Reduced urinary corin levels in patients with chronic kidney disease

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Abstract
Corin is a cardiac protease that regulates BP (blood pressure) by activating natriuretic peptides. Recent animal studies identified corin expression in the kidney where it may regulate renal function. In the present study, we tested the hypothesis that corin may be present in human urine and that urinary corin levels may be altered in patients with kidney disease. We obtained urine and kidney tissue samples from normal individuals and CKD (chronic kidney disease) patients. Using ELISA, we detected corin protein in human urine. In normal individuals, urinary corin levels did not correlate with that of plasma, indicating that urinary corin is probably of kidney origin. Compared with normal controls, CKD patients had markedly reduced urinary corin levels and this reduction correlated with disease severity. By immunostaining, human corin protein was identified on the epithelial cell surface in renal tubules. The renal corin mRNA and protein levels were significantly lower in CKD patients than non-CKD controls. The results indicate that renal tubular corin may be shed into urine and that urinary and renal corin levels were reduced in CKD patients. These data suggest that reduced corin levels in the kidney may reflect the underlying pathology in CKD.

Key words: atrial natriuretic peptide, chronic kidney disease (CKD), corin, protease, urine

INTRODUCTION
ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide) are evolutionarily conserved hormones that are essential for maintaining normal BV (blood volume) and electrolyte homeostasis [1,2]. Corin is a proteolytic enzyme discovered in the heart [3]. Corin regulates BP (blood pressure) and cardiac function by activating the natriuretic peptides [4,5]. In mice, corin deficiency led to spontaneous hypertension that was exacerbated by dietary salt loading [6,7]. In humans, gene variants that impaired corin function were reported in African Americans who had hypertension and cardiac hypertrophy [8–11]. Most recent studies also suggested that corin may have a local function in the pregnant uterus to promote spiral artery remodelling and prevent hypertension during pregnancy [12].

Structurally, corin is a type II transmembrane protein that contains a single-span transmembrane domain near the N-terminus, which anchors corin on the cell surface and restricts corin activity at the site of expression [13–15]. Such a membrane-anchoring mechanism is also employed in other type II transmembrane serine proteases such as enteropeptidase, hepsin and matriptases [16–21]. Recently, corin was found to be shed from the cardiomyocyte surface by metalloproteinase-mediated hydrolysis and corin autocleavage [22]. Apparently, shed corin molecules could enter the circulation. Soluble corin was detected in human blood [23–26]. In patients with heart failure, low plasma corin levels were associated with severe heart failure and poor clinical outcomes [27–29].

Corin is expressed primarily in cardiomyocytes [30–32]. Low levels of corin mRNA were reported in other tissues, including the kidney, bone, skin and brain [3,25,33–35]. Corin mRNA was also detected in human lung and uterus cancers [3,36]. In the kidney, corin expression was located in the proximal tubule, thick ascending limb, connecting tubule and collecting duct [35].

Abbreviations: BP, blood pressure; CGN, chronic glomerulonephritis; CKD, chronic kidney disease; CVD, cardiovascular disease; DBP, diastolic BP; GFR, glomerular filtration rate; NS, nephrotic syndrome; qRT-PCR, quantitative real-time PCR; SBR, systolic BP; SCR, serum creatinine; SLE, systemic lupus erythematosus.

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Polzin et al. [35] have reported that renal corin expression was significantly reduced in rat models of kidney disease, which may contribute to sodium retention in those animals [37]. The results suggest that corin may have a local function in the kidney in regulating sodium excretion. Consistent with this, impaired sodium excretion and salt-sensitive hypertension were found in corin knockout mice [7].

If corin is shed in the heart, a similar shedding process may occur in the kidney. In the present study, we tested the hypothesis that corin protein may be present in human urine. We also investigated the possibility that urinary and renal corin levels may be altered in patients with CKD (chronic kidney disease).

**MATERIALS AND METHODS**

**Patient samples**

The present study was approved by the ethics committee at the First Affiliated Hospital of Soochow University in Suzhou, People’s Republic of China. Plasma and urine samples were collected from 214 patients with CKD and 205 normal controls who underwent a routine health check-up and had no history of CVD (cardiovascular disease) or renal disease. All participants gave written informed consent. The diagnosis of CKD was based on the guidelines of the American National Kidney Foundation [38,39]. Patients with previous history of cancer and heart failure were excluded. Drugs taken by CKD patients included antihypertensives [ACEi (angiotensin-converting enzyme inhibitors), angiotensin receptor blockers and calcium channel blockers], cholesterol-lowering drugs (statins) and anticoagulants (low-molecular-mass heparin). Renal function in CKD patients was estimated by GFR (glomerular filtration rate) based on the Cockcroft–Gault formula: GFR (ml/min/1.73 m²) = ([140–age (year)]×weight (kg)×[SCR (serum creatinine) (mg/dl)]×0.85 if female. CKD stage classification was based on patients’ GFR according to the K/DOQI (Kidney Disease Outcomes Quality Initiative) guidelines [38,39]. Patients with GFR of ≥ 90, 60–90, 30–60, 15–30 and <15 ml/min per 1.73 m² were classified as CKD stages 1–5 respectively. Characteristics of normal controls and CKD patients are shown in Table 1.

**Measurement of plasma and urinary corin**

Venous blood samples were collected into tubes containing EDTA as an anticoagulant. Plasma was separated by centrifugation at 4 °C and stored at −80 °C until use. Spot and 24 h urine samples were collected into tubes without anticoagulant or protease inhibitors at room temperature, divided into aliquots, and stored at −80 °C until use. Corin protein in plasma or urine samples was quantified by Image Pro-Plus software (version 6.0; Media Cybernetics). For each section, at least three randomly selected fields under ×400 magnification were inspected under a light microscope (Olympus). The intensity of corin expression in these fields was quantified by Image Pro-Plus software (version 6.0; Media Cybernetics).

<table>
<thead>
<tr>
<th>Table 1 Characteristics of normal controls and CKD patients</th>
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<tbody>
<tr>
<td>*<em>Values are means ± S.D. or numbers (percentage). Age and sex distributions were not statistically different between the control and CKD patients. Urine tests were negative for proteinuria; 24 h urinary proteins were not determined (N/D). <em>P &lt; 0.001 compared with control.</em></em></td>
</tr>
<tr>
<td><strong>Characteristic</strong></td>
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<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Sex (n)</td>
</tr>
<tr>
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</tr>
<tr>
<td>Female</td>
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<tr>
<td>Medical history (n)</td>
</tr>
<tr>
<td>CGNs</td>
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<tr>
<td>NS</td>
</tr>
<tr>
<td>SLE</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
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<td>DBP (mmHg)</td>
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<td>GFR (ml/min/1.73 m²)</td>
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<tr>
<td>Antihypertensive drugs</td>
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<tr>
<td>Cholesterol-lowering drugs</td>
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<td>Anticoagulants</td>
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</table>

**Immunohistochemistry**

Kidney biopsies were obtained from CKD patients (n = 40), who received no steroids or immunosuppressants. Non-CKD control kidneys were from trauma patients who underwent surgery (n = 6). These non-CKD donors had no histories of CVD or renal disease, were not on anti-hypertensives or anti-coagulants, and had normal GFR. Tissues were fixed with 4 % (v/v) formaldehyde and embedded in paraffin. Sections of 4 μm in thickness were made and treated with xylene followed by rehydration in water solutions with gradually decreased concentrations of ethanol. Sections were incubated with a polyclonal anti-corin antibody (Berlex) followed by a secondary antibody conjugated with HRP (horseradish peroxidase) and counterstained with haematoxylin. Corin protein was visualized using a substrate solution containing 3,3’-DAB (3,3’-diaminobenzidine). For immunofluorescent staining, a second antibody conjugated with Alexa Fluor® 488 (green) (Invitrogen) was used. Tissue sections were mounted with DAPI (4’,6-diamidino-2-phenylindole)-containing media. In these experiments, no histological abnormalities were observed in non-CKD control kidneys. For immunostaining, negative controls were treated similarly but without the primary antibody. For each section, at least three randomly selected fields under ×400 magnification were inspected under a light microscope (Olympus). The intensity of corin expression in these fields was quantified by Image Pro-Plus software (version 6.0; Media Cybernetics).

**qRT-PCR (quantitative real-time PCR) analysis**

To examine corin mRNA expression in the kidney, biopsy samples from non-CKD controls (n = 6) and CKD patients (n = 10) were obtained. Total RNAs were isolated using RNeasy
kit (Qiagen) to make cDNAs using the RevertAid First Strand cDNA Synthesis kit (Fermentas) [40]. Corin mRNA expression was analysed by qRT-PCR using SYBR Green Master Mix (Applied Biosystems) in the PRISM 7500 Sequence Detection System (Applied Biosystems). The corin primers used were: 5\'-GCAAGCAGATGGTTAGGA-3' (sense) and 5\'-CCAGTTGGAGTGTAATGTCAGC-3' (antisense). In these experiments, samples were assayed in triplicate, and \( \beta \)-actin mRNA levels were used as an internal control. The relative levels of corin mRNA were presented as fold changes compared with the control.

**Statistical analysis**

Statistical analysis was done using the SPSS software. All data are presented as means \( \pm \) S.D. A Student’s \( t \) test or \( \chi^2 \) test was used to examine differences between the two groups. Comparisons among three or more groups were made using one-way ANOVA followed by LSD (least significant difference) post-hoc analysis. Multiple linear regression analysis was done to examine independent predictors for urinary and plasma corin levels in CKD patients. The correlation between plasma and urine corin levels was analysed using Pearson’s correlation coefficient test. A \( P \) value of \(<0.05 \) was considered to be statistically significant.

**RESULTS**

**Urinary corin levels in normal individuals**

By ELISA, we detected corin protein in human urine. In a pilot study with 30 individuals, corin levels were found similar in spot or 24 h urine samples \((143 \pm 29 \text{ compared with } 134 \pm 35 \text{ pg/ml; } P = 0.274)\). In these individuals, no significant correlation was found between urinary corin and urinary creatinine levels \((R^2 = 0.097, P = 0.073)\). Spot urine samples were used in subsequent studies. In samples from 205 normal individuals, corin levels were \(194 \pm 115 \text{ pg/ml} \). The levels in males \((227 \pm 107 \text{ pg/ml, } n = 101) \) were significantly higher than that in females \((161 \pm 108 \text{ pg/ml, } n = 104; P = 0.00003)\) (Figure 1A). In both males and females, urinary corin levels appeared similar in different age groups (Figures 1B and 1C).

**Correlation between urinary and plasma corin levels**

Plasma soluble corin has been detected in normal individuals and heart failure patients [23–28]. To understand the relationship between urinary and plasma corin levels in normal individuals
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Figure 2  Urinary corin levels in CKD patients
Data were from 101 normal males and 103 male patients (A), and 104 normal females and 111 female patients (B). Urinary corin levels in male and female patients with stages 1–5 CKD are shown in (C) and (D) respectively. *P < 0.05 and **P < 0.01 compared with the indicated groups.

Table 2  Multiple linear regression to predict urinary corin levels in CKD patients
The response variables were analysed from square root-transformed data. R² = 0.344. The coding for dummy variables was as follows: sex (male, 0; female, 1); chronic glomerulonephritis, nephrotic syndrome and SLE (yes, 1; no, 0); antihypertensive drugs, cholesterol-lowering drugs, anticoagulant drugs (yes, 1; no, 0).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>S.E.</th>
<th>t</th>
<th>P</th>
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<td>0.574</td>
<td>0.566</td>
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<tr>
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<td>&lt;0.001</td>
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<td>3.517</td>
<td>0.001</td>
</tr>
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<td>SBP (mmHg)</td>
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<td>DBP (mmHg)</td>
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<td>0.045</td>
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<td>GFR (ml/min per 1.73 m²)</td>
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<td>2.675</td>
<td>0.008</td>
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<td>SCR (μmol/l)</td>
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<td>0.530</td>
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<td>Anticoagulants</td>
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<td>30.589</td>
<td>−0.442</td>
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</tbody>
</table>

and CKD patients, we measured urinary and plasma corin levels in 134 normal controls and 165 CKD patients, from whom both urinary and plasma samples were available. In normal controls, no correlation was found between plasma and urinary corin levels (r = 0.004, P = 0.96) (Figure 4A). In CKD patients, urinary corin levels appeared positively correlated with plasma corin levels (r = 0.281, P < 0.001) (Figure 4B). We also performed multiple linear-regression analysis to identify variables that may independently predict plasma corin levels in CKD patients. The results indicated that gender (P = 0.012), proteinuria (P = 0.011) and SCR (P = 0.014) were independent predictors for plasma corin levels (Table 3).

Corin expression in kidney tissues
Previous studies showed that corin is expressed in renal tubules in rats [35]. We found similar results by immunostaining in human kidney biopsy samples. In sections from a non-CKD patient, strong corin staining was identified on the surface of epithelial cells in renal tubules (Figure 5A). Such staining was mostly absent in glomeruli (Figure 5A). In negative controls, in which the primary corin antibody was omitted, no positive corin staining was identified (results not shown). By qRT-PCR, corin mRNA expression was confirmed in human kidney biopsy samples. The level of corin mRNA was significantly lower in CKD patients than non-CKD controls (Figure 5B). When kidney corin mRNA
levels and urinary corin protein levels from the same individuals were examined, there appeared a weak correlation between the mRNA and the urinary protein levels (Figure 5C).

We did further experiments by immunohistochemistry. The results showed weaker corin staining in renal tubules in CKD patients than non-CKD controls in additional biopsy samples (Figure 6A). As a negative control, stomach sections had no positive brown corin staining (Figure 6B). By Image-Pro software analysis, corin staining (relative absorbance) was significantly weaker in samples from CKD patients with CGN (n = 21; P < 0.01), NS (n = 11; P < 0.01) or SLE (n = 8; P < 0.01), when compared with that of non-CKD controls (n = 6) (Figure 6C).

In these immunostaining experiments, no obvious gender difference in corin expression levels was observed in CKD patients and non-CKD controls.

**DISCUSSION**

Corin was identified as a cardiac enzyme [41]. Subsequent studies indicated that low levels of corin mRNA and protein were present in other tissues. Previously, Polzin et al. [35] have shown that corin expression in the kidney may play an important role in
Figure 5  Immunostaining of corin protein and corin mRNA expression in kidney tissues
(A) Kidney sections from non-CKD controls were stained with H&E (haematoxylin and eosin) (top panels) or immunostained for corin protein (lower panels). Corin-positive staining (green) was found on the surface of tubular epithelial cells but not in the glomerulus (red dashed circle). Scale bar, 20 μm. (B) qRT-PCR analysis of corin mRNA expression in kidney tissues from non-CKD controls (n = 6) and CKD patients (n = 10). Results are means ± S.D. (C) Correlation of renal corin mRNA levels, measured by qRT-PCR, and urinary corin protein levels, measured by ELISA.

Figure 6  Immunostaining of corin protein in kidney biopsies
(A) Kidney sections from non-CKD controls (control) or CKD patients with CGN, NS or SLE were stained for corin protein by IHC (immunohistochemistry). (B) Stomach sections stained with H&E (left-hand panel) or IHC (right-hand panel) were used as a negative control. (C) Quantitative data (means ± S.D.) were obtained by Image-Pro software analysis of stained sections from non-CKD controls (n = 6) and CKD patients with CGN (n = 21), NS (n = 11) or SLE (n = 8). **P < 0.001 compared with non-CKD control. Scale bar, 30 μm.
levels were reported previously in individuals of different age groups. In the present study, we tested the hypothesis that corin protein in the kidney may undergo proteolytic shedding and that corin may be present in urine. By ELISA, we detected soluble corin in human urine samples. Most likely, the primary source of urinary corin was the kidney, but not heart or plasma, because in normal individuals there was no direct correlation between urinary and plasma corin levels (Figure 4A). Interestingly, urinary corin levels were significantly higher in males than females (Figure 1A). A similar gender difference was found previously for plasma corin levels [23,25,27], although the underlying mechanism for such a gender difference remains unclear. Male hormones have been reported to promote the shedding of other membrane proteases [19,42]. Additional studies are needed to determine whether male hormones also stimulate corin shedding from the cell surface. Among the normal individuals, urinary corin levels appeared similar in different age groups (Figures 1B and 1C). Consistently, age was not an independent predictor in the multiple linear regression analysis (Table 2). Similar plasma corin levels were reported previously in individuals of different age groups [24]. More recently, higher levels of plasma corin were reported in older (>60 years) individuals [25]. Further studies are required to determine if plasma and urinary corin levels vary with age when larger sizes of samples are included.

An important finding of the present study is that urinary corin levels were significantly lower in CKD patients than normal controls. The reduction appeared to correlate with the disease severity, as progressively lower levels of urinary corin were found in CKD patients at more advanced disease stages (Figure 2). We also found that CKD patients with hypertension had lower levels of urinary corin than those in normal controls or CKD patients without hypertension (Figure 3). Consistent with these findings, multiple linear-regression analysis identified CGN, NS, SLE, GFR and DBP as strong independent predictors for urinary corin levels (Table 2). The analysis also indicated gender as an independent predictor (Table 2), which was consistent with the experimental data (Figure 1A).

By immunostaining, we detected human corin protein on the surface of epithelial cells of renal tubules (Figure 5A). The finding was similar to the report by Polzin et al. [35], which showed strong corin protein staining in rat renal tubules. Compared with non-CKD controls, CKD patients had weaker corin staining in their kidney biopsies (Figures 6A and 6C). Low levels of corin mRNA expression also were found in kidney samples from CKD patients (Figure 5B). These results suggest that low corin expression may reflect the underlying pathology in CKD. The results also indicate that low levels of renal corin expression may account for the low urinary corin levels in CKD patients. At this time, the mechanism responsible for the reduced renal corin expression in CKD patients remains unclear. Probably, inflammatory responses that often exist in CKD patients may suppress renal corin expression [43]. Unlike in normal individuals, there was a weak but significant correlation between urinary and plasma corin levels in CKD patients (Figure 4B). It is possible that, in CKD patients, inflammatory reactions may damage vessel walls and increase the glomerular permeability, allowing plasma corin molecules to enter into the urine.

Currently, the corin function in the kidney is not completely understood. Studies in rat models of kidney disease and corin knockout mice show that corin is important for maintaining normal renal function and sodium homeostasis [7,35]. In the present study, we report for the first time the presence of corin protein in human urine. More importantly, we found reduced urinary and renal corin levels in CKD patients. These findings suggest that corin deficiency may be part of the pathology in CKD. Our results should encourage additional studies to determine the role of corin in human kidney disease.

**CLINICAL PERSPECTIVES**

- The membrane protease corin regulates sodium excretion and BP by activating natriuretic peptides.
- In the present paper, we report that corin is present in human urine, and that urinary and renal corin levels were markedly reduced in CKD patients.
- The results indicate that reduced corin expression in the kidney may represent an underlying pathological change in CKD patients.

**AUTHOR CONTRIBUTION**

Chaodong Fang performed the experiments, analysed data and wrote the paper. Lei Shen collected the patient samples, analysed the medical data and wrote the paper. Liang Dong, Meng Liu and Sensen Shi performed the experiments; Ningzheng Dong and Qingyu Wu designed the experiments, analysed data and wrote the paper.

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