Urinary Angiotensinogen as a Potential Biomarker of Intrarenal Renin-angiotensin System Activity in Chinese Patients with Chronic Kidney Disease

Z Xu[1], B Xu[1], C Xu[2]

ABSTRACT

Urinary angiotensinogen (AGT) mainly derives from the AGT produced in the proximal tubular cells. Evidence exists that support the correlation between urinary AGT and circulating AGT. Previous studies measured urinary AGT by radioimmunoassay which is not convenient for clinical practice. In this study, we utilized an enzyme-linked immunosorbent assay (ELISA) based method to quantify urinary AGT. We analysed the relationship between urinary AGT and intrarenal angiotensin II (Ang II) activity in patients with chronic kidney disease (CKD). Urinary and plasma renin activity, AGT, Ang II and aldosterone were measured by radioimmunoassay or ELISA in 128 CKD patients. Furthermore, expression levels of intrarenal renin, AGT, Ang II and Ang II receptor were examined by immunohistochemistry staining (IHCS) in 72 CKD patients undergoing renal biopsy. Average urinary AGT was 2.02 ± 0.55 ng/(mg Cr). Hypertension, urinary protein, urinary Ang II and urinary Type IV collagen (Col IV) positively correlated with urinary AGT. Estimated glomerular filtration rate (eGFR), urinary sodium and serum AGT negatively correlated with urinary AGT. Multiple regression analysis indicated that low serum AGT, high urinary protein, urinary Ang II and urinary Col IV correlated significantly with high urinary AGT. Moreover, we observed positive correlation between urinary AGT and positive IHCS area of AGT, Ang II and Ang II Type 1 receptor in renal tissue. These data suggest that urinary AGT might be a potential biomarker of intrarenal angiotensin II activity in CKD patients.

Keywords: Angiotensin II, angiotensinogen, proteinuria, renin-angiotensin system, Type IV collagen

El Angiotensinógeno Urinario como Potencial Biomarcador de la Actividad del Sistema Renina-angiotensina Intrarrenal en Pacientes Chinos con Enfermedad Renal Crónica

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RESUMEN

El angiotensinógeno urinario (AGT) se deriva principalmente del AGT producido en las células tubulares proximales. Existen evidencias que apoyan la correlación entre el AGT urinario y el AGT circulante. Anteriores estudios midieron el AGT urinario mediante radioinmunoensayo, lo cual no es conveniente para la práctica clínica. En este estudio, se utilizó un método basado en el ensayo por inmunoabsorción ligado a enzimas (ELISA) para cuantificar el AGT urinario. Analizamos la relación entre la actividad del AGT urinario y la angiotensina II (Ang II) intrarenal en pacientes con nefropatía crónica (NC). La actividad de la renina en plasma y orina, el AGT, la Ang II, y la aldosterona fueron medidas por radioinmunoensayo o ELISA en 128 pacientes con nefropatía crónica. Además, los niveles de expresión de renina intrarrenal, AGT, Ang II y receptores de Ang II, fueron examinados por tinción inmunohistoquímica (IHQ) en 72 pacientes con nefropatía crónica sometidos a biopsia renal. El AGT urinario promedio fue 2.02 ± 0.55 ng /(mg Cr). La hipertensión, la proteína urinaria, la Ang II urinaria, y el colágeno tipo IV (Col IV) en orina tuvieron una correlación positiva con el AGT en orina. El índice

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de filtración glomerular estimado (IFGe), el sodio en orina y el AGT en plasma, guardaron una correlación negativa con el AGT urinario. El análisis de regresión múltiple indicó que un bajo nivel de AGT plasmático, una alto nivel de proteína urinaria, la Ang urinaria, y el Col IV en orina, guardaban una correlación significativa con un AGT urinario alto. Además, observamos una correlación positiva entre el AGT urinario y el área de la tinción de IHQ del AGT, la Ang II y el receptor de Ang II de tipo 1 del tejido renal. Estos datos sugieren que el AGT urinario podría ser un potencial biomarcador de la actividad de la angiotensina II intrarrenal en pacientes con nefropatía crónica.

Palabras claves: Angiotensina II, angiotensinógeno, proteinuria, sistema renina-angiotensina, colágeno tipo IV

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INTRODUCTION

Angiotensinogen (AGT) is a glycosylated globulin which, on hydrolysis by renin, gives rise to angiotensin. Most of the circulating AGT is produced and secreted by the liver but other tissues can also produce AGT (1). In kidneys, *AGT* mRNA and protein is mainly localized to proximal tubular cells, suggesting that intrarenal angiotensin II (Ang II) could be derived from locally formed and secreted AGT (2). Indeed, AGT produced in the proximal tubular cells seems to be secreted directly into the tubular lumen (3). This suggests that urinary AGT could originate from AGT produced in proximal tubular cells. Previous studies reported correlation between urinary AGT and circulating AGT (4, 5).

The crucial role of Ang II in the development of renal fibrosis and end-stage renal disease (ESRD) is widely recognized. It has been demonstrated that a significant relationship exists among urinary AGT and kidney Ang II content and renal Ang II immunostaining intensities (6–8). These results provide further evidence that urinary AGT may be a useful index of intrarenal Ang II activity. Until now, urinary AGT was measured by radioimmunoassay (RIA), which is not convenient for clinical practice. In this study, enzyme-linked immunosorbent assay (ELISA) was used to measure urinary AGT. We analysed the relationship of urinary AGT with intrarenal Ang II activity in patients with chronic kidney disease (CKD).

SUBJECTS AND METHODS

One hundred and twenty-eight CKD patients who were hospitalized in Zhongshan Hospital between January 2009 and December 2009 and gave informed consent were included in the study. The study protocol was approved by the ethics committee of Zhangshan Hospital (Fudan University, China). Patients had not received either angiotensin converting enzyme inhibitor or angiotensin II receptor blocker during the last two months. The patients included 54 men and 74 women with a mean age of 42.00 years (range 13 to 83 years). The estimated glomerular filtration rate (eGFR) ranged from 3.99 to 163.93 mL/min/1.73m^2 (mean 58.40 mL/min/1.73m^2). The eGFR was calculated using the Modification of Diet in Renal Disease Formula [eGFR = 175 × standardized serum creatinine$^{-1.154}$ × age$^{-0.203}$ × 0.741 [if Asian] × 0.742 (if female)] (9), which was found to correlate well with GFR corrected for body surface area in adults. The background renal diseases were minor lesion (n = 9), minimal change disease (n = 2), IgA nephropathy (n = 39), non-IgA mesangial proliferative glomerulonephritis (n = 3), membranous nephropathy (n = 9), focal segmental glomerulosclerosis (n = 8), focal proliferative glomerulonephritis (n = 17), membranoproliferative glomerulonephritis (n = 1), crescentic glomerulonephritis (n = 3), sclerosing glomerulonephritis (n = 1), lupus nephritis (n = 5), purpura nephritis (n = 3), diabetic nephropathy (n = 1), light chain deposition disease (n = 1), ankylosing spondylitis kidney damage (n = 1), lipoprotein nephropathy (n = 1), chronic interstitial nephritis (n = 2), kidney damage associated with tumours (n = 1), renal amyloidosis (n = 1), hepatitis B virus associated nephropathy (n = 1) and chronic kidney disease without renal biopsy data (n = 19).

Renal biopsies were performed in 72 patients during hospitalization. The patients included 33 men and 39 women with a mean age of 43.32 years (range 13 to 83 years). The eGFR ranged from 3.99 to 163.93 mL/min/1.73m^2 (mean 51.46 mL/min/1.73m^2). The background renal diseases were minor lesion (n = 7), IgA nephropathy (n = 21), non-IgA mesangial proliferative glomerulonephritis (n = 3), membranous nephropathy (n = 7), focal segmental glomerulosclerosis (n = 4), focal proliferative glomerulonephritis (n = 11), membranoproliferative glomerulonephritis (n = 1), crescentic glomerulonephritis (n = 3), sclerosing glomerulonephritis (n = 1), lupus nephritis (n = 5), purpura nephritis (n = 2), diabetic nephropathy (n = 1), light chain deposition disease (n = 1), lipoprotein nephropathy (n = 1), chronic interstitial nephritis (n = 2) and kidney damage associated with tumours (n = 1).

Seven healthy volunteers were included in this study. The volunteers included three men and four women with a mean age of 31.86 years (range 26 to 42 years).

The levels of plasma and urinary renin-angiotensin system (RAS) components were measured in blood samples that were collected at bed rest early in the morning and fresh urine samples that were collected after waking up. For measurements of plasma renin activity, plasma and urinary AGT, Ang II and aldosterone, blood and urine samples were collected in tubes and EDTA was added to the final concentration of 4 mM. Samples were cooled on ice,

centrifuged and stored at -20 °C. Urinary AGT, Ang II, aldosterone, and Type IV collagen (Col IV) levels are expressed per 1 mg of urinary creatinine (mg Cr).

Samples from renal biopsies were obtained under ultrasound guidance with a 16-gauge needle. Only biopsies providing sufficient amounts of sample for performing both the standard pathologic examination and molecular biology analysis were included in this study.

Serum and urinary concentration of AGT were measured with ELISA kits (Angiotensinogen, Immuno-Biological Laboratories, Gunma, Japan); 100 µl of human AGT standards (0.31~20 ng/mL diluted in ELISA buffer), plasma (1:8000 dilution in ELISA buffer), and urine (1:8 dilution in ELISA buffer) were added to each well of the plates and incubated at 37 °C for one hour. Plates were washed seven times with a washing buffer and incubated with horseradish peroxidase-labelled monoclonal antibody against human AGT (100 µl/well, 1:30 dilution in antibody solution) at 37 °C for 30 minutes. After washing nine times with washing buffer, plates were incubated with TMB solution (100 µl /well) in darkness at room temperature for 30 minutes. The reaction was stopped by addition of sulphuric acid (0.5 M, 100 µl/well) and absorbance was measured at 450 nm.

Immunoradiometric methods were used for the determination of active renin concentration in plasma. Plasma and urinary Ang II were measured by a radioimmunoassay kit according to manufacturer's recommendations (Angiotensin II, HTA Co Ltd, Beijing, China). Serum and urinary concentrations of aldosterone were measured with the ELISA kit (Aldosterone, Immuno-Biological Laboratories, Japan).

Serum concentration of creatinine, sodium, potassium and urinary concentration of sodium, protein and Col IV were measured in the clinical laboratory in Zhongshan Hospital of Fudan University.

Immunohistochemistry staining (IHCS) for renin, AGT, Ang II, angiotensin II Type 1 receptor (AT1R) and angiotensin II Type 2 receptor (AT2R) was performed in consecutive kidney sections. Microwave irradiation was used to enhance antigen retrieval. The primary antibodies were sheep anti-human renin (1:60; R&D systems, USA), rabbit anti-human angiotensinogen (1:80; Sigma-Aldrich, St Louis, MO, USA), rabbit anti-human angiotensin II (1:800; Phoenix Pharmaceutics, Burlingame, CA, USA), mouse anti-human AT1R (1:50; Abcam Ltd, Cambridge, UK) and rabbit anti-human AT2R (1:500; Abcam Ltd, Cambridge, UK). The secondary antibodies were donkey anti-rabbit IgG (1:100; DakoCytomation Ltd, Cambridge, UK), donkey anti-mouse IgG (1:100; DakoCytomation Ltd, Cambridge, UK) and rabbit anti-sheep IgG (1:100; KPL, Gaithersburg, Maryland, USA). Sections that were incubated with phosphate buffered saline instead of the primary antibodies served as negative controls. The immune reactivities were scored twice in a blind manner by Leica QWin V3 imaging analysis software. The inter assay variations were not significant.

Data represent mean ± SD or per cent. Logarithmic transformation for urinary AGT and common logarithmic transformation for other RAS components and urinary Col IV were performed because these variables did not exhibit normal distribution. Transformed variables were used in presentation and statistical analysis. Unpaired Student *t*-test was used to verify differences between two groups. Pearson single-regression analyses and Spearman single-regression analyses were used for parametric and non-parametric data, respectively. Standard least-squares method was used for multiple regression analyses. Statistical significance was set at $p < 0.05$. Computations, data management and statistical analyses were performed using SPSS software (v 17.0).

RESULTS

Average urinary AGT in 128 CKD patients was 2.02 ± 0.55 ng/(mg Cr) [range from 0.65 to 3.34 ng/(mg Cr)]. Urinary AGT correlated positively with hypertension ($\rho = 0.28$, $p < 0.01$), urinary protein ($r = 0.38$, $p < 0.01$), urinary Ang II ($r = 0.29$, $p < 0.05$), urinary Col IV ($r = 0.56$, $p < 0.01$) and negatively with eGFR ($r = -0.28$, $p < 0.01$), urinary sodium ($r = -0.22$, $p < 0.05$) and serum AGT ($r = -0.27$, $p < 0.01$). No significant correlation was found between urinary AGT and plasma renin activity (PRA), plasma Ang II, plasma and urinary aldosterone, body mass index (BMI) or gender (Table 1). Multiple regression analysis indicated that low serum AGT ($p < 0.01$), high urinary protein ($p < 0.01$), urinary Ang II ($p < 0.05$) and urinary Col IV ($p < 0.01$) correlated significantly with high urinary AGT (Table 2).

Average urinary AGT in healthy volunteers was 1.77 ± 0.40 ng/(mg Cr). Urinary AGT levels in hypertensive CKD patients were significantly higher in comparison to non-hypertensive CKD patients [1.84 ± 0.52 ng/(mg Cr) *vs* 2.11 ± 0.54 ng/(mg Cr), $p < 0.01$] (Fig. 1). There was no significant difference in urinary AGT between healthy volunteers and non-hypertensive CKD patients.

Urinary AGT level in CKD patients with eGFR > 90 mL/min/1.73 m^2 was significantly higher compared with CKD patients with eGFR < 90 mL/min/1.73 m^2 [1.65 ± 0.57 ng/(mg Cr) *vs* 2.05 ± 0.54 ng/(mg Cr), $p < 0.05$] (Fig. 2A). There was no difference in urinary AGT between healthy volunteers and CKD patients with eGFR > 90 mL/min/1.73 m^2. The sensitivity of > 1.80 ng/(mg Cr) of urinary AGT for prediction of moderate (eGFR < 90 mL/min/1.73 m^2) renal dysfunction was 72% and the specificity was 64%.

Urinary AGT level in CKD patients with eGFR > 30 mL/min/1.73 m^2 was significantly lower compared with CKD patients with eGFR < 30 mL/min/1.73 m^2 [1.93 ± 0.55 ng/(mg Cr) *vs* 2.37 ± 0.38 ng/(mg Cr), $p < 0.01$] (Fig. 2B). The sensitivity of > 2.11 ng/(mg Cr) of urinary AGT for prediction of severe (eGFR < 30 mL/min/1.73 m^2) renal dysfunction was 80% and the specificity was 65%.

Average urinary AGT in 72 CKD patients selected for renal biopsy was 2.25 ± 0.25 ng/(mg Cr) [1.69 to 2.86 ng/

Table 1: Correlation matrix between urinary angiotensinogen (AGT) and various parameters in 128 chronic kidney disease patients. Logarithmic transformation for urinary AGT, common logarithmic transformation for other renin-angiotensin system components and urinary Type IV collagen were performed

	Mean ± SD or %	*r* or *ρ*-value	*p*-value
Male (%)	42.2	-0.11	> 0.05
Age (years)	42.00 ± 14.77	0.10	> 0.05
BMI (kg/m^2)	23.69 ± 3.49	-0.07	> 0.05
Hypertension status (%)	65.6	0.24	< 0.01
SBP (mmHg)	126.63 ± 15.73	0.01	> 0.05
DBP (mmHg)	81.55 ± 11.24	-0.03	> 0.05
eGFR (mL/min/1.73 m^2)	58.40 ± 27.22	-0.28	< 0.01
Serum Na^+ (mM)	141.60 ± 3.23	-0.06	> 0.05
Serum K^+ (mM)	4.06 ± 0.43	-0.08	> 0.05
Proteinuria (g/24 h)	2.03 ± 2.66	0.38	< 0.01
Urinary Na^+ (mmol/24 h)	162.10 ± 81.94	-0.22	< 0.05
Urinary K^+ (mmol/24 h)	38.72 ± 16.28	0.01	> 0.05
PRA (pg/mL/h)	6.80 ± 1.30	0.01	> 0.05
Serum AGT (ng/mL)	11.32 ± 0.36	-0.27	< 0.01
Plasma Ang II (pg/mL)	4.09 ± 0.31	-0.08	>0.05
Serum aldosterone (pg/mL)	6.11 ± 0.57	0.07	> 0.05
Urinary Ang II [pg/(mg Cr)]	4.92 ± 0.70	0.28	< 0.01
Urinary aldosterone [pg/(mg Cr)]	8.50 ± 0.82	0.03	> 0.05
Urinary Col IV [ng/(mg Cr)]	5.19 ± 1.39	0.56	< 0.01

BMI – body mass index, SBP – systolic blood pressure, DBP – diastolic blood pressure, eGFR – estimated glomerular filtration rate, Na – sodium, K – potassium, PRA – plasma renin activity, AGT – angiotensinogen, Ang II – angiotensin II, Col IV – Type IV collagen

Table 2: Multiple regression analysis for urinary angiotensinogen in 128 chronic kidney disease patients*

Variable	Partial regression coefficient	SE	Standard partial regression coefficient	*T* value	*p*-value
Hypertension	0.105	0.082	0.091	1.278	0.204
eGFR (mL/min/1.73 m^2)	-0.002	0.001	-0.111	-1.547	0.125
Proteinuria (g/24 h)	0.045	0.016	0.219	2.821	0.006
Urinary Na^+ (mmol/24 h)	0.000	0.000	-0.108	-1.558	0.122
Serum AGT (ng/mL)	-0.299	0.106	-0.197	-2.827	0.006
Urinary Ang II [pg/(mg Cr)]	0.142	0.059	0.182	2.413	0.017
Urinary Col IV [ng/(mg Cr)]	0.153	0.032	0.376	4.788	0.000

*R^2 = 0.480, $p < 0.01$. Log-transformed values were adopted for analysis of urinary AGT and common logarithmic transformed values were adopted for analysis of serum AGT, urinary Ang II and Col IV levels.

eGFR – estimated glomerular filtration rate, Na – sodium, AGT – angiotensinogen, Ang II – angiotensin II, Col IV –Type IV collagen

(mg Cr)]. Representative IHCS pictures of renin, AGT, Ang II, Ang II Type 1 receptor, Ang II Type 2 receptor in biopsies from patients with IgA nephropathy and membranous nephropathy are shown in Fig. 3. In IgA nephropathy, Ang II staining was detected mainly in distal tubules. Mild Ang II staining was also noted in brush borders, some proximal tubular cells, Bowman's epithelial cells and some glomerular cells. In membranous nephropathy, increased Ang II staining was observed in the glomerular and tubulointerstitial lesions. Some interstitial mononuclear cells were also positive for Ang II staining. Urinary AGT correlated positively with intrarenal immunostaining intensities of AGT ($r = 0.46$, $p < 0.01$), Ang II ($r = 0.56$, $p < 0.01$) and Ang II Type 1 receptor ($r = 0.32$, $p < 0.05$). Urinary AGT did not correlate significantly with intrarenal immunostaining intensities of renin and Ang II Type 2 receptor.

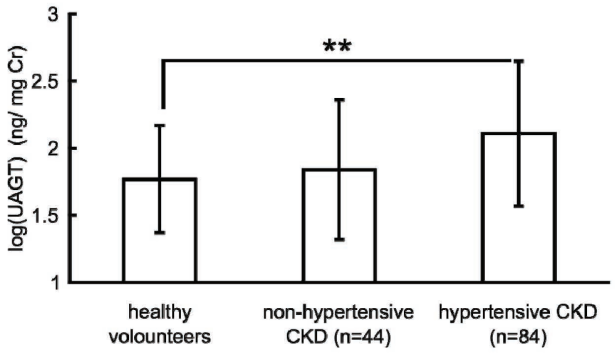

Fig. 1: Urinary angiotensinogen (UAGT) in volunteers and chronic kidney disease (CKD) patients with or without hypertension. Data represent mean ± SD. $^{**}p < 0.01$.

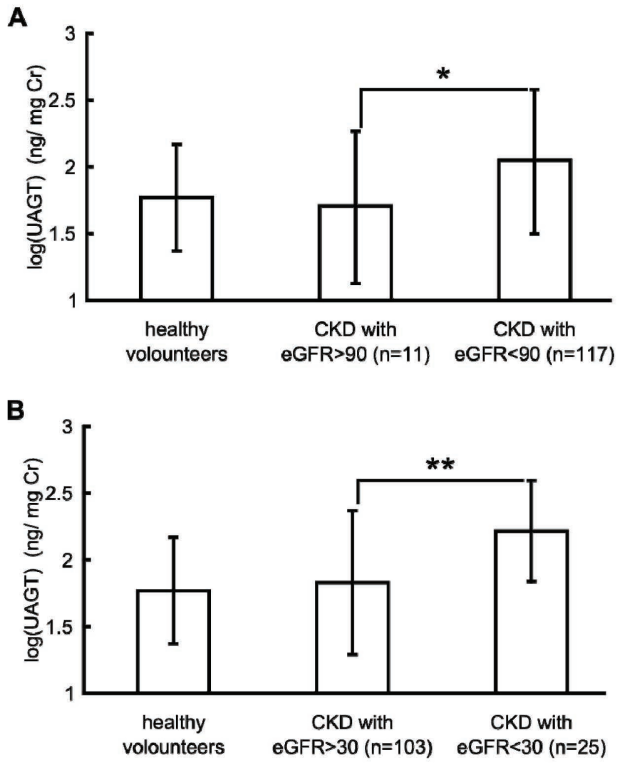

Fig. 2: Urinary angiotensinogen (UAGT) in volunteers and chronic kidney disease (CKD) patients with different estimated glomerular filtration rate (eGFR). Estimated glomerular filtration rate is displayed as mL/min/1.73m^2. Data represent mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

DISCUSSION

Recently, focus on the role of RAS in blood pressure regulation and sodium homeostasis shifted to emphasis on the involvement of organ-specific RAS components in organ fibrosis. Intrarenal *AGT* mRNA and protein is mainly localized to proximal tubular cells, suggesting that the intrarenal Ang II could be derived from locally formed and

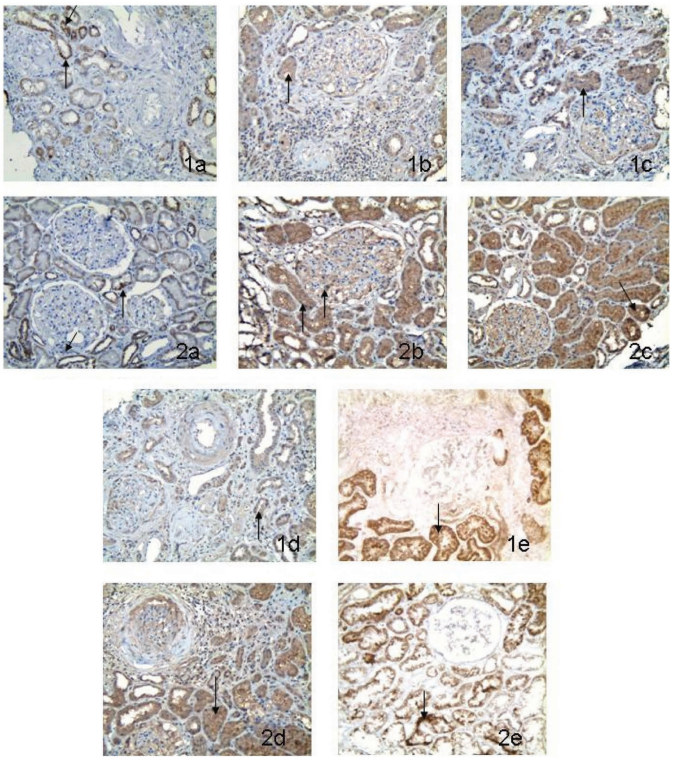

Fig. 3: Immunostaining of consecutive kidney sections for renin (a), angiotensinogen (b), angiotensin II (c), angiotensin II Type 1 receptor (d) and angiotensin II Type 2 receptor (e) in IgA nephropathy (1) and membranous nephropathy (2) [×200].

secreted AGT (2). Chronic Ang II infusion to normal rats significantly increased the urinary excretion rate of AGT in a time- and dose-dependent manner that was associated with elevation in kidney AGT and Ang II levels but not with plasma Ang II concentrations (6). To determine whether the increase in urinary AGT excretion was simply a nonspecific consequence of the proteinuria and hypertension, further studies were done in rats made hypertensive with desoxycorticosterone acetate (DOCA) salt plus high-salt diet. Although urinary protein excretion in DOCA salt-induced volume-dependent hypertensive rats was increased to the same or to a greater extent, urinary AGT was significantly lower in volume-dependent hypertensive rats than in Ang II-dependent hypertensive rats and was not greater than in control rats (7).

Recent clinical study with 80 CKD patients demonstrated significant relationship between urinary AGT and renal Ang II immunostaining intensities. Urinary AGT is positively correlated with renal Ang II IHCS staining intensities (8). These results indicate that urinary AGT may be a potential marker of intrarenal Ang II activity.

In previous studies, urinary AGT levels were measured by RIA. Radioimmunoassay of urinary AGT requires three steps. First, samples are incubated with and without exogenous renin. Then angiotensin II concentrations in

paired test tubes are measured by RIA. Finally, converted angiotensin II is calculated as the difference between paired angiotensin II concentrations. This assay is not suitable in large-scale clinical practice because it involves time-consuming procedures (~2 days), indirect measurements, radioisotopes and expensive reagents. In contrast to RIA, the newly developed ELISA kits provide simple, quick and inexpensive method for direct measurements of human AGT.

The results of our experiments demonstrated that urinary AGT level was higher in CKD patients with high proteinuria, urinary Ang II and Col IV excretion. Positive correlation between urinary AGT and proteinuria was reported earlier by Yamamoto *et al* in 80 CKD patients (8). In contrast, studies by Kobori and Nishiyama in rats reported different results (7). These differences can be explained by different models. Animal studies focussed on hypertensive rat models, whereas clinical studies focussed on patients with chronic kidney disease. Variation between different models indicates that the relationship between urinary AGT and proteinuria in humans still requires further investigation.

Multiple regression analysis in our study showed that urinary AGT positively correlated with urinary Ang II. Yamamoto *et al* found no correlation between these parameters (8). Different from our study, Yamamoto *et al* measured AGT by RIA. In addition, it is possible that urinary Ang II derives from both circulating Ang II filtration through the glomerular basement membrane and intrarenal Ang II secretion into the tubules (11). With the increase in proteinuria, filtration of circulating Ang II will elevate at the same time. Our study demonstrated that urinary AGT positively correlated with proteinuria, supporting the hypothesis that proteinuria might be the reason for the positive correlation of urinary Ang II and urinary AGT (11). Type IV collagen is directly involved in the composition of the extracellular matrix in the form of precollagen and is a marker of renal extracellular matrix production and renal fibrosis. Because of its molecular weight, serum Type IV collagen cannot pass through the glomerular basement membrane into the urine. Urinary Col IV is mainly derived from intrarenal Col IV secretion into the tubules. Elevated urinary AGT level may therefore indicate kidney injury severity, especially chronic kidney injury severity in CKD patients.

Our study differed from Yamamoto *et al* (8), as we found the negative correlation between urinary AGT and serum AGT, which still supports the postulation that urinary AGT derives mainly from the intrarenal AGT secretion (7, 8). However, the mechanism by which the negative relationship is established is still unclear. It has become widely accepted that the kidney contains a local RAS that is independent of the circulating RAS (8). This local system seems to be regulated independently and seems to express distinct levels of hydrolytic enzymes and angiotensin peptide receptors (12). The intrarenal AGT does not have the direct negative feedback regulation on circulating AGT. Our speculation is that the intrarenal AGT might play a negative feedback on circulating AGT through Ang II mediated blood pressure regulation and sodium homeostasis but direct evidence is still needed to support this hypothesis.

Although we found that hypertension positively correlated with urinary AGT in the univariate analysis, hypertension was excluded in multiple regression analysis, different from the previous studies in a hypertensive population (13) and hypertensive rat models (6). Urinary AGT had no correlation with systolic and diastolic blood pressure in our study. The reason might be the high treatment rate (85.71%) and control rate (73.81%) of hypertension in our study population. Systolic and diastolic blood pressure could not reflect blood pressure before treatment. Furthermore, single analysis of hypertension status reduced the efficiency of statistical analysis but our study still demonstrated that urinary AGT level of non-hypertensive CKD patients was lower than hypertensive CKD patients and had no difference with healthy volunteers.

In the univariate analysis, we also found negative correlation between eGFR and urinary AGT, but eGFR was excluded in the multiple regression analysis. The relationship between eGFR and urinary AGT is controversial (8, 14, 15). Renal fibrosis is a major reason, but not the only one for decline in glomerular filtration rate. Given the significant correlation between urinary AGT and urinary Col IV, urinary AGT might associate closely with renal fibrosis. The effect of renal fibrosis on eGFR decline might differ because of the difference in the study population. In our study, urinary AGT level in CKD patients with eGFR > 90 and 30 mL/min/1.73 m^2 was significantly higher compared with those with eGFR < 90 and 30 mL/min/1.73 m^2; however, the specificity and sensitivity of urinary AGT in distinguishing mild and severe renal dysfunction is not satisfactory.

We also found that urinary sodium negatively correlated with urinary AGT in the univariate analysis but it had no correlation with urinary AGT in multiple regression analysis. There was also no correlation between urinary AGT and serum sodium and potassium. These data suggest that no relationship exist between urinary AGT and sodium and potassium intake.

Urinary AGT levels correlated positively with renal Ang II immunostaining intensities by IHCS. This suggests that urinary AGT can reflect the intrarenal Ang II activity and could be used as a potential non-invasive marker of intrarenal Ang II activity. Previously, animal studies demonstrated that urinary AGT mainly derives from proximal tubular cells (4, 5) and positively correlates with renal AGT content (6). Urinary angiotensinogen is the only known substrate of renin. Urinary angiotensinogen levels in human and rat are close to the Michaelis constant (16), so both the changes of AGT (substrate) and renin (enzyme) can affect the activity of the RAS. Intrarenal activity of Ang II can be influenced by the intrarenal AGT expression. The correlation between urinary AGT and intrarenal Ang II activity is based on

physiological characteristics of intrarenal AGT generation, transformation and excretion.

As the most important function receptor of Ang II, Ang II Type 1 receptor is mainly localized in the brush border of proximal tubular cells, mediating endocytosis and uptake of Ang II which is suggested to be the most important mechanism of Ang II accumulation in the kidney (17, 18). We also observed that intrarenal Ang II Type 1 receptor staining was mainly detected in the proximal tubular cells and correlated positively with urinary AGT, which supports the role of Ang II Type 1 receptor in the Ang II accumulation in the kidney.

It has already been confirmed that the intrarenal Ang II plays an important role in renal fibrosis through Ang II Type 1 receptor in numerous studies (19). Our results demonstrated that urinary AGT reflects intrarenal Ang II and Ang II Type 1 receptor expression and correlates with urinary Col IV collagen, which confirms the important role of intrarenal Ang II and Ang II Type 1 receptor in renal fibrosis. This topic needs further investigation. Because of scarcity of human kidney sample, we used IHCS to evaluate intrarenal RAS components expression, which had the shortcomings of low sensitivity and high susceptibility to subjective factors.

In conclusion, we have demonstrated in this study that urinary AGT levels are elevated in CKD patients with high proteinuria, urinary Ang II and Col IV excretion. Urinary AGT could reflect the intrarenal Ang II activity and might be used as a non-invasive marker of intrarenal Ang II activity.

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