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ARTICLE

Urinary mRNA expression of *ACE* and *ACE2* in human type 2 diabetic nephropathy

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Abstract
Aims/hypothesis The interplay of ACE and type 2 ACE (ACE2) has been recognised as playing an important role in the tissue renin–angiotensin system within the kidney. In the present study, we measured urinary mRNA expression of *ACE* and *ACE2* in patients with type 2 diabetic nephropathy.
Methods We studied 50 patients with diabetic nephropathy: 26 were being treated by ACE inhibitor (ACEI) alone (ACEI group), the other 24 by ACEI and angiotensin-receptor blocker (ARB) (ACEI+ARB group). mRNA expression of *ACE* and *ACE2* was measured by real-time quantitative RT-PCR at 0 and 12 weeks. All patients were then followed for 56 weeks.
Results Proteinuria correlated significantly with urinary *ACE* (r=0.454, p=0.001) and *ACE2* expression (r=0.651, p<0.001). Urinary *ACE2* expression correlated with estimated GFR (r=−0.289, p=0.042). In the ACEI group, there was a significant inverse correlation between the rate of GFR decline and urinary *ACE2* expression at baseline (r=−0.423, p=0.031) as well as at 12 weeks (r=−0.395, p=0.046). In contrast, there was no significant correlation between the rate of GFR decline and urinary *ACE2* expression at baseline or at 12 weeks in the ACEI+ARB group. The rate of GFR decline did not correlate with the baseline urinary *ACE* expression of either group.
Conclusion/interpretation There was a relationship between urinary mRNA expression of *ACE2* and the degree of proteinuria. The physiological implication and possibility of clinical application of quantifying urinary *ACE2* expression require further study.

Keywords ACE2 · Diabetes mellitus · Urinalysis

Abbreviations

ACE2	type 2 ACE
ACEI	ACE inhibitor
ARB	angiotensin-receptor blocker
RAS	renin–angiotensin system
RT-QPCR	quantitative RT-PCR

Introduction

Activation of the renin–angiotensin system (RAS), especially intrarenal RAS, is an important pathogenetic mechanism of diabetic nephropathy [1, 2]. Besides ACE, type 2 ACE (ACE2) has been recently described. It has 60% sequence similarity with ACE but acts to reduce the level of angiotensin II [3–6]. Recent studies have revealed a possible role of the interplay of ACE and ACE2 in diabetic nephropathy [7, 8]. Intrarenal localisation of these two enzymes in diabetes has been examined by a number of investigators [8–10] and RAS blockade has been shown to affect the tissue *ACE2* expression [11, 12].

The study of human intrarenal *ACE* and *ACE2* expression has long been hampered by the need for renal biopsy. In the last few years, with the development of a reliable RNA

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extraction technique from urinary sediment and real-time quantitative RT-PCR (RT-QPCR), measurement of mRNA expression in urinary sediment has become an emerging tool for the study of kidney diseases [13, 14]. In the present study we aimed to examine whether urinary expression of *ACE* and *ACE2* could be used as a non-invasive marker of renal injury in diabetic nephropathy.

Methods

Patient selection and follow-up From 2004 to 2005, we studied 24 patients with diabetic nephropathy in the Li Ka-Shing Specialty Clinic, Prince of Wales Hospital, Hong Kong. All patients had type 2 diabetes and were receiving ACE inhibitor (ACEI) therapy for diabetic nephropathy, which had been diagnosed clinically based on a long history of diabetes, proteinuria and the absence of clinical or laboratory evidence of other kidney disease. They were treated with an addition of an angiotensin-receptor blocker (ARB; irbesartan 300 mg/day) to ACEI (ACEI+ARB group). We studied another 26 patients with diabetic nephropathy who were continued on ACEI alone (ACEI group). The study was approved jointly by the Chinese University of Hong Kong New Territories East Cluster Clinical Research Ethics Committee. The study protocol adhered to the Declaration of Helsinki and informed consent had been obtained from all individuals for specimen and information collection.

All patients were followed every 8 weeks for 56 weeks. Clinical data including serum creatinine, urea, electrolytes, albumin, liver enzymes and 24 h urine protein were measured at each visit. GFR was estimated by a standard equation [15]. Disease progression was measured by the rate of GFR decline [16], which was calculated by the least-square regression method. Other treatments for individual patients were determined by the responsible physician and not affected by this study. All physicians were blinded from the results of urinary gene expression.

Serial measurement of urinary gene expression A whole-stream early morning urine specimen was collected by each patient at 0 and 12 weeks for gene expression study. Urinary mRNA extraction was performed according to the previously described method [13]. In brief, urine specimens were centrifuged at 3,000 *g* for 30 min and at 13,000 *g* for 5 min at 4°C shortly after collection. The supernatant fraction was then discarded and the urinary cell pellets were lysed by RNA lysis buffer (Qiagen, Mississauga, ON, Canada). Specimens were stored in −70°C until use. RNeasy mini kits (Qiagen) were used to extract total RNA according to the manufacturer's protocol. DNase was used to digest probable genomic DNA. We confirmed the purity of RNA by the relative absorbance at 260/280 nm ratio using a spectrometer. Our previous data have shown that the integrity of RNA isolated from urinary sediment by this method is adequate for RT-QPCR [17]. For reverse transcription, 5 μl total RNA was mixed with 1 μl random primers (150 ng), 1 μl dNTP mix (10 mmol/l each), 4 μl 5× first-strand buffer, 2 μl dithiothreitol (0.1 mol/l), 1 μl Superscript II RNase H Reverse Transcriptase (all from Invitrogen, Life Technologies, Philadelphia, PA, USA) made up to 20 μl with water. Reverse transcription was performed at 65°C for 5 min, 25°C for 10 min, 42°C for 50 min and then inactivation reaction at 70°C for 10 min. The resulting cDNA was stored in −70°C until use.

In this study we quantified urinary gene expression of *ACE* and *ACE2* using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Commercially available Taqman primers and probes, including two unlabelled PCR primers and one FAM dye-labelled TaqMan MGB probe were used for all the target genes (all from Applied Biosystems). The primer and probe sets were designed by the manufacturer and commercially available. They were deliberately designed across the intron–exon boundary so as not to detect possible genomic DNA. For RT-QPCR, 10 μl universal master mix, 1 μl primer and probe set, 2 μl cDNA and 7 μl water were mixed to make a 20 μl reaction volume. Each sample was run in triplicate. RT-QPCR were performed at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. 18S rRNA (Applied Biosystems) was used as a housekeeping gene to normalise the mRNA expression level of each target gene. Results were analysed with Sequence Detection Software version 1.7 (Applied Biosystems). In order to calculate the differences in expression level for each target genes among samples, the $\Delta\Delta C_T$ method for relative quantification was used according to the manufacturer's manual [18]. Because the amplification efficiency of all TaqMan Gene Expression Assays is equivalent to any other target assay, it is not necessary to validate the PCR efficiencies of the target and endogenous control(s) [19]. We also recruited nine healthy individuals as normal controls. The average expression level of the normal control individuals was defined as 1.

Statistical analysis Statistical analysis was performed by SPSS for Windows software version 11.0 (SPSS, Chicago, IL, USA). All the results were presented as medians (lower quartile, upper quartile) unless otherwise specified. Since data of target gene expression levels were highly skewed, either log transformation or a non-parametric statistical method was used. We used the Mann–Whitney *U* test to compare gene expression levels between groups, Wilcoxon signed-ranks test to compare gene expression levels within groups and Spearman's rank-order correlations to test associations between gene expression levels and clinical

Table 1 Demographic and clinical information for the patients

Demographics	ACEI group	ACEI+ARB group	Healthy volunteers
Number of patients	26	24	9
Age (years)	56.8±8.6	60.0±9.3	31.4±2.5
Sex (*n*, male:female)	22:4	19:5	7:2
Body height (cm)	166.3±9.8	163.3±8.4	166.8±8.8
Body weight (kg)	60.8±12.1	57.5±9.3	57.8±12.1
BMI (kg/m^2)	21.9±3.6	21.5±2.7	22.4±4.6
BP (mmHg)			
Systolic	146.5±12.6	150.4±20.4	121.0±13.7
Diastolic	81.2±9.5	77.4±10.5	75.9±8.5
Pre-existing comorbidity, no. of cases (%)			
Hypertension	19 (73.08)	21 (87.50)	–
Cerebrovascular disease	2 (7.70)	2 (8.33)	–
Coronary heart disease	7 (26.92)	6 (25.00)	–
Charlson's comorbidity index	3.92±1.32	4.13±1.78	–
ACEI, no. of patients (dosage, mg/day)			
Lisinopril	21 (9.88±5.21)	14 (13.57±6.02)	–
Ramipril	4 (3.75±4.21)	8 (4.22±2.75)	–
Captopril	1 (50)	–	–
Perindopril	–	2 (8)	–

Data are presented as means± SD unless stated otherwise

parameters. If data distribution became normal after log transformation, Pearson's correlation and Student's *t* test were also used as appropriate. A p value <0.05 was considered statistically significant. All probabilities were two-tailed.

Results

Demographic and clinical information of study individuals are displayed in Table 1. There was no significant difference in age, sex, BMI, BP and pre-existing comorbidities between the ACEI and ACEI+ARB groups. Baseline and follow-up data on renal function, proteinuria and urinary gene expression are summarised in Table 2. In brief, there was no significant difference in baseline proteinuria or baseline urinary *ACE* or *ACE2* expression between the groups, while baseline GFRs of patients in the ACEI+ARB group were slightly lower than those in the ACEI group (p=0.037). Three months after treatment, proteinuria improved significantly in the ACEI+ARB group (Wilcoxon signed-ranks test, p=0.014) but remained static in the ACEI group.

Baseline urinary gene expression and baseline clinical parameters Although not statistically significant, a tendency for internal correlation was observed between baseline urinary expression of *ACE* and *ACE2* (r=0.267, p=0.061) (Fig. 1). Baseline proteinuria strongly correlated with both urinary *ACE* (r=0.454, p=0.001) and *ACE2* expression (r=0.651, p<.001) (Fig. 2). Baseline estimated GFR had a

Table 2 Baseline and follow-up data on renal function, proteinuria and urinary gene expression

	ACEI group		ACEI+ARB group	
	0 weeks	12 weeks	0 weeks	12 weeks
Renal function				
Proteinuria (g/day)	1.77±1.48	1.75±1.42	1.53±1.42	1.04±0.99*
Serum creatinine (μmol/l)	149.35±27.70	155.54±31.14	162.46±29.96	172.04±36.88
Estimated GFR (ml min^{-1} 1.73 m^{-2})	44.35±8.60	42.09±8.05*	39.76±8.97†	37.63±10.05
Urinary gene expression[a]				
ACE	1.69 (0.89–6.19)	3.17 (1.06–4.80)	2.59 (0.97–9.57)	3.01(1.44–4.79)
ACE2	2.44 (1.05–5.83)	1.63 (0.81–4.07)	2.54 (1.11–5.27)	3.00 (1.74–6.29)

[a] Data are presented as medians (interquartile range) or means±SD
*p<0.05 vs 0 weeks; †p=0.037 vs ACEI group

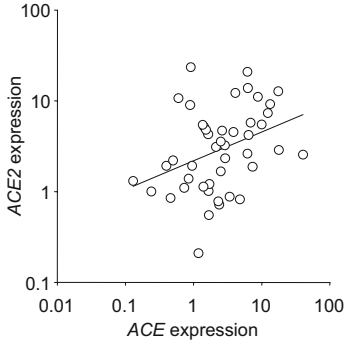

Fig. 1 Relationship between baseline urinary mRNA expression of *ACE* and *ACE2*. Data are compared by Spearman's rank correlation coefficient; r=0.267, p=0.061

modest but statistically significant correlation with urinary *ACE2* (r=−0.289, p=0.042) but not *ACE* expression (Fig. 3).

Change in urinary expression of target genes between groups Urinary mRNA expression of *ACE* and *ACE2* at 0 and 12 week is summarised in Table 2. There was no significant difference between urinary expression of *ACE* or *ACE2* at 0 and 12 weeks in either the ACEI or ACEI+ARB groups. Compared with the ACEI group, the ACEI+ARB group had a marginally lower percentage increase in *ACE* expression after 12 weeks of treatment (median 28.04 vs 20.96, Mann–Whitney U test, p=0.080), but their percentage change in *ACE2* expression was similar. There was no correlation between the percentage reduction in proteinuria and the change in urinary expression of *ACE* or *ACE2* (details not shown).

Urinary gene expression and renal function decline All patients were followed for an average of 12.88±2.84 months. Although GFR decreased significantly after 12 months in both groups, the rate of GFR decline was significantly slower in the ACEI+ARB group than in the ACEI group (0.25±0.42 vs 0.78±0.63 ml min^{-1} 1.73 m^{-2} month^{-1}, p=0.002).

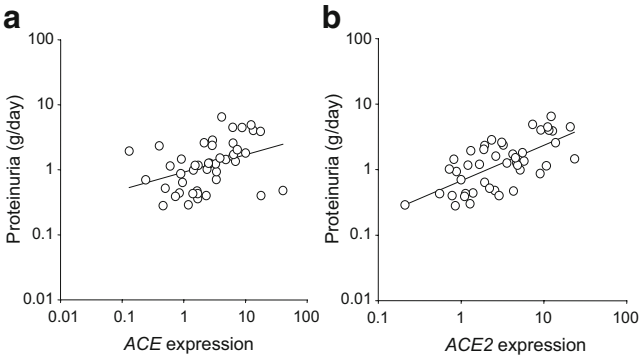

Fig. 2 Relationship between baseline proteinuria and urinary mRNA expression of *ACE* (r=0.454, p=0.001) (**a**) and *ACE2* (r=0.651, p<0.001) (**b**). Data are compared by Spearman's rank correlation coefficient

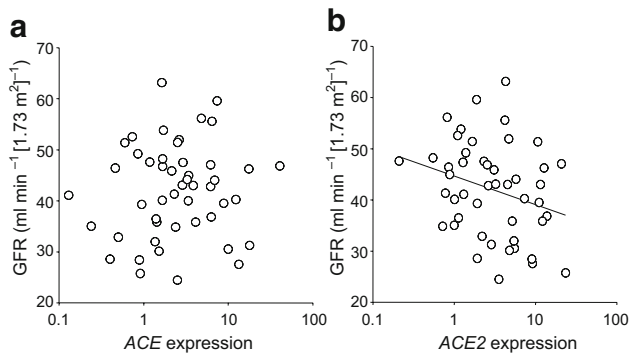

Fig. 3 Relationship between baseline estimated GFR and urinary expression of *ACE* (r=0.056, p=0.699) (**a**) and *ACE2* (r=−0.289, p=0.042) (**b**). Data are compared by Spearman's rank correlation coefficient

In the ACEI group, there was a significant inverse correlation between the rate of GFR decline and urinary *ACE2* expression at baseline (r=−0.423, p=0.031) as well as at 12 weeks (r=−0.395, p=0.046; Fig. 4b). In contrast, there was no significant correlation between the rate of GFR decline and urinary *ACE2* expression at baseline (r=−0.330, p=0.12) or at 12 weeks (r=−0.102, p=0.6) in the ACEI+ARB group. The rate of GFR decline did not correlate with the baseline urinary *ACE* expression of the ACEI group (r=−0.174, p=0.4) or the ACEI+ARB group (r=−0.197, p=0.4).

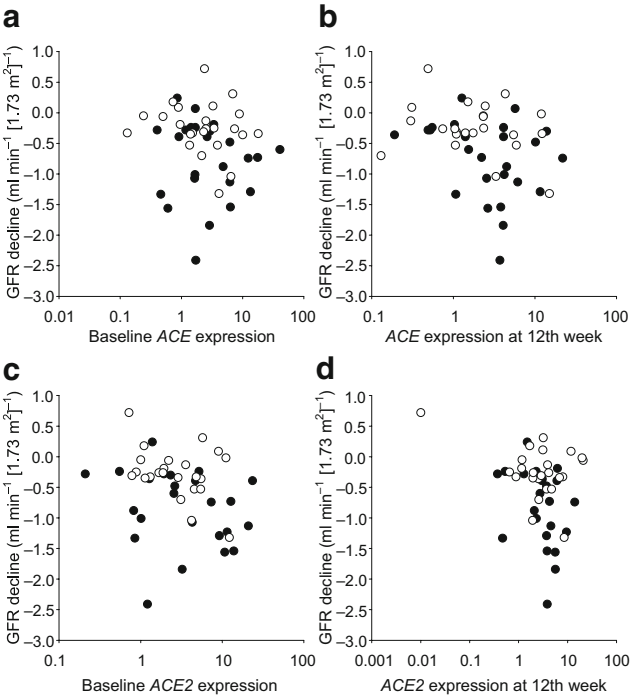

Fig. 4 Relationship between the rate of GFR decline in 1 year and urinary expression of *ACE* (**a**, **b**) and *ACE2* (**c**, **d**). Black circles, ACEI group patients; white circles, ACEI+ARB group patients. See text for separate analysis of the two groups

Discussion

Recent discoveries have identified ACE2 as an important enzyme that generates angiotensin 1–7, which, by binding the G protein-coupled receptor Mas, acts as the main counterregulator against the traditional ACE–angiotensin II axis [20]. In animal models, ACE2 has been proved to be indispensable for maintaining normal renal structure [21]. The intrarenal balance of the RAS components is pivotal in renal disease progression and extensive evidence has shown that local rather than systemic RAS plays the determining role within the kidney [22, 23]. In fact, all known major components of local RAS have been proved to be present in renal parenchymal cells [24]. The present study identified a close relationship between urinary mRNA expression of *ACE2* (and *ACE*) and proteinuria, and to a lesser extent, estimated GFR and GFR decline rate in patients with diabetic nephropathy.

A number of studies have reviewed the relationship between RAS and proteinuria [25, 26]. RAS is a causal factor of many pathophysiological processes of proteinuria [25]. A recent study showed that *ACE2* mRNA expression in human renal tissue marginally correlated with the degree of proteinuria [27]. The strong correlations between proteinuria and urinary expression of *ACE* and *ACE2* observed in our study further supports the role of intrarenal RAS. The inverse correlations between urinary expression of *ACE*/*ACE2* and renal function and its rate of decline imply that urinary expression of RAS-related genes is related to the degree of renal damage. Furthermore, the positive correlation between proteinuria and urinary *ACE2* expression suggests that *ACE2* expression is a compensatory response of renal tissue to insults. In fact, increased renal *ACE2* expression is often regarded as being a renoprotective mechanism [21, 28]. Although the present data cannot affirm the cellular origin of *ACE* and *ACE2* expression in urinary sediment, we believe proximal tubular cells are the main source because other renal cells that can express these genes are not commonly found in the urinary sediment [28]. Although cells of glomerular origin—such as parietal and visceral epithelial cells—do express *ACE2*, previous studies in the *db*/*db* mouse model showed markedly reduced glomerular *Ace2* but high tubular *Ace2* expression in type 2 diabetes [7, 8, 28]. In fact, the correlations we observed may partly reflect an increase in detachment of podocytes and tubular cells in patients with severe renal damage. Although we used 18S RNA to control for each measurement, this housekeeping gene merely safeguards an equal total number of cells in each reaction; any change in the proportion of podocytes or tubular cells would not be detected. Nonetheless, it remains possible that there was an increase in gene expression per cell. Taken together, the present study shows a close relationship between urinary expression of *ACE* and *ACE2* and clinical parameters of renal damage.

There have been inconsistent reports on the change in intrarenal *ACE* and *ACE2* mRNA expression in diabetes. Wysocki et al. [8] recently reported that renal *Ace* mRNA expression, as well as its post-transcriptional production level, was decreased in animal models of diabetes, while *Ace2* expression levels increased significantly. In human renal tissue, however, *ACE* mRNA expression actually increased in diabetes, while *ACE2* mRNA expression did not change [29]. In the present study, we did not observe a significant difference in urinary *ACE* or *ACE2* mRNA expression between diabetic patients and normal control volunteers. Nonetheless, it is important to note that urinary expression levels of *ACE* and *ACE2* are probably different from those in renal tissue because the cellular composition is different [30]. Furthermore, we quantified only the mRNA expression but not the actual enzymatic activity—the result of these may not always parallel each other—of *ACE* and *ACE2*. Although technically demanding, a microplate-based fluorometric method for the concurrent determination of ACE and ACE2 activity in tissue samples has been developed by Wysocki et al. [8], and simultaneous quantification of multiple peptidase activities of ACE, ACE2 and neprilysin was recently described by Shaltout et al. [31]. It is, however, important to note that this fluorometric method has not been tested in urinary sediment, and a single whole-stream urine sample, as in our study, does not yield sufficient sediment for the quantification of enzyme activity.

We did not observe any effect of ARB therapy on the transcriptional level of urinary *ACE* and *ACE2* expression in 12 weeks. Published data on this area are somewhat conflicting. For example, Tikellis et al. [10] found that ACEI prevented the reduction in tubular *ACE2* expression in diabetes and Ferrario et al. [12] found that renal cortex ACE2 activity was significantly augmented in rats treated with lisinopril or losartan but was not changed in those given the combination, while Jessup et al. [32] found increased renal *ACE2* gene expression and activity with either lisinopril or losartan treatment in a hypertensive model (but the effect of combination therapy was not reported). Since we aimed to examine the relationship between the early change in urinary gene expression after initiation of ARB therapy and the subsequent decline in renal function, we did not measure urinary gene expression at later time-points. In this study, we found an inverse correlation between GFR decline and urinary *ACE2* expression in the ACEI group but not the ACEI+ARB group. The reason for this discrepancy is not well explained. On one hand it may reflect the difference in the ACEI—more lisinopril in the ACEI group, but ramipril and perindopril in the ACEI+ ARB group. Theoretically, quantification of urinary gene expression after 1 year of treatment would provide

additional information on the relationship between decline of kidney function and urinary *ACE* or *ACE2* expression. In our present study, the ACEI+ARB group had less proteinuria and a slower GFR decline rate compared with the ACEI group. However, there are currently no data on the change in intrarenal *ACE* and *ACE2* mRNA expression in response to therapy, and the subject needs further investigation. In fact, the correlation between urinary *ACE*/*ACE2* expression and GFR decline was weak, and the effect of therapy could only be directly addressed by an untreated control group, which is unlikely to be possible on ethical grounds.

In conclusion, there was a relationship between urinary mRNA expression of *ACE2* and the degree of proteinuria. The physiological implication and possibility of clinical application of quantifying urinary *ACE2* expression require further study.

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

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