Urinary angiotensinogen reflects the activity of intrarenal renin–angiotensin system in patients with IgA nephropathy

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Abstract

Background. A potential contribution of local activation of the renin–angiotensin system (RAS) to the pathogenesis of renal injury has been indicated by evidence for blood pressure-independent renoprotective effects of angiotensin II (AngII) receptor blockers (ARBs). The present study was performed to test the hypothesis that urinary angiotensinogen provides a specific index of intrarenal RAS status in patients with immunoglobulin A (IgA) nephropathy.

Methods. This paper is a survey of urine specimens from three groups: healthy volunteers, patients with IgA nephropathy and patients with minor glomerular abnormality (MGA). Patients with hypertension, diabetes, reduced glomerular filtration rate and/or who were under any medication were excluded from this study. Urinary angiotensinogen levels were measured by a sandwich enzyme-linked immunosorbent assay system.

Results. Urinary angiotensinogen levels were not different between healthy volunteers and patients with MGA. However, urinary angiotensinogen expression and AngII immunoreactivity were significantly higher in patients with IgA nephropathy than in patients with MGA. Baseline urinary angiotensinogen levels were positively correlated with renal angiotensinogen gene expression and AngII immunoreactivity but not with plasma renin activity or the urinary protein excretion rate. In patients with IgA nephropathy, treatment with an ARB, valsartan (40 mg/day), significantly increased renal plasma flow and decreased filtration fraction, which were associated with reductions in urinary angiotensinogen levels.

Conclusion. These data indicate that urinary angiotensinogen is a powerful tool for determining intrarenal RAS status and associated renal derangement in patients with IgA nephropathy.

Keywords: angiotensin II; angiotensinogen; IgA nephropathy; urinary biomarker; valsartan

Introduction

In recent years, the role of the renin–angiotensin system (RAS) in the pathophysiology of renal injury has received much attention with emphasis on the activity of the local RAS in the kidney. Treatment with angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II (AngII) type I receptor blockers (ARBs) elicits blood pressure-independent renoprotective effects in both hypertensive and normotensive chronic kidney disease (CKD) patients [1–5]. Therefore, most national guideline groups have recommended the use of ARBs or ACEIs in preference to other antihypertensive agents for hypertensive patients with diabetic nephropathy [6–9]. Experimental studies have also revealed that intrarenal AngII is regulated in a manner distinct from circulating AngII concentrations [10,11] and that inappropriately elevated intrarenal AngII levels lead to renal functional derangement and tissue injury [11]. In the kidney, AngII is derived from its locally formed substrate, angiotensinogen [10,12]. Experimental studies have demonstrated that angiotensinogen levels in renal tissues reflect the activity of intrarenal RAS [10–13]. We have also demonstrated a significant relationship between the urinary excretion rate of angiotensinogen and the intrarenal production of AngII in several animal models of renal injury [11,14,15]. Recently, Yamamoto et al. [16] used radioimmunoassay to measure angiotensin I that is generated after incubation of samples with excess recombinant renin and showed that urinary angiotensinogen levels are increased in patients with CKD. However, this method is impractical for clinical examinations and diagnoses because
of its complicated procedures. Furthermore, there is no direct evidence that urinary angiotensinogen actually reflects the angiotensinogen originating from the kidney.

Most recently, we have developed a sandwich enzyme-linked immunosorbent assay (ELISA) system to directly measure human angiotensinogen [17]. In the present study, we tested the hypothesis that an easy measurable urine-based test for angiotensinogen using this new ELISA kit should provide a simple and noninvasive diagnostic test to identify patients with activated intrarenal RAS. To test this hypothesis, we proposed exploratory studies in normotensive patients with moderately proteinuric immunoglobulin A (IgA) nephropathy and normal glomerular filtration rate for the following reasons: (i) IgA nephropathy is a common primary glomerulonephritis and is a major cause of end-stage renal disease [18]; (ii) treatment with ACEIs or ARBs is successful in mitigating IgA nephropathy, independent of blood pressure changes [19–21]; (iii) we recently demonstrated that angiotensinogen levels in renal tissues are increased in mice [22] and patients [23,24] with IgA nephropathy.

Materials and methods

Participants and protocols

Between January 2006 and December 2007, 52 patients were diagnosed with IgA nephropathy by clinical course and renal biopsy in accordance with clinical guidelines for immunoglobulin A nephropathy in Japan, second version [25], at Osaka City General Hospital. Detailed information is available as Online Data Supplements. Patients with hypertension (systolic blood pressure >140/90 mmHg), diabetes (fasting blood glucose >7.8 mmol/L or random glucose >11.1 mmol/L), reduced creatinine (Cr) clearance (<60 mL/min), hepatic diseases, infections or malignancies were excluded from this study. Patients who were taking any medication, had previously received ACEIs or ARBs and who had previously had any cardiovascular events were also excluded. Finally, 11 hospitalized patients with IgA nephropathy were recruited for the present study. Applying the exclusion criteria described above, we also recruited, from Osaka City General Hospital between January 2006 and December 2007, four hospitalized patients with minor glomerular abnormality (MGA) including two with minimal change disease who had intermittent or persistent proteinuria. In this study, urine samples were also collected from 14 age-matched healthy volunteers who had not been taking any medication and had provided written informed consent between May and November 2007.

Sample collection

We recently demonstrated that the daily urinary angiotensinogen excretion rate is highly correlated with the ratio of urinary angiotensinogen concentration to urinary Cr concentration (U_{AGT}/U_{Cr}) in humans [17]. Therefore, spot urine samples were collected to analyse U_{AGT}/U_{Cr}. Within a few days before each renal biopsy, second-morning urine samples were collected from the patients with IgA nephropathy and MGA. Blood samples were also collected into EDTA-containing tubes for both groups of patients. In all patients with IgA nephropathy, urine and blood samples were also collected 2–8 weeks after the administration of valsartan at 40 mg/day. Prednisolone was co-administered (at 1 mg/kg for the first 2 weeks and was reduced by 5 mg/day every 2 weeks thereafter) in 4 of the 11 patients with IgA nephropathy. These four patients had diffuse mesangial cell proliferation and increased matrix in more than 80% of the biopsied glomeruli or crescent formation or adhesion to Bowman’s capsule with acute inflammatory changes in more than 10% of the biopsied glomeruli.

Urine angiotensinogen, immunohistochemistry of AngII and gene expression of angiotensinogen

Urinary angiotensinogen levels were measured using a novel sandwich ELISA system, as previously described [17]. Detailed information is available as Online Data Supplements. As mentioned above, renal biopsy was performed in all patients for diagnosis. In 4 of the 11 patients with IgA nephropathy, renal biopsy was also performed after treatment with valsartan. Renal biopsy specimens were fixed with 10% formalin (pH 7.4), embedded in paraffin, sectioned into 3-µm slices and stained with periodic acid–Schiff or periodic acid–Schiff–methenamine silver reagent to evaluate the index scores of glomerular sclerosis, as previously described [26,27]. In all sections, immunohistochemistry for AngII was performed by a robotic system (Dako, Autostainer) to apply exactly the same condition on all slides, and sections were counter-stained with haematoxylin–eosin, as previously described [23,24]. The primary antibody against AngII was purchased from Phoenix Pharmaceuticals (6H-002-12), and the concentration for immunohistochemistry was 1:3000 [23,24].

Using real-time PCR with a Laser Capture Microdissection, we measured mRNA expression of angiotensinogen in the biopsy samples [28,29]. Detailed information is available as Online Data Supplements. We previously demonstrated that, in the kidney, angiotensinogen is predominantly expressed in proximal tubular cells [10–15]. Therefore, we dissected cortical tubulointerstitial tissues from the frozen tissue sections using an Olympus LM200 system (Olympus, Tokyo).

Blood pressure and other parameters

Systemic blood pressure was measured three consecutive times using a Hawksley random zero sphygmomanometer after the patients had rested for at least 15 min; the mean of the lowest two readings was recorded. Blood pressure was measured three times a day in a supine position, and the means of values obtained 3 days before and 3 days after the start of treatment were recorded. Detailed information for methods of other parameters is available as Online Data Supplements.

Statistical analyses

The number of enrolled subjects who met the eligibility criteria was 29. The data are expressed as mean ± SD. Logarithmic transformation was performed for variables that did not exhibit normal distribution. Transformed variables were used only in the statistical analysis, while the original values were used for presentation. Paired t-tests were used for comparisons of two dependent points and unpaired t-tests for differences between two groups. When the number of groups to be compared exceeded two, one-way analysis of variance (ANOVA) with Newman–Keuls’ test was used for the comparison of the mean value among the groups. Time-dependent data were analysed by repeated measures ANOVA to test whether group and time had significant effects on the measured variables. When a significant F ratio was obtained, differences between the groups were isolated using the post hoc Newman–Keuls multiple comparisons test. The relationship between the gene expression of angiotensinogen/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U_{AGT}/U_{Cr} was assessed using linear regression analysis. Multiple regression was used to detect U_{AGT}/U_{Cr} and the relevancy of each parameter. P-values < 0.05 (two-tailed) or less were considered statistically significant. The present exploratory study does not include a calculation of sample size.

Results

Baseline participant profiles

Baseline characteristics, including gender, age, body mass index, SBP and DBP were not different among healthy volunteers, the patients with MGA and the patients with IgA nephropathy. Plasma renin activity (PRA), aldosterone and C-reactive protein levels were also not different between the patients with MGA and those with IgA nephropathy (Table 1). Basal renal parameters, including serum blood urea nitrogen (BUN) and Cr and urinary protein excretion rate (U_pV) were not different between the patients with MGA and those with IgA nephropathy (Table 1). The index score for glomerular sclerosis of patients with IgA nephropathy was 105 ± 76, whereas patients with MGA showed normal glomeruli (index score 0 ± 0).
Basal urinary angiotensinogen, renal tissue angiotensinogen gene expression and AngII immunoreactivity

Figure 1 shows the urinary angiotensinogen levels in healthy volunteers and patients with MGA and IgA nephropathy. Baseline $U_{AGT}/U_{Cr}$ in healthy volunteers averaged 10 ± 4 µg/g, which was not significantly different from that of patients with MGA (13 ± 6 µg/g). However, IgA nephropathy patients showed markedly elevated $U_{AGT}/U_{Cr}$ (39 ± 31 µg/g). Similarly, basal mRNA levels of angiotensinogen were 2.4 ± 1.6-fold higher in patients with IgA nephropathy as compared with those in patients with MGA (Figure 2A).

As shown in Figure 3A, AngII immunoreactivity of renal biopsy samples was significantly higher in patients with IgA nephropathy (5282 ± 5973 arbitrary units) than in patients with MGA (415 ± 71 arbitrary units). Representative images of AngII immunostaining in patients with MGA and IgA nephropathy are shown in Figure 4A–C and D–F, respectively.

**Table 1.** Baseline data and the effects of treatment with valsartan

<table>
<thead>
<tr>
<th></th>
<th>Healthy volunteers ($n=14$)</th>
<th>MGA ($n=14$)</th>
<th>IgA nephropathy ($n=11$)</th>
</tr>
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<tr>
<td>Male/Female ($n$)</td>
<td>4/10</td>
<td>1/3</td>
<td>2/9</td>
</tr>
<tr>
<td>Age (year)</td>
<td>33 ± 6</td>
<td>43 ± 19</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>20.4 ± 4.4</td>
<td>20.1 ± 1.5</td>
<td>20.6 ± 3.8</td>
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<td>SBP (mmHg)</td>
<td>112 ± 8</td>
<td>120 ± 13</td>
<td>118 ± 13</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>67 ± 8</td>
<td>69 ± 6</td>
<td>74 ± 9</td>
</tr>
<tr>
<td>PRA (ng/mL/h)</td>
<td>–</td>
<td>1.5 ± 1.7</td>
<td>5.5 ± 4.5</td>
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<tr>
<td>Aldosterone (ng/dL)</td>
<td>–</td>
<td>11 ± 5</td>
<td>18 ± 10</td>
</tr>
<tr>
<td>C-reactive protein (mg/dL)</td>
<td>–</td>
<td>0.03 ± 0.01</td>
<td>0.07 ± 0.07</td>
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<tr>
<td>BUN (mg/dL)</td>
<td>–</td>
<td>19 ± 12</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Cr (mg/dL)</td>
<td>0.9 ± 0.6</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>UpV (mg/day)</td>
<td>3080 ± 3617</td>
<td>1338 ± 1265</td>
<td>435 ± 460*</td>
</tr>
</tbody>
</table>

*P<0.05; IgA nephropathy (pretreatment) vs IgA nephropathy + valsartan.

**Fig. 1.** $U_{AGT}/U_{Cr}$ in age-matched healthy volunteers and patients with MGA and IgA nephropathy are shown. Patients with IgA nephropathy were treated with an angiotensin II receptor blocker, valsartan.
Effects of an ARB on renal parameters and RAS in patients with IgA nephropathy

In patients with IgA nephropathy, valsartan (40 mg/day) significantly decreased SBP, DBP and $U_pV$ but did not change serum BUN or Cr levels (Table 1). On the other hand, valsartan significantly increased renal plasma flow (RPF) from $479 \pm 98$ to $611 \pm 147$ mL/min and decreased the filtration fraction from $0.26 \pm 0.03$ to $0.20 \pm 0.04$ (Figure 5), whereas the index scores of glomerular scler-
Osis were not changed by 2–8 weeks of valsartan treatment (data not shown). Valsartan also significantly increased PRA and decreased plasma aldosterone levels, whereas serum C-reactive protein levels were not changed (Table 1).

Treatment with valsartan significantly decreased $\frac{U_{\text{AGT}}}{U_{\text{Cr}}}$ in all patients with IgA nephropathy. The average $\frac{U_{\text{AGT}}}{U_{\text{Cr}}}$ in IgA nephropathy patients treated with valsartan was $12 \pm 15 \mu g/g$, which was similar to the levels of

![Representative images of AngII immunostaining in patients with MGA and IgA nephropathy.](image)

Fig. 4. Representative images of AngII immunostaining in patients with MGA and IgA nephropathy. Representative images of immunostaining of AngII in renal tissues from biopsy samples in patients with MGA (A–C) and IgA nephropathy (D–F) are shown. In four of the 11 patients with IgA nephropathy, renal biopsy samples were also collected after treatment with an AngII receptor blocker, valsartan, and AngII immunostaining was determined. Each pair of the pictures D and G, E and H, and F and I comes from one IgA nephropathy patient each (pretreatment: D, E and F vs post treatment: G, H and I). Immunoreactivity of AngII in tubules appeared to be higher in patients with IgA nephropathy than in patients with MGA. Furthermore, treatment with valsartan decreased immunoreactivity of AngII in tubules from patients with IgA nephropathy. Original magnification, ×400.

![Effects of valsartan on RPF and filtration fraction in patients with IgA nephropathy.](image)

Fig. 5. Effects of valsartan on RPF and filtration fraction in patients with IgA nephropathy.
healthy volunteers and patients with MGA at baseline, respectively (Figure 1). In 4 of the 11 patients with IgA nephropathy, we were able to perform renal biopsy again after valsartan treatment. In these patients, we collected renal biopsy samples within a few days of the urinary angiotensinogen sampling period. Basal mRNA levels of angiotensinogen in the renal tissues of these four patients with IgA nephropathy were 2.2 ± 1.6-fold higher than those of baseline values in patients with MGA. Treatment with valsartan significantly decreased mRNA levels of angiotensinogen in the renal tissues of these four patients with IgA nephropathy (Figure 2B). Similarly, valsartan significantly decreased AngII immunoreactivity from 7943 ± 9084 to 2237 ± 1490 arbitrary units in the renal tissues of the four patients with IgA nephropathy (Figure 3B). Representative images of AngII immunostaining in valsartan-treated IgA nephropathy patients are shown in Figure 4G–I. Immunoreactivity of AngII in tubules appeared to be higher in patients with IgA nephropathy than in patients with MGA. Furthermore, treatment with valsartan decreased immunoreactivity of AngII in tubules from patients with IgA nephropathy. Each pair of pictures, D and G, E and H, and F and I, comes from the same IgA nephropathy patient (pretreatment: D, E and F vs post treatment: G, H and I). On the other hand, treatment with valsartan did not significantly alter the index score of glomerular sclerosis in patients with IgA nephropathy (from 74 ± 42 to 48 ± 18).

Correlation with urinary angiotensinogen

Correlation between UAGT/UCr and other patient data at baseline were evaluated by stepwise selection methods. Using uni-variate analysis, UAGT/UCr was correlated with mRNA levels of angiotensinogen/GAPDH in renal tissues \( r = 0.77, P = 0.002 \). Age, BUN, serum levels of Cr or C-reactive protein, \( U_r \), RPF, filtration fraction, PRA and plasma levels of aldosterone were not correlated with UAGT/UCr. In subsequent multivariate analyses, UAGT/UCr was not significantly correlated with age \( (r = 0.18, P = 0.230) \) or plasma C-reactive protein \( (r = 0.17, P = 0.277) \). Data for DBP, serum levels of BUN and Cr, RPF, \( U_r \), PRA and plasma aldosterone levels were rejected from the model. On the other hand, correlations between UAGT/UCr and SBP, filtration fraction or AngII immunoreactivity in renal tissues were statistically significant \( (SBP: r = 0.68, P \leq 0.001; \text{filtration fraction: } r = 0.41, P = 0.016; \text{AngII: } r = 0.35, P = 0.045) \).

Discussion

The present exploratory study showed that (i) urinary angiotensinogen levels correlated with renal tissue gene expression of angiotensinogen and AngII immunoreactivity; (ii) urinary angiotensinogen levels, renal tissue angiotensinogen gene expression and AngII immunoreactivity were much higher in patients with IgA nephropathy than in MGA; (iii) urinary angiotensinogen levels positively correlated with renal tissue angiotensinogen expression and AngII immunoreactivity but not with PRA; (iv) in patients with IgA nephropathy, treatment with an ARB reduced urinary angiotensinogen levels, renal tissue angiotensinogen gene expression and AngII immunoreactivity; and (v) in these patients, changes in urinary angiotensinogen levels significantly correlated with changes in filtration fraction. These data support the hypothesis that urinary angiotensinogen provides a specific index of intrarenal RAS status in patients with IgA nephropathy.

Intrarenal AngII is regulated in a manner distinct from circulating AngII concentrations [10,11] and is involved in a derangement of renal functions and the progression of renal injury when its levels are inappropriately elevated [11]. Therefore, assessment of intrarenal RAS status is essential to understand the mechanisms that mediate the pathophysiology of renal function and injury [10,11]. Intrarenal AngII is rapidly degraded by high activity of renal peptidases [11]. On the other hand, substantial preclinical evidence suggests that intrarenal RAS activity is regulated by changes in local angiotensinogen levels [10–13] and that urinary excretion of angiotensinogen reflects intrarenal angiotensinogen production [11,14,15]. These observations from the preclinical studies prompted us to investigate whether urinary angiotensinogen acts as a specific index of intrarenal RAS status in humans, which must be analysed in a clinical laboratory. However, the standard method for measuring angiotensinogen is impractical for clinical examinations and diagnoses because of its complicated procedures. Thus, we have developed a specific, simple and accurate quantification system for human angiotensinogen using a microtiter plate-based sandwich-type ELISA [17,30]. By using this method, we have recently shown that urinary angiotensinogen levels were significantly increased in hypertensive patients [30]. Nevertheless, it is still not clear whether urinary angiotensinogen actually reflects the angiotensinogen that originates from the kidney. The present study showed that urinary angiotensinogen levels were highly correlated with renal tissue angiotensinogen gene expression. These data are consistent with the hypothesis based on our earlier animal studies [10–15] that urinary angiotensinogen is a useful biomarker to evaluate intrarenal angiotensinogen levels in humans.

The activation of intrarenal RAS during the progression of IgA nephropathy has been suggested, largely based on the ability of ACEIs and ARBs to reduce proteinuria [19–21,31]. However, some evidence exists suggesting the possible augmentation of intrarenal RAS activity in patients with IgA nephropathy. For example, similar to the observations in the present study, acute administration of an ACEI decreased the filtration fraction consequently to an increase in the effective RPF, suggesting the activation of intrarenal RAS [32]. We recently demonstrated that renal angiotensinogen immunoreactivity is elevated in patients with IgA nephropathy [23,24]. In the present study, patients with IgA nephropathy showed elevated urinary angiotensinogen levels. Furthermore, these levels were associated with increases in renal tissue angiotensinogen expression and AngII immunoreactivity, but not with PRA. These data indicate that, in patients with IgA nephropathy, intrarenal RAS is activated through local augmentation of angiotensinogen production.
In the present exploratory study, we were not able to determine the precise mechanisms by which patients with IgA nephropathy show intrarenal augmentation of angiotensinogen. However, several possible mechanisms are indicated in other studies. In vitro studies show that proximal tubular AngII production is enhanced by conditioned culture medium from human mesangial cells activated by IgA [33]. In mice [22] and patients [23] with IgA nephropathy, the augmentation of intrarenal angiotensinogen expression is associated with increases in reactive oxygen species, suggesting the potential roles of reactive oxygen species in the local production of angiotensinogen in patients with IgA nephropathy. Thus, it is possible that the activated intrarenal reactive oxygen species–angiotensinogen axis has a role in the progression of IgA nephropathy.

It is well known that blockade of AngII with ARBs results in increases in PRA and circulating AngII levels, which are associated with increases in renin release from juxtaglomerular cells [11]. Meanwhile, our animal studies have revealed that treatment with ARBs decreased, rather than increased, AngII levels in the kidney through blockade of AngII type 1 receptor-mediated stimulation of intrarenal angiotensinogen production [10–12]. In the present study, treatment with an ARB increased PRA but consistently reduced urinary angiotensinogen levels, renal tissue gene expression of angiotensinogen and AngII immunoreactivity in patients with IgA nephropathy. These data demonstrate that treatment with an ARB decreases intrarenal angiotensinogen and AngII levels in humans and further support the hypothesis that urinary angiotensinogen is a useful marker to monitor the changes in intrarenal RAS activity.

In patients with IgA nephropathy, the basal values of filtration fraction were significantly correlated with urinary angiotensinogen levels. Furthermore, before and after treatment with an ARB, reductions in filtration fraction were associated with decreases in urinary angiotensinogen levels. These data suggest that the beneficial effects of ARBs on renal function are accompanied by their inhibitory effects on intrarenal RAS activity. In these patients, changes in proteinuria were also positively correlated with changes in urinary angiotensinogen levels both before and after treatment with an ARB. As described above, however, baseline urinary angiotensinogen levels were not correlated with proteinuria in both patients with IgA nephropathy and MGA. These data are consistent with those of our recent finding [30] that not all the individual urinary angiotensinogen levels fall under their correlation with urinary albumin or protein levels in hypertensive patients, suggesting that urinary angiotensinogen excretion is not a simple consequence of the proteinuria. Thus, it can be speculated that, in patients with IgA nephropathy, some antiproteinuric effects of ARB are mediated through its inhibitory effects on intrarenal RAS activity. In the present study, urinary angiotensinogen was highly correlated with SBP. These data are consistent with our recent finding [30] that urinary angiotensinogen is highly correlated with SBP in hypertensive patients. In transgenic mice that express systemic human renin and human angiotensinogen in just the kidney, the development of hypertension and renal injury is associated with increases in intrarenal AngII levels [13]. Collectively, these data suggest that increases in intrarenal AngII production induced by the augmentation of local angiotensinogen expression contribute to the development of hypertension in patients with CKD. It should also be noted that we failed to observe a positive correlation between urinary angiotensinogen and index scores of glomerular injury in patients with IgA nephropathy treated with an ARB. Obviously, further studies with a larger number of patients and a longer observation period are needed to address this issue.

In summary, the present study indicates, for the first time, that increases in urinary angiotensinogen levels are correlated with augmented intrarenal angiotensinogen gene expression and AngII levels in normotensive patients with moderately proteinuric IgA nephropathy. Furthermore, after treatment with an ARB, decreases in renal tissue AngII levels were associated with decreases in angiotensinogen gene expression and urinary angiotensinogen levels. Urinary angiotensinogen levels were also significantly correlated with filtration fraction. These data support the hypothesis that urinary angiotensinogen is a powerful tool for determining intrarenal RAS status and associated renal derangement. However, there are several limitations in this exploratory study. Future evaluation of urinary angiotensinogen in prospective studies with a larger number of patients and a longer observation period is needed.

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Conflict of interest statement. None declared.

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