translationally modified, raising the possibility that some peptides would be subject to inefficient ionization or fall outside the expected *m/z* range. Further analyses are necessary to better understand this observation.

3.2. *Characterization of the differentially excreted proteins between T2DM and DN patients*

2DICAL analysis identified 62 decreased proteins and 42 increased proteins in DN3–4 patients compared with T2DM. To characterize these proteins, we performed network analysis using Ingenuity Pathway Analysis as previously described [21]. The top-rated interaction networks were metabolic disease, neurological disease, and psychological disorders, with 27 proteins differentially excreted between DN and T2DM (Supplementary Fig. 1). Upstream regulator analysis suggested that SERPINA1, SERPINA3, orosomucoid 1, haptoglobin, angiotensinogen, complement component 3, and cadherin would be modulated by increased expression of IL6 (z-score = 2.115, *P* value of overlap = 4.63E−08).

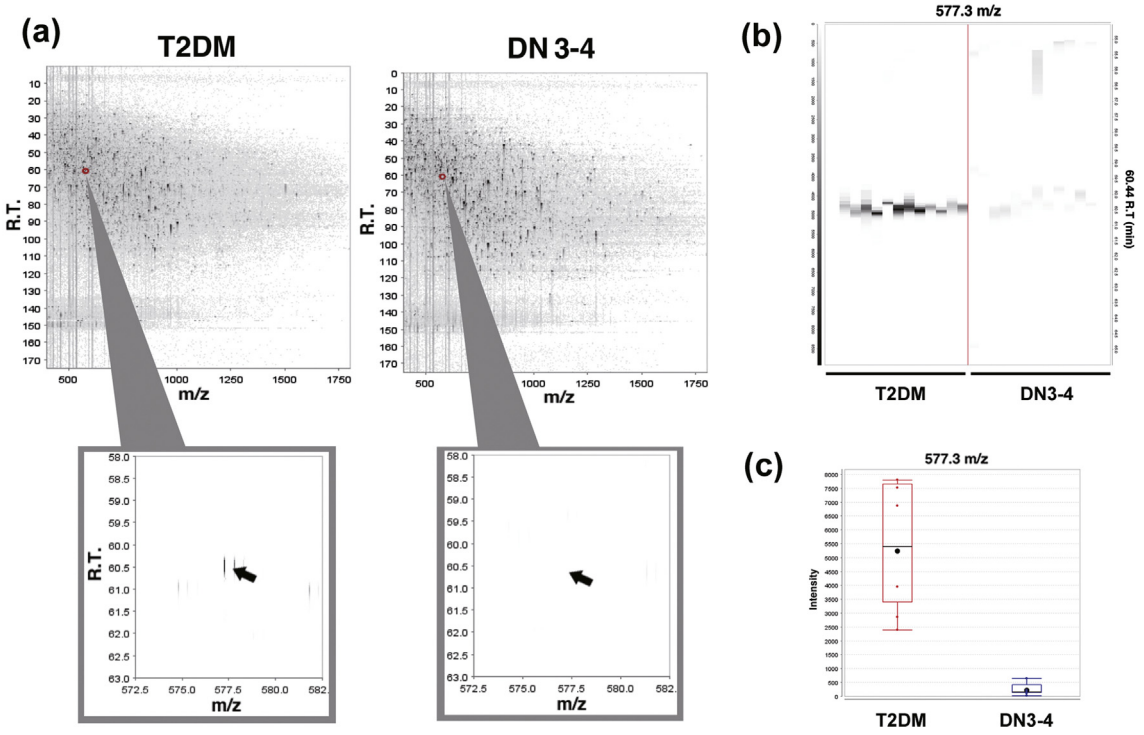

Fig. 1 – (a) Representative two-dimensional display of all MS peaks in 2-dimensional image-converted analysis of liquid chromatography and MS (2DICAL). Insets, representative peak spot images (peak identification number 168). (b) MS peak intensity map, and (c) box plot of the peak intensity derived from peak identification number 168 (577.3 *m/z*). T2DM, type 2 diabetes mellitus; DN3–4, stages III to IV diabetic nephropathy.

3.3. *Validation of differentially excreted urinary proteins by MRM*

To validate the results of discovery phase proteome analysis by 2DICAL, we performed relative quantification of the identified proteins in MRM analysis. We focused on 50 proteins containing the peptides that showed more than 10-fold significant changes. We finally constructed nine peptides for the detection of nine proteins: α-1-acid glycoprotein 1 (A1AG), α-1-antichymotrypsin (AACT), α-1-antitrypsin (A1AT), α-1B-glycoprotein (A1BG), afamin, CD44, Ig α-1 chain C region, LAMP2, and serotransferrin (Supplementary Table 6). Supplementary Fig. 2 shows a typical extracted ion chromatogram overlay of each peptide of the target proteins. Predetermined MRM transitions for each protein were confirmed to detect the product ions of the target peptides (Supplementary Fig. 3).

We initially performed MRM analysis using an independent sample set obtained from T2DM ($n = 19$) and DN3–4 ($n = 15$) patients (Supplementary Table 2). The nine proteins showed significant differences between the two groups, which validated the results obtained in the 2DICAL analysis (Table 1.1). To examine the association of these proteins with DN, we further performed relative quantification of the nine proteins using the additional sample set including healthy control subjects ($n = 24$) and DN2 patients ($n = 19$) (Supplementary Table 2). A1AG, AACT, A1AT, A1BG, afamin, Ig α-1-chain C region, and serotransferrin increased according to DN progression. In contrast, CD44 and LAMP2 decreased according to DN progression (Table 1.1). Additionally, afamin was significantly increased in T2DM patients compared with healthy control subjects ($P = 0.024$).

3.4. *Correlations of ACR and eGFR with the identified urinary proteins*

We performed correlation analysis between the levels of the nine proteins and ACR and between the levels of the nine proteins and eGFR. Afamin, CD44, and LAMP2 were significantly correlated with both ACR and eGFR. Afamin was positively correlated with ACR and negatively correlated with eGFR. CD44 and LAMP2 were negatively correlated with ACR and positively correlated with eGFR (Table 1.2). We then compared the urinary levels of the nine proteins between patients with an eGFR ≥ 60 and patients with an eGFR < 60. Compared with the eGFR ≥ 60 group, A1AG, afamin, and serotransferrin were significantly increased in the eGFR < 60 group, and CD44 and LAMP2 were significantly decreased (Table 1.3).

3.5. *Afa/Cre increases according to DN severity*

Among the proteins associated with both ACR and eGFR, urinary afamin was the only one to show significant differences between T2DM patients and healthy control subjects. We thus further analyzed the association of afamin with the progression of DN. We assayed afamin levels by ELISA, finding that they were significantly correlated with those assayed by MRM analysis ($r = 0.877$, $P = 1.4E{-}24$). We assessed urinary afamin levels in the DN patients enrolled in the cohort study

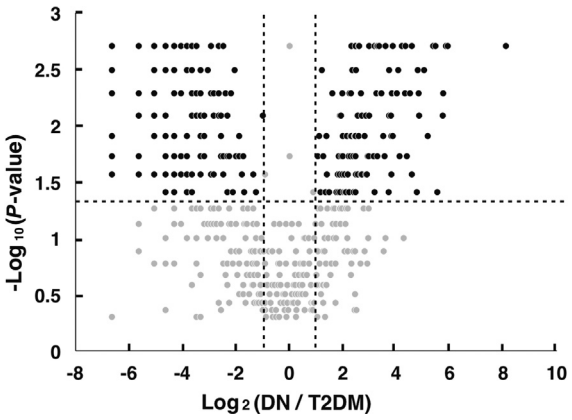

Fig. 2 – Volcano plot showing the distribution of the diabetic nephropathy (DN)/type 2 diabetes mellitus (T2DM) ratios (log_2) vs the *P* values (log_{10}) for all of the peptides identified in 2DICAL analysis. Black circles indicate significantly different peptides between DN and T2DM patients (*U* test *P* value <0.05, fold change ≥ 2). Grey circles indicate the peptides not reaching the level of statistical significance.

(Supplementary Tables 3 and 4). The baseline Afa/Cre was significantly increased in patients who progressed from T2DM to DN2 ($n = 42$) compared with patients who did not ($n = 80$; Fig. 3a). Furthermore, the baseline Afa/Cre was significantly increased in the DN2 → DN3–4 group ($n = 11$) compared with the DN2 → DN2 group ($n = 52$) and was significantly decreased in the DN2 → T2DM group ($n = 18$) compared with the DN2 → DN2 group ($n = 52$; Fig. 3b). Stepwise multivariate regression analysis showed that Afa/Cre was significantly associated with ACR ($\beta = 0.794$, $P = 4.0E{-}61$) and eGFR ($\beta = -0.079$, $P = 0.024$), which was consistent with the results of the validation assay (Supplementary Table 7).

ROC analysis was performed to assess the predictive value of afamin for the progression of DN (Supplementary Table 8). The AUC of Afa/Cre was higher than that of ACR, implying that Afa/Cre predicts DN progression (Supplementary Fig. 4). Diagnostic measures, including sensitivity, specificity, positive predictive values, and negative predictive values, were substantially dependent on the chosen cutoff points. Logistic regression analysis also showed that Afa/Cre was a significant predictor of the progression of DN (Table 2).

3.6. *Afa/Cre is associated with early renal function decline*

Finally, we analyzed the association of Afa/Cre with early renal function decline. Early renal function decline was defined as a ≥ 3.3% decline in eGFR per year according to previous work [7]. Afa/Cre was significantly increased in patients with a ≥3.3%/year decline in eGFR (median: 12.38, interquartile range: 4.34–38.52; $n = 66$) compared with patients with a <3.3%/year decline in eGFR (median: 6.43, interquartile range: 2.62–20.20; $n = 137$) ($P = 0.030$).

4. Discussion

In this study, we performed label-free urine proteome analysis to identify proteins associated with DN. One-hundred-

Table 1.1 – Validation of the differentially excreted proteins in the multiple reaction monitoring analysis.

Accession No.	Protein name	H	T2DM	DN2	DN3–4
P02763	Alpha-1-acid glycoprotein 1	4.66 ± 1.06	5.20 ± 0.92	6.20 ± 0.83[b,d]	6.97 ± 0.47[c,e]
P01011	Alpha-1-antichymotrypsin	2.58 ± 0.66	2.48 ± 1.20	3.26 ± 1.09	4.15 ± 0.86[c,e]
P01009	Alpha-1-antitrypsin	2.93 ± 0.80	2.29 ± 0.94	3.75 ± 1.23[b,d]	5.73 ± 1.09[c,e,f]
P04217	Alpha-1B-glycoprotein	1.67 ± 0.90	1.94 ± 0.95	2.90 ± 0.73[b,d]	3.82 ± 0.70[c,e,f]
P43652	Afamin	0.03 ± 0.84	0.79 ± 0.91[a]	2.21 ± 0.78[b,d]	3.46 ± 0.85[c,e,f]
P16070	CD44 antigen	5.61 ± 0.59	5.11 ± 0.66	4.86 ± 0.64[b]	3.98 ± 1.04[c,e,f]
P01876	Ig alpha-1 chain C region	2.14 ± 0.93	1.88 ± 0.73	2.64 ± 0.84[d]	3.47 ± 1.01[c,e,f]
P13473	Lysosome-associated membrane glycoprotein 2	1.50 ± 0.77	1.23 ± 0.55	1.37 ± 0.45	0.65 ± 0.93[c]
P02787	Serotransferrin	3.93 ± 0.63	4.44 ± 0.79	5.95 ± 0.75[b,d]	7.28 ± 0.74[c,e,f]

Values represent the normalized area under the most intense peak of the target peptides. Data were log-transformed before analysis and are represented as the mean ± SD. Duplicate analyses were performed for each of the subjects. H, healthy control subjects; T2DM, type 2 diabetes mellitus; DN2, stage II diabetic nephropathy; DN3–4, stages III to IV diabetic nephropathy.

[a] *P* value <0.05 (H vs T2DM).
[b] *P* value <0.05 (H vs DN2).
[c] *P* value <0.05 (H vs DN3–4).
[d] *P* value <0.05 (T2DM vs DN2).
[e] *P* value <0.05 (T2DM vs DN3–4).
[f] *P* value <0.05 (DN2 vs DN3–4).

Table 1.2 – Correlation of 9 urinary proteins with the urinary albumin to creatinine ratio (ACR) or eGFR.

Accession number	Protein name	ACR		eGFR	
		r	*P* value	r	*P* value
P02763	Alpha-1-acid glycoprotein 1	0.20	0.09	−0.15	0.18
P01011	Alpha-1-antichymotrypsin	0.27	1.8E−02[a]	−0.01	0.96
P01009	Alpha-1-antitrypsin	0.47	1.5E−05[b]	−0.05	0.65
P04217	Alpha-1B-glycoprotein	0.28	1.4E−02[a]	−0.08	0.48
P43652	Afamin	0.34	2.0E−03[b]	−0.23	0.04[a]
P16070	CD44 antigen	−0.61	3.3E−09[b]	0.40	2.7E−04[b]
P01876	Ig alpha-1 chain C region	0.31	7.1E−03[b]	−0.07	0.52
P13473	Lysosome-associated membrane glycoprotein 2	0.48	1.1E−05[b]	0.30	7.0E−03[b]
P02787	Serotransferrin	0.37	1.0E−03[b]	−0.19	0.1

[a] *P* value <0.05.
[b] *P* value <0.01.

Table 1.3 – Urinary levels of nine proteins in patients with an eGFR ≥ 60 and with an eGFR < 60 validated in the multiple reaction monitoring assay.

Accession No.	Protein name	eGFR ≥ 60	eGFR < 60	*P* value
P02763	Alpha-1-acid glycoprotein 1	251.45 (91.82–679.18)	500.9 (246.3–711.05)	0.034[a]
P01011	Alpha-1-antichymotrypsin	19.59 (11.17–37.75)	27.17 (11.59–79.71)	0.598
P01009	Alpha-1-antitrypsin	25.52 (10.6–68.72)	55.15 (6.61–383.4)	0.423
P04217	Alpha-1B-glycoprotein	11.14 (4.53–23.87)	17.37 (7.94–53)	0.083
P43652	Afamin	2.99 (1.16–11.69)	11.01 (3.55–33.7)	0.015[a]
P16070	CD44 antigen	166.59 (110.44–277.96)	80.17 (31.95–113.24)	0.004[b]
P01876	Ig alpha-1 chain C region	12.28 (4.94–18.87)	12.22 (5.28–56.48)	0.310
P13473	Lysosome-associated membrane glycoprotein 2	3.72 (2.53–5.38)	2.59 (0.73–4.25)	0.045[a]
P02787	Serotransferrin	101.36 (55.09–464.3)	581.13 (137.35–1691)	0.017[a]

The values represent the normalized area under the most intense peak of the target peptides. Data were log-transformed before analysis and are represented as median (interquartile range).

[a] *P* value <0.05.
[b] *P* value <0.01.

four proteins were identified as differentially excreted proteins in the discovery phase (Fig. 2 and Supplementary Table 5) and nine of these proteins – A1AG, AACT, A1AT, A1BG, afamin, CD44, Ig α-1 chain C region, LAMP2, and serotransferrin – were confirmed in the validation phase (Table 1.1). Of these proteins, afamin, CD44, and LAMP2 were correlated with both ACR and eGFR (Table 1.2). Furthermore, when subjects were grouped by eGFR (eGFR ≥ 60 mg min^{-1}

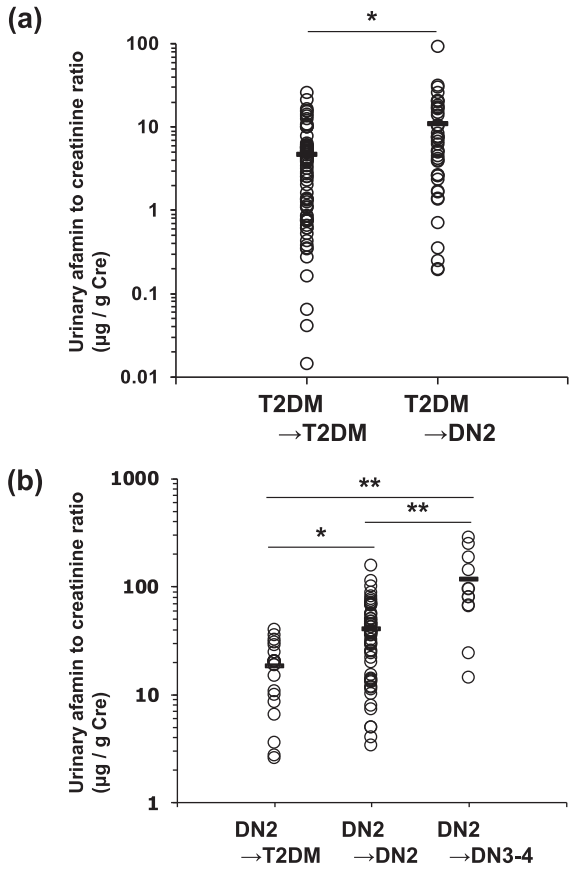

Fig. 3 – (a) Baseline urinary afamin to creatinine ratio (Afa/Cre) in patients who progressed from type 2 diabetes mellitus (T2DM) to stage II diabetic nephropathy (DN) (T2DM → DN2) and in patients who did not (T2DM → T2DM). (b) Baseline Afa/Cre in patients who progressed from stage II to stage III–IV DN (DN2 → DN3–4), in patients who regressed from stage II DN to T2DM (DN2 → T2DM), and in patients who remained in stage II DN (DN2 → DN2). The vertical line represents the average Afa/Cre in each group. *P < 0.05, **P < 0.01.

1.73 m^{-2} and eGFR < 60 mg min^{-1} 1.73 m^{-2}), the levels of A1AG, afamin, and serotransferrin were significantly increased in the eGFR < 60 group, and the levels of CD44 and LAMP2 were significantly decreased in the eGFR < 60 group (Table 1.3). Among the urinary proteins validated by MRM analysis, several published studies have also reported the differential excretion of A1AG, AACT, A1AT, afamin, Ig α-1 chain C region, and serotransferrin in T2DM patients with microalbuminuria compared with those with normoalbuminuria [22–24]. However, to our knowledge, no studies have reported the association of urinary A1BG, CD44, and LAMP2 levels with DN.

CD44 encodes a cell surface glycoprotein that acts as an immune cell receptor and is involved in inflammatory cell migration and activation. CD44 also protects against apoptotic factors such as UV radiation, tumor necrosis factor, and carcinostatic agents [25]. CD44 knockout mice are protected from insulin resistance and adipose tissue inflammation during diet-induced obesity [26]. In obese subjects with insulin resistance, serum CD44 concentrations are also increased, and CD44 gene expression is also induced in subcutaneous adipose tissue [27]. In contrast, in the current study, the urinary CD44 level was decreased as DN progressed. In the MRM analysis, we selected unmodified peptides of CD44 for quantitation. However, it is possible that the target peptides would contain post-translationally modified residues such as those subject to glycosylation, lipidation, and proteolysis, which are poorly detected by the predetermined MRM transitions. Therefore, although post-translationally modified residues of CD44 proteins need to be analyzed, post-translationally modified CD44 could still be a biomarker for the progression of DN.

Table 2 – Multivariate assessment of the effect of urinary afamin level on the progression of diabetic nephropathy.

	OR[b]	95% CI	P value
Model 1			
Age (year)	0.86	0.76–0.98	0.03[b]
Afa/Cre (μg/g Cre)[a]	8.44	2.15–33.20	0.002[b]
Model 2			
Age (year)	0.86	0.76–0.98	0.03[c]
eGFR (mL/min/1.73 m^2)	0.96	0.91–1.01	0.10
ACR (mg/g Cre)[a]	1.54	0.25–9.60	0.64
Afa/Cre (μg/g Cre)[a]	6.37	1.37–29.77	0.02[c]

[a] Log-transformed before analysis.
[b] per 1-unit increase.
[c] P value <0.05. Afa/Cre, urinary afamin to creatinine ratio; ACR, urinary albumin to creatinine ratio.

LAMP2 is a highly glycosylated protein that, along with LAMP1, constitutes the membrane proteins in the lysosome. LAMP2 has three isoforms: LAMP2A, LAMP2B, and LAMP2C [28]. In humans, LAMP2 deficiency causes Danon's disease-related hypertrophic cardiomyopathy [29]. Expression of *Lamp2* gene is reported to be reduced in diabetic islets [30]. Bhensdadia et al. [31] have reported the association of the urinary LAMP2 level with DN progression; however, the results were not significant. LAMP2 is critical for autophagy, with LAMP2 deficiency leading to the accumulation of autophagic vacuoles in several tissues, including the liver, pancreas, spleen, kidney, and skeletal and heart muscle. Insufficient autophagy is also observed in the podocytes in DN, leading to massive proteinuria [32]. Our present study showed that the urinary LAMP2 level was decreased in DN3–4 patients compared with T2DM patients. The lower level of urinary LAMP2 in DN patients may reflect the impaired autophagic activity in DN, which should be assessed by further functional studies.

In this study, afamin was suggested to be involved in the progression of DN. Afamin, also known as α-albumin or α_1T-glycoprotein, is a member of the albumin protein family including albumin, α-fetoprotein, and vitamin D-binding protein [33] and is also reported to be a vitamin E-binding glycoprotein [34]. The human afamin gene is predominantly expressed in the liver and is slightly expressed in the kidney [35]. Plasma afamin levels are significantly lower in patients with ovarian cancer than in healthy controls [36–38]. In a large-scale human epidemiological study, Kronenberg et al. [39] reported that afamin is strongly associated with the prevalence and development of metabolic syndrome and that afamin transgenic mice display increased body weight and serum concentrations of lipids and glucose. They also

reported that plasma afamin levels were associated with insulin resistance and incidence of T2DM on the basis of eight prospective cohort studies [40]. In polycystic ovary syndrome patients with insulin resistance, serum afamin concentrations were also increased compared with patients without insulin resistance [41]. We thus assessed the serum concentrations of afamin in the DN2 patients enrolled in the cohort study. However, no significant differences were observed in serum afamin levels between the DN2 → DN3–4 group (11.0 ± 4.2 pg/mL) and the DN2 → DN2 group (15.6 ± 9.0 pg/mL, *P* value = 0.29). We have also observed increased expression of afamin in the kidneys of *db/db* mice, an animal model of diabetic nephropathy (data not shown). Taken together, increased expression of afamin in the kidney may cause the development of DN, although we could not exclude the possibility that increased expression of afamin in the liver may cause insulin resistance, leading to the development of T2DM and renal function decline. There is also a possibility that an increase in some specific fragments might be generated by a mechanism underlying the onset of DN. However, we did not quantify multiple peptides derived from afamin in the present study. Further analyses are required to clarify the involvement of afamin in the onset of DN.

One limitation of this study is that the present MRM measurements were mostly restricted to the detection of only 9 of 104 differentially excreted proteins in the validation phase proteome analysis. In the MRM analysis, there were two possible reasons why not all identified proteins were measured. They include (1) the absence of appropriate peptide regions of the target proteins meeting the criteria described in the Methods, and (2) the difficult detection of the MS peaks of target proteins due to high background noise. To validate the 95 candidate proteins, we need to analyze urinary levels by other methods such as parallel reaction monitoring, ELISA, or western blotting. By validating all of the urinary proteins identified in the present study, we could further investigate the potential association between these proteins and DN, leading to the development of highly accurate diagnostic and/or predictive markers for DN.

In summary, a combination of two MS-based analyses—non-labeled quantitative analysis and MRM analysis—enabled the identification of several differentially excreted proteins in DN, including afamin, CD44, and LAMP2. In particular, urinary afamin levels were associated with DN and may predict DN progression. The proteins identified in our study could provide valuable information for better understanding the mechanisms underlying DN.

5. Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

6. Contribution statement

Y.K. designed the research, obtained the human urine samples and contributed to data collection and verification, and wrote the manuscript. E.T. conducted experiments, acquired and analyzed data, and wrote the manuscript. H.K., S.Y., R. Y., T.S., A.O., Y.F., N.S., K.T., M.M., and M.N. obtained the human urine samples and contributed to data collection and verification. A.G. contributed to the statistical analysis. H.U. designed the research and reviewed and revised the manuscript.

7. Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.diabres.2018.02.034.

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