Development of enzyme-linked immunosorbent assays for urinary thiazide-sensitive Na-Cl cotransporter measurement

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The Na-Cl cotransporter (NCC) in the distal convoluted tubules in kidney is known to be excreted in urine. However, its clinical significance has not been established because of the lack of quantitative data on urinary NCC. We developed highly sensitive and specific enzyme-linked immunosorbent assays (ELISAs) for urinary total NCC (tNCC) and its active form, phosphorylated NCC (pNCC). We first measured the excretion of tNCC and pT55-NCC in urinary exosomes in pseudohypoaldosteronism type II (PHAII) patients since PHAII is caused by NCC activation. Highly increased excretion of tNCC and pNCC was observed in PHAII patients. In contrast, the levels of tNCC and pNCC in the urine of patients with Gitelman’s syndrome were not detectable or very low, indicating that both assays could specifically detect the changes in urinary NCC excretion caused by changes in NCC activity in the kidney. Then, to test whether these assays could be feasible for a more general patient population, we measured tNCC and pNCC in the urine of outpatients with different clinical backgrounds. Although urinary protein levels >30 mg/dl interfered with our ELISA, we could measure urinary pNCC in all patients without proteinuria. Thus we established highly sensitive and quantitative assays for urinary NCC, which could be valuable tools for estimating NCC activity in vivo.

WNK kinase; hypertension; pseudohypoaldosteronism type II; exosome

EXOSOMES ARE NANOVESICLES (40–100 nm) released by cells on fusion of multivesicular bodies with the plasma membrane (5, 26). Pisikun et al. (22, 23) reported that urine also contains exosomes. Urinary exosomes are derived from all types of cells facing the urine flow along the nephrons, and they contain not only membrane proteins but also cytosolic proteins trapped during the formation of multivesicular bodies (23, 33). Therefore, urine is regarded as the most attractive biomarker sample for the early detection of kidney diseases and their therapeutic monitoring. Recently, to search for biomarkers of kidney diseases, such as IgA nephritis (17), minimal change nephrotic syndrome (30), and Lupus nephritis (19, 24), several proteomic analyses of urinary exosomes were conducted (10, 15, 18, 23). Although immunoblots are usually used to evaluate these exosomal biomarkers, this method is too labor intensive and not easily scaled up for larger clinical samples. Accordingly, another efficient, sensitive, and quantitative measurement of urinary exosomal proteins is necessary.

In the present study, we focused on the thiazide-sensitive sodium chloride cotransporter (NCC). NCC localizes to the apical membrane of the distal convoluted tubules in the kidney (4, 6, 9) and is responsible for reabsorbing 5–10% of the filtered load of sodium chloride. Pseudohypoaldosteronism type II (PHAII) is an autosomal dominant hereditary hypertensive disease characterized by hyperkalemia and metabolic acidosis (11). By analysis of PHAII model mice that carry a heterozygous D561A missense mutation in the Wnk4 gene, which corresponds to the PHAII-causing D564A mutation in the human WNK4 gene, we identified a new signal cascade, namely the WNK-OSR1/SPAK kinases, which phosphorylate NCC (2, 32). When phosphorylated, NCC becomes functionally active and is concentrated on the plasma membranes of the distal convoluted tubules. Mayan et al. (14) reported that the abundance of urinary NCC was increased in patients with the PHAII Q565E mutation in the WNK4 gene. Conversely, in Gitelman syndrome, NCC is genetically inactivated and absent or reduced in urinary exosomes (12). The excretion of phosphorylated NCC (pNCC) in urinary exosomes is increased in patients with low-salt intake and primary hyperaldosteronism (27) since the WNK-OSR1/SPAK kinase cascade is regulated by aldosterone (3). Although these investigations indicated that the excretion of total NCC (tNCC) and pNCC in urinary exosomes could be biomarkers to estimate NCC activity in vivo, the levels of urinary tNCC and pNCC were evaluated only by immunoblots.

To establish the clinical significance of urinary tNCC and pNCC measurements, a large amount of data from patients with different backgrounds must be collected. To gather such data, an efficient, sensitive, and quantitative assay is absolutely
necessary. For this purpose, we sought to establish sandwich ELISA systems to measure the absolute amount of urinary tNCC and pNCC. The measurements in the urine of patients with PHAII and Gitelman syndrome convinced us that these assays can adequately evaluate the activity of NCC in the kidney. Furthermore, we confirmed that these assays are feasible for outpatients with different clinical backgrounds.

METHODS AND MATERIALS

Urine collection and urinary exosome isolation. Mid-stream urine specimens (spot urine samples) were collected from normal volunteers and patients. In most cases, urine was collected in the morning. Urinary exosomes were prepared by an ultracentrifugation method optimized for ELISA. Twenty-five milliliters of urine samples treated with a protease inhibitor (0.5 tablet/0.25 ml urine; Complete Protease Inhibitor Cocktail; Roche Applied Science, Penzberg, Germany) were centrifuged at 3,000 \( g \) for 20 min at room temperature to remove cells and debris. Then, the supernatants were transferred to 26.3-ml polycarbonate ultracentrifuge tubes (no. 355618; Beckman Coulter, Brea, CA) and ultracentrifuged (Ultra Beckman Coulter OPTIMATM L-90K Ultracentrifuge, Beckman Type 70 Ti rotor; Beckman Coulter) at 240,000 \( g \) for 1 h at 25°C. The pellets were resuspended in 600 µl of TBS buffer containing 100 mM DTT. This suspension was incubated at 37°C for 2 min and then at 95°C for 2 min. After the incubation, the samples were centrifuged again at 17,000 \( g \) for 2 min to remove debris and large membrane fractions. The 17,000-g supernatants were diluted with 24.4 ml TBS buffer and ultracentrifuged at 240,000 \( g \) for 1 h at 25°C. The resulting pellets were resuspended in 300 µl TBS-0.02% Tween20 and centrifuged at 17,000 \( g \) for 2 min. The supernatants were the final samples for ELISA. The Medical Research Ethics Committee of Tokyo Medical and Dental University approved this human study (No. 1031). Urine samples from the Wnk4P651A/+ knockin (PHAII model) mice were collected for 24 h, and the urine exosomes were prepared as described above. The animal experiment was approved by the Animal Care and Use Committee of Tokyo Medical and Dental University.

Immunoblotting and antibodies. Urinary exosomal samples prepared for ELISA were boiled with SDS sample buffer (Cosmo Bio, Tokyo, Japan) and subjected to SDS-PAGE. Blots were probed with the following primary antibodies: rabbit anti-tNCC (Millipore, Temecula, CA), rabbit anti-phosphorylated T55 NCC (3), goat anti-aquaporin 2 (anti-AQP2; Santa Cruz Biotechnology, Dallas, TX), mouse anti-Tamm-Horsfall protein (Cerderlane Laboratories, Hornby, ON, Canada), and mouse anti-TSG101 (Abcam, Cambridge, UK).

A guinea pig anti-NCC antibody was previously generated by using the same peptide sequence as the antigen (residues 75–90: QPGEPKRKVRPTLADLH) that was used for the commercially avail-

Fig. 1. Development of sandwich ELISA for total (tNCC) and phosphorylated Na-Cl cotransporter (pNCC). A: optimization of sample preparation method for ELISA. A urine sample was obtained from a healthy human volunteer. Exosomes were prepared by 2 different methods from the same urine sample. The urinary pNCC signal could not be detected by the original method for exosome preparation without DTT treatment (23). However, a urinary pNCC signal was detected in the sample obtained by our ultracentrifugation method optimized for ELISA. B: summary of sandwich ELISA for tNCC and pNCC. For tNCC, the epitopes of the capture antibody and detection antibody are the residues from 75 to 90 and from 1 to 16 of the NCC protein, respectively. For pNCC, the epitope of the capture antibody is the residues from 51 to 59, where 55T is phosphorylated. The detection antibody recognizes the residues from 75 to 90. The capture antibodies are from rabbit, and the detection antibodies are from guinea pig. The standard protein for tNCC was prepared as a GST-fusion protein of the amino terminal portion of NCC (GST-NCC; 1–121) expressed in bacteria. For pNCC, we prepared the phosphorylated peptide (pNCC peptide; 51–91, 55ST) corresponding to the epitope of anti-pNCC antibody and also containing the epitope of the detection antibody.
standard proteins, respectively. OD260, optical density at 260.

detected as little as 3 pmol/ml and 100 fmol/ml of pNCC. The sandwich ELISA for tNCC and pNCC

Fig. 3. Sensitivity of sandwich ELISA for tNCC and pNCC. Without the capture and detection antibodies, the signals for tNCC (A) were not detected. B: validation of sandwich ELISAs for tNCC and pNCC. Without the capture and detection antibodies, the signals for tNCC (A) and pNCC (B) were not detected. C: phosphospecificity of sandwich ELISA for pNCC. GST-NCC and pNCC peptide were used as standards for sandwich ELISA for tNCC and pNCC, respectively. GST-NCC was not phosphorylated. Our sandwich ELISA for pNCC did not detect GST-NCC but did detect the pNCC peptide.

able rabbit anti-NCC (Millipore; Ref. 20). In the present study, we generated another guinea pig anti-NCC antibody by immunizing animals with keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to residues 1–16 of NCC (MAELPTTETPG-DATLC). The serum was affinity purified with the antigen peptides. We used the following formula to calculate fractional excretion of sodium (FE\textsubscript{Na}): FE\textsubscript{Na} [%] = \frac{[\text{serum Cr} \times \text{urinary sodium}] - [\text{serum sodium} \times \text{urinary Cr}]}{[\text{serum Cr}]} \times 100.

RESULTS AND DISCUSSION

Development of sandwich ELISA for tNCC and pNCC. We needed to modify an ultracentrifugation method of urine exosome preparation for our sandwich ELISA since the original kDa were quantified by using ImageJ software (National Institutes of Health, Bethesda, MD).

Sandwich ELISA. Rabbit anti-tNCC (75–90) (0.18 μg/well) and anti-pT55NCC (0.9 μg/well) antibodies in 100 μl of bicarbonate buffer (15 mM Na\textsubscript{2}CO\textsubscript{3}, 35 mM NaHCO\textsubscript{3}) were applied to 96-well ELISA plates (Iwaki, Tokyo, Japan) and incubated at 4°C for 96 h for immobilization. The anti-NCC antibodies used in the assays share no antigenic homology with other proteins including homologous NKCC1 and NKCC2. After being washed with 200 μl of wash buffer (TBS-0.02% Tween20) three times and being blocked with 200 μl of 1.5% (wt/vol) albumin in TBS-0.02% Tween20 for 30 min at room temperature, 100 μl of urinary exosome samples or standard proteins for TNCC and pNCC were applied to the wells. After a 2-h incubation at 37°C under gentle shaking, the plates were washed six times with a wash buffer (TBS-0.02% Tween20). Then, 100 μl of guinea pig anti-tNCC antibody (1–16; 0.16 μg/well) and anti-tNCC antibody (75–90; 0.38 μg/well) in EIA buffer (TBS-0.02% Tween20 and 0.2% BSA) were applied to each well for the tNCC and pNCC assays, respectively, for 2 h at 37°C under gentle shaking. Plates were washed with wash buffer again for seven times, and 100 μl AP-labeled anti-guinea pig antibody in EIA buffer (1:2,000 dilution) were added as a secondary antibody and incubated for 1 h at 37°C. After being washed eight times, 100 μl of BluePhos (KPL, Gaithersburg, MD) were applied and incubated for 10 min. The signals at 620 nm were obtained by a plate reader (Spectrafluor; Tecan Japan, Kanagawa, Japan). All of the antibodies used in this study are available in small amounts upon request.

Patients. Between July 2012 and March 2013, we recruited 93 patients who were treated as chronic kidney disease patients in the nephrology department of Tokyo Medical and Dental University. The Medical Research Ethics Committee of Tokyo Medical and Dental University approved this study (Approval No. 1031).

Clinical data collection. Medical records were used to collect data on age, sex, blood pressure (BP), serum albumin, sodium, potassium, creatinine (Cr), chloride, and urine Cr, urine protein, and urine sodium. Estimated glomerular filtration rates (eGFRs) were calculated with the following previously published Japanese eGFR equation (13): eGFR (ml/min\cdot1.73 m\textsuperscript{2}) = 0.741 \times 175 \times (age\textsuperscript{-0.287} \times (serum Cr\textsuperscript{-1.094} + 0.739) (if female).

We used the following formula to calculate fractional excretion of sodium (FE\textsubscript{Na}): FE\textsubscript{Na} [%] = \frac{[\text{serum Cr} \times \text{urinary sodium}] - [\text{serum sodium} \times \text{urinary Cr}]}{[\text{serum Cr}]} \times 100.

Statistical methods. Data are expressed as means ± SD. Comparisons between the two groups were performed with unpaired t-tests. The relationship of urinary tNCC and pNCC with continuous variables was examined by Pearson’s correlation coefficient. The multivariable regression analysis was created based on the variables that had a simple correlation of P < 0.05 to eGFR and FE\textsubscript{Na}.
ultracentrifugation method (23) results in the formation of tight pellets that cannot be dissolved in a buffered solution for ELISA. Tamm-Horsfall protein (THP), also known as uromodulin, is the most abundant protein in human urine (22), and it is known to form polymeric meshwork (31). Accordingly, we speculated that the THP polymers could make the pellet insoluble, and we incorporated a DTT treatment step into the original ultracentrifugation method to disrupt the networks, as described previously (7, 23). We also modified the centrifugation speed and optimized the buffer and the detergent for exosome preparation for our ELISA assays. As for the detergent, we judged that 0.02% Tween20 was enough to demolish the exosomes and to extract NCC since we did not observe any significant changes of signals in our ELISA if the concentrations of Tween20 was increased up to 0.1% or a more potent detergent, 0.1% TritonX-100, was used instead of Tween20.

This modified procedure allowed us to detect a pNCC signal (Fig. 1A). We determined reproducibility of this exosome preparation by analyzing a single urine sample in six different tubes. NCC and other exosome-associated proteins (THP, TSG101, and AQP2) were reproducibly detected by immunoblots. The average coefficient of variation was <10%.

The principle of our sandwich ELISA is summarized in Fig. 1B. As shown in Fig. 2, A and B, the elimination of either the capture or detection antibody in the assays resulted in no signal detection, confirming that there were no nonspecific signals in these assays. Then, we tested the phosphospecificity of sandwich ELISA for pNCC. Although we previously demonstrated the phosphospecificity of the antibody to detect p55T in NCC protein (3), we also verified it in our sandwich ELISA. As shown in Fig. 2C, while the pNCC peptide was detected by ELISA for pNCC, GST-NCC, which was not phosphorylated (confirmed by immunoblot with anti-p55T antibody, data not shown), was not detected. This result indicated that the sandwich ELISA for pNCC maintained high phosphospecificity.

We further evaluated the sensitivities of these sandwich ELISA systems. The sandwich ELISAs for tNCC and pNCC detected as little as 3 pmol/ml and 100 fmol/ml of each standard, respectively (Fig. 3). Since the active form of NCC is the phosphorylated form, pNCC in urine may be a better parameter than tNCC to estimate the activity of NCC in the kidney. In this respect, it was fortunate for us that the ELISA for pNCC showed a higher sensitivity than the tNCC ELISA.
which might be due to the high sensitivity of anti-p-T55 antibody used for capturing pNCC.

Correlation between immunoblots and sandwich ELISA. We examined the correlation between sandwich ELISA and NCC immunoblot, which has been used to compare the relative abundance of urinary NCC proteins (12, 14, 27). Spot urine samples were collected from 13 healthy volunteers. After the preparation of exosomes, the samples were divided into sandwich ELISA and immunoblot samples. The immunoblot signals of tNCC and pNCC (150 kDa) were obtained by the capture antibodies that were used in ELISA and were analyzed by densitometry. The amounts of tNCC and pNCC measured by the sandwich ELISAs were highly correlated with the immunoblot data ($r = 0.94$, $r = 0.97$, respectively; Fig. 4), validating that the ELISA assays could measure the amounts of full-length NCC and pNCC although the epitopes of capture and detection antibodies were relatively closely located. The very close correlation of pNCC ELISA and its immunoblot data also verified the phosphospecificity of pNCC ELISA in urine samples.

Urinary exosomal NCC can be normalized with urinary Cr. To evaluate the amount of urinary tNCC and pNCC in spot urine samples, we investigated a method to correct the amount of tNCC and pNCC in spot urine samples from a single subject. Normalization of urinary biomarkers is an unresolved issue. Fernández-Llama (7) previously reported that exosomal proteins can be normalized by THP. Likewise, urinary exosome markers, such as TSG101, Alix, or Hsp70 (7, 23, 33), may be used for the normalization of urinary exosomal proteins. However, it is unknown if these proteins are constant under various conditions.

Table 1. Urinary excretion of total and phosphorylated NCC in Gitelman’s syndrome

<table>
<thead>
<tr>
<th>Mutation found in NCC gene</th>
<th>L849H (hetero)</th>
<th>V578M (hetero)</th>
<th>delTT1 (hetero)</th>
<th>V578M (hetero)</th>
<th>delTT1 (hetero)</th>
<th>T180K (homo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NCC, fmol/mg Cr</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Phosphorylated NCC, fmol/mg Cr</td>
<td>0.30</td>
<td>ND</td>
<td>ND</td>
<td>2.51</td>
<td>ND</td>
<td>1.2</td>
</tr>
</tbody>
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NCC, Na-Cl cotransporter; homo, homozygous mutation; hetero, heterozygous mutation; Cr, creatinine; ND, not detected. 12-bp (TT) deletion at 2543–2544.
also discussed that normalization by urine Cr is currently the best option for clinical study since normalization of urine flow rate is theoretically the best but rarely practical. Therefore, we tested urinary Cr for normalization in our assays. We measured pNCC in five different spot urine samples within a day from three different subjects. The pNCC concentrations corrected by the Cr level were almost equal among the five spot urine samples (Fig. 5) from the same subject. This result suggests that a single spot urine sample can be used to estimate the total excretion of pNCC for 24 h.

Measurements of tNCC and pNCC in patients with PHAII and Gitelman’s syndrome. To evaluate whether the activity of NCC in the kidney would reflect the excretion of tNCC and pNCC in urinary exosomes, we first collected urine samples from two PHAII families and measured the excretion of tNCC and pNCC in urinary exosomes since NCC is constitutively activated in PHAII. Very recently, we reported that WNK4 is a target of Cullin3-KLHL3 E3 ligase and that the impaired ubiquitination and subsequently increased level of WNK4 in the kidney could activate downstream OSR1/SPAK-NCC signaling, which is the major common pathogenesis of PHAII by mutations in three different genes (29). In family A, the patient carried a novel de novo mutation in the KLHL3 gene

Table 2. Main characteristics of participants

<table>
<thead>
<tr>
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<th>Total NCC (n = 39)</th>
<th>Phosphorylated NCC (n = 61)</th>
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<tr>
<td>Age, yr</td>
<td>65.0 ± 14.7</td>
<td>63.5 ± 14.6</td>
</tr>
<tr>
<td>Male, %</td>
<td>25 (64)</td>
<td>39 (64)</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>8 (21)</td>
<td>11 (18)</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>29 (75)</td>
<td>47 (77)</td>
</tr>
<tr>
<td>eGFR, ml/min·1.73 m⁻²</td>
<td>49.7 ± 17.9</td>
<td>50.3 ± 18.1</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>124.9 ± 16.0</td>
<td>126.3 ± 17.7</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>75.4 ± 11.3</td>
<td>76.0 ± 11.9</td>
</tr>
<tr>
<td>Serum albumin, mg/dl</td>
<td>4.3 ± 0.3</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Serum sodium, mEq/l</td>
<td>140.8 ± 2.0</td>
<td>140.3 ± 1.9</td>
</tr>
<tr>
<td>Serum potassium, mEq/l</td>
<td>4.4 ± 0.3</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>Serum chloride, mEq/l</td>
<td>106.0 ± 2.6</td>
<td>106.0 ± 2.7</td>
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Values are means ± SE. eGFR, estimated glomerular filtration rate; BP, blood pressure.

pathophysiological conditions, and their quantitative measurements are still not easy for a large number of clinical samples. Although normalization of urinary biomarkers to urine Cr has been recognized to be misleading especially when GFR is changing (28), most clinical urine sample measurements are usually corrected by urine Cr. Zhou et al. (33) reported that specific exosome-associated proteins normalized by Cr are almost equal within samples from the same person, and they
(heterozygous T386A; Fig. 6A). Since this mutation is located within the hot spot of PHAII mutations in the kelch domain (1), and there was genotype-phenotype correlation within the family, we regarded this mutation as a PHAII-causing mutation (a case report is in preparation). In family B, a heterozygous novel de novo mutation in intron 8 of the CUL3 gene [splice acceptor (-6) T > G] was identified in the patient (Fig. 6B). The skipping of exon 9, postulated as a consequence of the CUL3 gene mutations (1), was confirmed in the patient’s white blood cells by RT-PCR (21). As shown in Fig. 6, A and B, the excretion of tNCC and pNCC in urinary exosomes was robustly increased in the family members who carried each mutation in the two families.

We also confirmed this finding in our PHAII model mice (Wnk4\textsuperscript{D561A/+} knockin mice) in which NCC is constitutively phosphorylated and activated. We only measured urinary pNCC in the PHAII model mice since the guinea pig antibody used in the sandwich ELISA for tNCC only recognizes human NCC. As shown in Fig. 6C, the level of pNCC was highly increased in the urine of the PHAII model mice.

Furthermore, we corrected urine samples from four Gitelman syndrome patients (16) and measured the excretion of tNCC and pNCC (Table 1). The loss-of-function of NCC is the cause of Gitelman’s syndrome (25), and there was already preliminary data that the urinary excretion of NCC was significantly reduced (12). In all of the patients that we tested, the level of tNCC was under the detection limit, and pNCC levels were under the detection limit in two patients and low in the other two patients (0.30 and 2.51 fmol/mg Cr, respectively) compared with the mean values in outpatients with eGFR \( 60 \text{ ml·min·1.73 m}^2 \) (8.1 ± 5.4 fmol/mg Cr; \( n = 13 \)).

These data in PHAII and Gitelman’s syndrome patients indicate that urinary excretion of tNCC and pNCC reflects NCC activity in the kidney and that our ELISA assays are detecting NCC specifically. Phosphorylation of NCC in the kidney is now widely recognized as an excellent marker to reflect the in vivo activity of NCC (8). However, it is poorly understood at present how the pNCC localized to the apical plasma membranes of the distal tubules is excreted in urine, especially into the exosomes. Nonetheless, previous studies (12, 17, 27) and the present study clearly suggest that tNCC and pNCC in the urine may reflect the amounts in the kidney.

**Analysis of tNCC and pNCC in urinary exosomes in outpatients.** Since the initial purpose of developing ELISA assays for urinary NCC was to establish assays to predict thiazide sensitivity as well as to help to diagnose PHAII and Gitelman’s syndrome, we sought to gather more data on urinary NCC excretion not only in the specific genetic diseases but also in a more general population of patients with different clinical backgrounds. We measured urinary tNCC and pNCC in 93 outpatients from the Nephrology Department of Tokyo Medical and Dental University. Most of these outpatients had chronic kidney diseases. The 32 patients with proteinuria were excluded since urinary protein levels >30 mg/dL interfered with our ELISA. We measured urinary tNCC and pNCC in 61 patients. We could detect urinary pNCC in all 61 patients, but the measurement of tNCC was successful in only 39 patients, probably due to the lower sensitivity of the tNCC assay. Patient characteristics are shown in Table 2. The participants were predominantly male (64% in both groups) with a mean (±SD) age of 65.0 ± 14.7 yr in the tNCC group and 63.5 ± 14.6 yr in the pNCC group. The overall mean eGFR was 49.7 ± 17.9 ml/min·1.73 m\(^2\) in the tNCC group and 50.3 ± 18.1 ml/min·1.73 m\(^2\) in pNCC group. Eight (21%) and eleven (18%) patients in the tNCC and pNCC groups had diabetes mellitus, respectively. Twenty-nine (75%) patients in the tNCC group and forty-seven (77%) patients in the pNCC group had hypertension.

We found interesting correlations between the clinical data and the excretion of NCC in urinary exosomes (Fig. 7). We found that excretion of tNCC and pNCC in urinary exosomes was positively correlated with eGFR (\( r = 0.36, P < 0.05; r = 0.56, P < 0.0001 \), respectively; Fig. 7A). This decrease of tNCC and pNCC excretion may come from the decreased number of nephrons. Thus analyses of nephron segment-specific proteins in urine exosomes would be new tools to evaluate kidney function from viewpoints other than GFR. We also found that the excretion of tNCC and pNCC in urinary exosomes negatively correlates with the FENa (\( r = -0.47, P < 0.01, r = -0.62, P < 0.001 \), respectively; Fig. 7B). The correlation was still evident when the analysis was limited in the patients with similar eGFR (40–60 ml/min·1.73 m\(^2\); Fig. 7C). Further studies are necessary to elucidate the significance of these correlations. Systolic BP, diastolic BP, serum albumin, serum sodium, serum potassium, and serum chloride were not correlated with tNCC or pNCC excretion (data not shown).

In summary, we developed sandwich ELISAs for tNCC and pNCC in urinary exosomes. This assay enabled us to estimate the activity of NCC in the human kidney and may be useful not only for the diagnosis of PHAII and Gitelman syndrome but also for predicting thiazide sensitivity in hypertensive patients with normal GFR in the future.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


