

Synergistic Expression of Angiotensin-Converting Enzyme (ACE) and ACE2 in Human Renal Tissue and Confounding Effects of Hypertension on the ACE to ACE2 Ratio

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# Synergistic expression of ACE and ACE2 in human renal tissue and a confounding effects of hypertension on the ACE/ACE2 ratio

Short title: ACE/ACE 2 expression ratio in human renal tissue

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Key terms: renin-angiotensin system, ACE, ACE2, hypertension, renin, kidney

#### Abstract

Angiotensin-converting enzyme 2 (ACE2), a newly emerging component of the renin-angiotensin system (RAS), is presumed to be a counter-regulator against angiotensin-converting enzyme (ACE) in generating and degrading angiotensin II. It remains to be elucidated how mRNA levels of these 2 genes are quantitatively regulated in the kidney and also what kind of clinico-pathological characteristics could influence the gene expressions in humans. Seventy-eight cases of biopsy-proven renal conditions were examined in details. Total RNA from a small part of each renal cortical biopsy specimen was reverse-transcribed and the resultant cDNA was amplified for ACE, ACE2 and GAPDH with a real time PCR system. Then, we investigated the relationship between clinico-pathological variables and mRNA levels adjusted GAPDH. Statistically significant correlation was not observed between for anv clinico-pathological variables and either of the gene expressions by pairwise comparison. However, a strong correlation was observed between the gene expressions of ACE and those of ACE2. Moreover, the ACE/ACE2 ratio was significantly higher in subjects with hypertension (HT) than that in subjects without HT. While parameters of renal function, e.g., UPE and Ccr, are not significantly related with the ACE/ACE2 ratio as a whole, the HT status may reflect disease-induced deterioration of renal function. That is, UPE and Ccr of subjects with HT are significantly different from those without HT, and where a significant correlation is also observed between UPE and Ccr. Finally, stepwise regression analysis further revealed that only the HT status is an independent confounding determinant of the ACE/ACE2 ratio among the variables tested. Our data suggest that ACE2 might play an important role in maintaining a balanced status of local RAS synergistically with ACE by counter-regulatory effects confounded by the presence of hypertension. Thus ACE2 may exert pivotal effects on cardiovascular and renal disease conditions.

# Introduction

The renin-angiotensin system (RAS) plays important roles in the pathophysiology of cardiovascular and renal conditions  $(1, 2)^{1,2}$  {Hackenthal, 1990 #42; Corvol, 1997 #41}(1, 2). Recently, the existence of the local RAS in tissues such as heart, kidney and blood vessel has been a focus of investigation  $(3)^3$ {Zisman, 2003 #16}(3). Various studies have reported the pivotal characterizations of the tissue RAS (4-6)<sup>4-6</sup>{Sigmund, 2001 #9; Vio, 2003 #2; Mezzano, 2003 #1}(4-6). Angiotensin-converting enzyme (ACE) is one of the most important components in the RAS. From a genetic viewpoint, ACE variants have been conducted tested for their association with cardiovascular and renal diseases, resulting in with the controversy (7-13)<sup>7-13</sup>{Rigat B, 1990 #14; Danser AH, 2000 #37; McNamara DM, 2001 #22; Ortiz MA, 2003 #20; Sakka Y, 2004 #19; Samuelsson O, 2000 #36; Konoshita T, 2001 #38}(7-13). On the other hand, as a novel component of the RAS, a homologue of ACE, designated as ACE2, has been isolated (14, 15)<sup>14,15</sup>{Donoghue M, 2000 #11; Tipnis SR, 2000 #10}(14, 15). This enzyme is highly expressed in the endothelial cells of heart, kidney and testis (16)<sup>16</sup>{Harmer D, 2002 #4}(16) and in contrast to ACE activity, ACE2 mainly degrades angiotensin II (angiotensin II (Ang II)Ang II), which acts as a strong vasoconstrictor and is involved in cellular proliferation, to Angiotensin 1-7 (Ang 1-7), which is thought to act as a vasodilator and to be involved in apoptosis and growth arrest in contrast to ACE activity of generating Ang II and degrading Ang 1-9. Thus, ACE2 might be a potent counter-regulator against ACE and might play a significant role in the regulation of cardiovascular and renal conditions (17-21). Especially, a recent report suggested that deletion of the ACE2 gene leads to the development of angiotensin II-dependent glomerular injury (22){Donoghue M, 2003 #5; Boehm M, 2002 #13; Crackower MA, 2002 #7; Goulter AB, 2004 #28; Lely AT, 2004 #40}(17-21). However, no quantitative, comparative assessment was made for human renal tissue, thereby; the gene expressions of ACE and ACE2, and hence it is expressions are of interest for the elucidation ofto characterize the tissue RAS especially in human renal tissue.

In this study, we evaluated, first, the relationship between clinico-pathological variables and

renal tissue expression of the 2 genes; second, the relationship between ACE and ACE2 gene expressions; finally, the relationship between clinico-pathological variables and the ACE/ACE2 ratio of human renal tissue expression in humans. For this sake, real time PCR with a very small part of renal biopsy specimen was applied, making an accurate quantification of mRNA possible, in spite of the inability in similar protein evaluation because of the limitation of specimens' quantity.

## Methods

## Subjects and clinico-pathological evaluation

Subjects were 78 patients (38 male and 40 female subjects) with biopsy-proven renal diseases. The study was approved by the ethics committee of Fukui University (No.17-12) and informed consent for participation was obtained from all individuals. For each subject, salt-intake was standardized to 10 g daily in hospitalization. Their basal clinical characteristics was as follows (values are means  $\pm$  SD); age 39.1 $\pm$ 19.8 years old, systolic blood pressure (SBP) 123.2 $\pm$ 22.4 mmHg, diastolic blood pressure (DBP) 72.9 $\pm$ 14.4 mmHg, urinary sodium excretion (UNaE) 129.2 $\pm$ 74.5 mEq/gCr, urinary protein excretion (UPE) 1,435 $\pm$ 2,652 mg/gCr, serum creatinine level (s-Cr) 0.87 $\pm$ 0.43 mg/dl, creatinine clearance (Ccr) 96.6 $\pm$ 52.3 ml/min, plasma renin activity (PRA) 2.4 $\pm$ 3.4 ng/ml/hr, plasma aldosterone concentration (PAC) 100.4 $\pm$ 55.9 pg/ml and serum angiotensin converting enzyme (s-ACE) 11.1 $\pm$ 4.1 (IU/l).

The numbers of patients administering each class of antihypertensive drugs at the admission for biopsy were as follows: calcium channel blocker (CCB); 7,  $\alpha$ -blocker; 3, diuretics; 9, ACE inhibitor (ACEI); 1, angiotensin receptor blocker (ARB); 1. However, Administered ACEIs and ARBs were replaced by CCB or  $\alpha$ -blocker at least 1 week before biopsy as ACEIs and ARBs are known to have a significant effect on ACE2 gene expression (23).

The histological diagnosis of the subjects consisted of 7 with minor renal abnormalities, 7 with benign nephrosclerosis, 41 with primary glomerulonephritis including 4 with minimal change nephrotic syndrome, 8 with diabetic nephropathy, 12 with lupus nephritis and 3 with

others. Serum ACE levels were measured in 60 cases by Kasahara's method  $(24)^{22}$ . After 30 minutes' rest in the supine position, blood samples were drawn for the measurement of plasma renin activity (PRA) and plasma aldosterone concentration (PAC). Hypertension (HT) was defined as BP above 140/90 measured in the sitting position on two separate occasions and/or under antihypertensive medication. Glomerular injury was classified into 4 grades (I, II, III and IV) according to Jackson's method  $(25)^{23}$ . Tubulo-interstitial injury was also classified into 3 grades (I, II and III) according to Bertani's method  $(26)^{24}$ .

## Quantification of mRNA of ACE and ACE2 in human renal tissues

A small part of the renal cortex specimen (about 2 mm) was obtained from each subject by echographically-guided percutaneous renal biopsy with 18G needle. Each specimen was presumed to contain about 20-30 glomeruli in this procedure. Immediately after obtaining the biopsy specimen, total RNA was extracted using RNA-Bee (TEL-TEST, INC., USA) according to the protocol recommended for a small amount of samples followed by DNase treatment with Deoxyribonuclease (RT grade) (NIPPON GENE CO., LTD, Japan) by the manufacturers' instructions. Single strand cDNA was synthesized by a reverse transcriptase reaction with 500 ng/µl Oligo-dT (TOYOBO CO. LTD., Japan), 1.0 U/µl RNase inhibitor (TOYOBO CO. LTD., Japan), 50 mM dithiothreitol (DTT) and M-MLV reverse transcriptase (TOYOBO CO. LTD., Japan) and incubated for 1 hour at 37°C. The resultant cDNA was amplified for ACE and ACE2, as well as a house-keeping gene, GAPDH. To access genomic DNA contamination, controls without reverse transcription were included.

Primer sequences were adopted from previous reports for ACE, ACE2(16) and GAPDH (27). The primer for ACE were 5'-CCGAAATACGTGGAACTCATCAA-3' (sense) and 5'-CACGAGTCCCCTGCAT -CTACA-3' (anti-sense) that located from 2430 to 2451 bp (exon 17) and from 2475 bp to 2495 (exon 18) respectively(28, 29) and amplifies a 68 bp region of the mRNA. The primer for ACE2 were 5'-CATTGGAGCAAGTGTTGGATCTT-3' (sense) and 5'-GAGCTAATGCATGCCATTCTCA-3' (anti-sense) that located from 2864 to 2886 bp (exon 18) and from 2950 bp to 2971 (exon 18) respectively (15) and amplifies a 107 bp region of the mRNA. The primer for GAPDH were 5'-CCCATCACCATCTTCCAGGAG-3' (sense) and 5'-GTTGTCATGGATGACCTTGCC-3' (anti-sense) that located from 211 to 231 bp (exon 4/exon5) and from 475 bp to 495 (exon 7) respectively (30, 31) and amplifies a 284 bp region of the mRNA. The real time PCR took place with a final volume of 20µl containing 0.5 mM of forward and reverse primer, and 2µl of single strand cDNA template in 2xQuantiTect SYBR Green PCR Master Mix (QIAGEN Inc., Japan). The highly specific measurement of mRNA was carried out for ACE, ACE2 and GAPDH using the LightCycler system (Roche Diagnostics Inc., Japan). Each sample was run and analyzed in duplicate. The absolute quantification was performed using the samples of known concentration prepared from amplified DNA fragments extracted and purified from agarose gel for electrophoresis for ACE, ACE2 and GAPDH. With this method, 6 orders linearity was attained in serial dilutions of the sample. The mRNA levels of ACE and ACE2 were adjusted for as the values relative to those of GAPDH, which is the most standard house keeping gene for mRNA assessment.

#### Statistical analysis

Statistical analyses were performed with SPSS Version 11.0J (SPSS Japan, Inc., Japan). When the distribution of measurement values was significantly deviated, the values were transformed so as to be consistent with the normal distribution prior to statistical analysis; square root transformation was performed for PRA and logarithmic transformation was performed for UNaE, UPE, and ACE and ACE2 gene expressions (after adjustment with GAPDH). Because the distribution of the [net ACE / net ACE2] ratio was significantly deviated (kurtosis=3.42, skewness=13.1, Kolmogorov-Smirnov test z=2.52, p<0.01), we alternatively adopted the ratio of each log-transformed value, i.e., the [log ACE / log ACE2] ratio, (kurtosis=0.16, skewness=1.70, Kolmogorov-Smirnov test z=0.65, p=0.80) as the ACE/ACE2 gene expression ratio for statistical analysis. Still, its distribution was platykurtic but no further transformation was thought to be adequate. Firstly, linear regression analysis was performed to test the correlation between a pair of variables under investigation. One-way ANOVA followed by the 2-tailed Student *t* test was then performed for the nominal and ordinal data. Stepwise regression analysis was further performed to evaluate the confounding factors for the ACE/ACE2 gene expression ratio with the entry threshold of test variables set to be a p-value of 0.05. Data are expressed as means  $\pm$ SD, unless otherwise indicated.

## **Subjects and Methods**

## Subjects and clinico-pathological evaluation

Subjects were 78 patients (38 male and 40 female subjects) with biopsy-proven renal diseases. The study was approved at by the ethics committee of Fukui University (No.17-12) and informed consent for participation was obtained from all individuals for inclusion onto the study. For each subject, Saltsalt-intake was standardized to 10 g daily in hospitalization. Their basal clinical characteristics was as follows (values are means  $\pm$  SD); male/female 38/40, age 39.1 $\pm$ 19.8 years old, systolic blood pressure (SBP) 123.2 $\pm$ 22.4 mmHg, diastolic blood pressure (DBP) 72.9 $\pm$ 14.4 mmHg, urinary sodium excretion (UNAE) 129.2 $\pm$ 74.5 mEq/gCr, urinary protein excretion (UPE) 1,435 $\pm$ 2,652 mg/gCr, serum creatinine level (s-Cr) 0.87 $\pm$ 0.43 mg/dl, creatinine clearance (Ccr) 96.6 $\pm$ 52.3 ml/min, plasma renin activity (PRA) 2.4 $\pm$ 3.4 ng/ml/hr, plasma aldosterone concentration (PAC) 100.4 $\pm$ 55.9 pg/ml and serum angiotensin converting enzyme (s-ACE) 11.1 $\pm$ 4.1 (IU/l).

The total patients' numbers of patients administered administering each class of antihypertensive drugs depressors at the admission for biopsy were as follows: calcium channel blocker (CCB); 7,  $\alpha$ -blocker; 3, diuretics; 9, ACE inhibitor (ACEI); 1, angiotensin receptor blocker (ARB); 1. When Administered ACEI and/and or ARB had been prescribed to hypertensive patients, they were replaced by CCB or  $\alpha$ -blocker prior to biopsy.

The histological diagnosis of the subjects consisted of 7 with minor renal abnormalities, 7 with benign nephrosclerosis, 41 with primary glomerulonephritis including 4 with minimal change nephrotic syndrome, 8 with diabetic nephropathy, 12 with lupus nephritis and 3 with others. Serum ACE levels were measured in 60 cases by Kasahara's method (22). After 30 minites' rest in the supine position, Blood blood samples for plasma renin activity (PRA) and plasma aldosterone concentration (PAC) measurement were drawn for the measurement of plasma renin activity (PRA) and plasma aldosterone concentration (PAC) after 30minites'supine position. Hypertension (HT) was defined as BP above 140/90 measured in the supine position on two separate occasions and/or under antihypertensive therapymedication. Glomerular injury was

classified into 4 grades (I, II, III and IV) according to Jackson's method (23). Tubulo-interstitial injury was also classified into 3 grades (I, II and III) according to Bertani's method (24).

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The primer sequences for primers ACE and ACE2 were as follows; ACE: 5'-CCGAAATACGTGGAACTCATCAA-3' (sense) and 5'-CACGAGTCCCCTGCAT -CTACA-3' (anti-sense); ACE2: 5'-CATTGGAGCAAGTGTTGGATCTT-3' (sense) and 5'-GAGCTAATGCATGCCATTCTCA-3' (anti-sense). The real time PCR reaction took place with a final volume of 20µl containing 0.5 mM of forward and reverse primer, and 2µl of single strand cDNA template in 2xQuantiTect SYBR Green PCR Master Mix (QIAGEN Inc., Japan). The highly specific Measurement measurement of specific mRNA of ACE, ACE2 and GAPDH was carried out for ACE, ACE2 and GAPDH using the LightCycler system (Roche Diagnostics Inc., Japan). Each sample was run and analyzed in duplicate. The absolute quantification was absolutely performed using the samples of known concentration in each run. With this method, 6 orders linearity was obtainedattained. The mRNA levels of ACE and ACE2 were adjusted for as relative the values relative to those of GAPDH mRNA.

# Statistical analyses analysis

Statistical analyses were performed with SPSS Version 11.0J (SPSS Japan, Inc., Japan). In the case of measures that When the distribution of measurement values werewas significantly not confirmed to be normally distributed deviated, values the values were transformed so as to be consistent with the normal distribution prior to statistical analysis.; To normalize the data distribution for analysis, square root transformation was performed for PRA and logarithmic transformation was performed for UNaE, UPE, and ACE/ GAPDH gene expressions and ACE2/GAPDH gene expressions (after adjustment with GAPDH). Because[net / net]ratio was kurtosis=skewness=alternatively log-,i.e., the [log log ],kurtosis=skewness=as the ACE/ACE2 gene expression ratio .Still, itsbut furtherFirstly, Linear linear regression analysis of analysis was used performed to determine test the correlation between 2 a pair of variables under investigation. One-way ANOVA followed by the 2-tailed Student t test was then used performed for the nominal and ordinal data. Stepwise regression analysis was further performed to evaluate the confounding factors for ACE gene expression and the ACE/ACE2 gene expression ratiothe entry threshold of test variables be a p-value of . Data are expressed as means  $\pm$  standard deviation (SD, unless otherwise indicated).

## **Results Results**

#### Clinico-pathological variables and ACE, ACE2 gene expression

First, the relationship between clinico-pathological variables and the tissue ACE, and ACE2 gene expressions was assessed (Tables 1, Table and 2). Though not statistically significant, An an interesting non-significant tendency for of correlation was observed between UPE and ACE2 gene expression (r=-=-0.231, p=0.051). However, no No significant correlation was, however, observed between any other variables and the 2 genes expressions (Table 1). No significant difference of in the 2 genes expressions was observed when each of them was tested for association with clinico-pathological among variables including histological scores (Table 2).

# Relationship between ACE and ACE2 gene expressions

Next, We we evaluated the a potential correlationship between ACE and ACE2 gene expressions. The This relationship is thought to be of interest because the 2 genes' products have the opposite effects on the generation of Ang II, i.e. the opposite action to regarding tissue damage. As a result,Our data showed that there was a significant correlation was revealed between the expression of ACE and ACE2 gene expressionss (r=0.396, P<0.001) (Figure 1). A similar significant correlation was observed in glomerulonephritis group (n=41, r=0.382, P<0.014).

#### Clinico-pathological aspects and ACE/ACE2 gene expression ratio

Based on this finding of a significant correlation, we further evaluated confounding factors (or perturbation) on the ACE/ACE2 ratio of human renal tissue expression. No significant correlation was observed between clinical variables and the ACE/ACE2 gene expression ratio (Table 3). Neither, did histological scoring for glomeruli and interstitial injury show significant relationship with the ACE/ACE2 gene expression ratio (Table 4). However, as for the HT status, the ACE/ACE2 ratio was significantly higher in subjects with HT than in subjects without HT (p=0.0496) (Table 4). Stepwise regression analysis also revealed that only the HT status was an independent determinant of the ACE/ACE2 ratio among the clinico-pathological variables tested, which explained a total of 5.7% of the variance in this measure ( $R^2$ =0.057, p=0.045) (Table 5).

# Clinico-pathological aspects and ACE/ACE2 gene expression ratio

Thus, the evaluation has proceeded to the assessment of ACE/ACE2 ratio of human renal tissue expression. No significant correlation was observed between clinical variables and the genes expression ratio (Table 3). Histological scoring for glomeruli and interstitial injury showed no special relationship with the genes expression ratio (Table 4). However, it was shown that the ACE/ACE2 ratio of subjects with HT was significantly higher than those without HT (p=0.0496) (Table 4). Finally, stepwise regression analysis revealed that only HT was the independent

determinant of the ACE/ACE2 ratio among major clinico-pathological variables, explaining a total of 5.7% of the variance in this measure ( $r^2$ =0.057) (Table 5).

## **Discussion Discussion**

The RAS is a coordinated hormonal cascade and the a central regulator of cardiovascular, renal and adrenal functions that regulates fluid and electrolyte balance and arterial pressure (1, 2)<sup>1,2</sup>{Hackenthal, 1990 #42; Corvol, 1997 #41}(1, 2). Besides, the RAS plays a key role in the pathophysiology of various diseases of the cardiovascular and renal systems (32, 33){Dostal DE, 1999 #32; Dzau, 2001 #33}(25, 26). ACE removes two carboxyl-terminal amino acids from angiotensin I (Ang I) giving rise to Ang II, which is degraded only by peptidases at different amino acid sites to form fragments in the classical concept for the RAS. Recently, an enzyme that cleaves Ang I and Ang II directly into Ang 1-9 and Ang 1-7, respectively, was identified (14, 15)<sup>14,15</sup>{Donoghue M, 2000 #11; Tipnis SR, 2000 #10}(14, 15). This enzyme, a homologue of ACE, is designated as ACE2. ACE and ACE2 have been reported to be highly expressed in the endothelial cells of heart, kidney and testis (14-16)<sup>14-16</sup>{Donoghue M, 2000 #11; Tipnis SR, 2000 #10; Harmer D, 2002 #4}(14-16). The ACE2 mainly degrades angiotensin II (Ang II), which acts as a strong vasoconstrictor and is involved in cellular proliferation, to Angiotensin 1-7 (Ang 1-7), which is thought to act as a vasodilator and to be involved in apoptosis and growth arrest in contrast to ACE activity of generating Ang II and degrading Ang 1-7. Thus, ACE2 might be a potent counter-regulator against ACE. As local Ang II is thought to be a pivotal mediator in cardio-vascular and renal injuryinjuries, the ACE2 has emerged as a potential inhibitor of Ang II generation against the action of ACE. Recently, Some some knowledge of the functional roles of ACE2 in the cardiac RAS has recently been provided for functional roles of ACE2 in the cardiac and renal RAS (17-22){Donoghue M, 2003 #5; Boehm M, 2002 #13; Crackower MA, 2002 #7; Goulter AB, 2004 #28}(17-20).

As an assessments for of the human intra-renal RAS other than animal models (27, 28), there is only one report it was only reported that in primary and secondary renal disease

conditions and renal transplants, "neo-"expression of ACE2 was found in several renal conditions glomerular and peritubular capillary endothelium in human study based on unnon-quantitative histological evaluation in humans so far  $(21)^{21}$  {Lely AT, 2004 #40}(21) other than animal models (34, 35){Tikellis C, 2003 #6; Minghao, 2004 #44}(27, 28). The role and the significance of ACE2 in the regulation of the RAS in human renal tissue must be yetremain to be well well elucidated with quantitative evaluations. Moreover, clinical and pathological factors modulating the expression of ACE and ACE2 must remain to be well well elucidated. We have already reported an up-regulation of the ACE expression in diabetic nephropathy among major components of the RAS in a our recent previous study (36){Konoshita, 2006 #45}(Diabetes Care in press). So In this line, first, we first assessed the relationship between clinico-pathological characteristics other than diabetes and the tissue gene expression of ACE and ACE2 in human renal tissues. However, iIn this the current study, we were unable tocould not detect any significant relationship between clinico-pathological characteristics, i.e., including HT, UPE, Ccr or and pathological grade of glomerular-interstitial changes, and each of the 2 gene expressions when accessed with solely each genebeing assessed individually. As Consequently, the evaluation of the relationshipwe could detect a significant correlation between ACE and ACE2 gene expressions, revealed a significant correlation. We we consider that the this result finding is very important for elucidating the mechanisms of the interplay between ACE and ACE2, and for establishing the role of ACE2 as a counter-regulator against ACE. Especially, the significance of the results showing positive but not negative correlation would be controversial. Our data suggest the ACE2 gene expression is not actively involved in alteration of the local RAS status, rather, it is can be speculated that ACE and ACE2 gene expressions might may be regulated in a balanced induction statemanner, or synergistically, which is mediated via the local Ang II concentration. The exact mechanism for this regulation is not clear so far and future study including transcriptional analysis would clarify the issue. A notable point here is that not only ACE but also ACE2 is notmay be responsible for the local Ang II generation, while a balanced expression of ACE2 exerts a counter action to against ACE activities. ThusWith reference to the above

speculation, the role of ACE2 must be taken into consideration for the pathogenesis of renal tissue injury.

At the same time, tThis synergistic expression implies has prompted us to test a the possibility that some clinico-pathological characteristics have substantial effects on the ACE/ACE2 gene expression ratio. ThusIndeed, the relationship between clinico-pathological characteristics and the ratio was assessed. It wasour assessment of the relationship between clinico-pathological variables and the ACE/ACE2 ratio of renal tissue expression revealed that ACE/ACE2 ratio of renal tissue expression the ACE/ACE2 ratio is significantly related withinfluenced by the HT status. While parameters of renal function, e.g., UPE and Ccr, are not significantly related with the ACE/ACE2 ratio as a whole (Table 5), the HT status may reflect disease-induced (or disease-specific) deterioration of renal function. That is, UPE and Ccr of subjects with HT are significantly different from those without HT (UPE: 3.16±0.69 vs. 2.34±0.67, p<0.0001; Ccr: 63.1±41.5 vs. 108.2±50.9, p<0.0001, respectively), and where a significant correlation is also observed between UPE and Ccr (r=-=-0.396, p<0.001). Finally, Stepwise regression analysis has further revealed that only the HT status wasis the an independent determinant of the ACE/ACE2 ratio among major clinico-pathological variables tested. Which of the HT status and ACE/ACE2 ratio is the cause and the result must be an issue. It seems that the HT status should be the cause for the alteration of the ratios, however, this is difficult to address and additional assessments, for example, repeated biopsies and/or prospective follow-up, would be needed for solve the issue. Anyway, these results suggest that HT as a cardiovascular risk factor might effect oninfluence the renal ACE/ACE2 expression ratio and consequently might hereby play a pivotal role in renal conditions, presumably, affecting the tissue concentrations of Ang II and Ang 1-7.

In summary, the gene expressions of ACE and ACE2 were assayed with a very small quantity amount of human renal tissues by quantitative methods and conducted assessed for the relationship with clinico-pathological characteristics. A significant correlation was observed between the gene expressions of ACE and those of ACE2. The ACE/ACE2 ratio is significantly

related withinfluenced by the HT status. These findings suggest that besides ACE, ACE2, a newly-emerging component of the RAS, ACE2 mightis likely to play an important role in maintaining a balanced status of local RAS synergistically with ACE by counter-regulatory effects confounded by the presence of hypertension. in renal tissue and may have take a pivotal role part in the pathophysiology for of cardiovascular and renal and presumably cardiovascular conditions. Further investigation focusing also on ACE2 inhibitor (37){Dales NA, 2002 #17}(29) and ACE2 genetic variants (38){Benjafield, 2004 #27}(30) would provide important data for aenable us to better understanding of the roles of these enzymes–ACE and ACE2– in cardiovascular and renal conditions.

(29)(30)(31)

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Figure 1. Relationship between ACE and ACE2 mRNA levels in renal tissues. The mRNA levels were adjusted as relative values to GAPDH mRNA. To normalize the data distribution for statistical analysis, logarithmic transformation was performed for ACE and ACE2 gene expressions (after adjustment with GAPDH). logarithmic transformation was performed for ACE/GAPDH gene expressions and ACE2/GAPDH gene expression. (r=0.396, P<0.001).

|                | ACE   |      | _ | ACE2  |       |
|----------------|-------|------|---|-------|-------|
|                | r     | р    |   | r     | р     |
| Age (years)    | 0.11  | 0.36 |   | 0.08  | 0.50  |
| UNaE (mEq/gCr) | 0.05  | 0.70 |   | 0.11  | 0.36  |
| UPE (mg/gCr)   | -0.04 | 0.72 |   | -0.23 | 0.051 |
| Ccr (ml/min)   | 0.18  | 0.12 |   | 0.19  | 0.09  |
| PRA (ng/ml/hr) | -0.11 | 0.37 |   | -0.10 | 0.80  |
| PAC (pg/ml)    | 0.18  | 0.14 |   | 0.18  | 0.13  |
| s-ACE (IU/I)   | 0.05  | 0.71 |   | 0.04  | 0.77  |

Table 1. Correlation between clinical variables and renal tissue mRNA levels of ACE and ACE2

UNaE: urinary sodium excretion, UPE: urinary protein excretion, Ccr: creatinine clearance, PRA: plasma renin activity, PAC: plasma aldosterone concentration, s-ACE: serum angiotensin converting enzyme. To normalize the data distribution for statistical analysis, square root transformation was performed for PRA and logarithmic transformation was performed for UNAE, UPE, and ACE and ACE2 gene expressions (after adjustment with GAPDH).square root transformation was performed for UNAE, UPE, ACE/GAPDH gene expressions and ACE2/GAPDH gene expression. \*p <0.05.

|                    | ACE         |                    | ACI             | E2                 |
|--------------------|-------------|--------------------|-----------------|--------------------|
| Variables (n)      | mean±SD     | р                  | mean±SD         | р                  |
| Gender             |             |                    |                 | ·                  |
| male (38)          | 2.12±0.65   | 0.48               | 2.90±0.71       | 0.34               |
| female (40)        | 2.02±0.10   |                    | 3.04±0.57       |                    |
| нт                 |             |                    |                 |                    |
| - (58)             | 2.04±0.57   | 0.48               | 3.02± 0.61      | 0.19               |
| + (20)             | 2.15±0.62   |                    | $2.80 \pm 0.73$ |                    |
| Glomerular scler   | osis score* |                    |                 |                    |
| l (36)             | 2.10±0.55   | 0.73 (vs. II)      | 3.00±0.70       | 0.80 (vs. II)      |
| II (29)            | 2.05±0.61   | 0.48 (vs. III, IV) | 3.01±0.63       | 0.40 (vs. III, IV) |
| III and IV (12)    | 1.91±0.49   | 0.33 (vs. I)       | 2.82±0.50       | 0.51 (vs. I)       |
| Tubulo-interstitia | l score**   |                    |                 |                    |
| l (25)             | 2.01±0.78   | 0.56 (vs. II)      | 2.97±0.59       | 0.87 (vs. II)      |
| II (39)            | 2.10±0.52   | 0.95 (vs. III)     | 2.95±0.72       | 0.81 (vs. III)     |
| <u>III (13)</u>    | 2.10±0.31   | 0.71 (vs. I)       | 3.00±0.16       | 0.92 (vs. I)       |

Table 2. Clinico-pathological variables and renal tissue mRNA levels of ACE and ACE2

HT: hypertension, Glomerular injury was classified into 4 grades (I, II, III and IV) according to Jackson's method (25)<sup>23</sup>{Jackson B, 1988 #30}(23) Tubulo-interstitial injury was classified into 3 grades (I, II and III) according to Bertani's method (26)<sup>24</sup>{Bertani T, 1986 #31}(24). One of samples was not adequate for glomerular assessment and the other one was not adequate for tubulo-interstitial assessment.{Jackson B, 1988 #30}(23) To normalize the data distribution for statistical analysis, logarithmic transformation was performed for ACE and ACE2 gene expressions (after adjustment with GAPDH).logarithmic transformation was performed for ACE and ACE2 gene expressions and ACE2/GAPDH gene expression.

|                | ACE// | ACE2 |
|----------------|-------|------|
|                | r     | р    |
| Age (years)    | 0.10  | 0.37 |
| UNaE (mEq/gCr) | -0.10 | 0.41 |
| UPE (mg/gCr)   | 0.17  | 0.15 |
| Ccr (ml/min)   | 0.01  | 0.91 |
| PRA (ng/ml/hr) | 0.06  | 0.61 |
| PAC (pg/ml)    | -0.04 | 0.75 |
| s-ACE (IU/I)   | 0.11  | 0.42 |

Table 3. Correlation between clinical variables and the renal ACE /ACE2 expression ratio

UNaE: urinary sodium excretion, UPE: urinary protein excretion, Ccr: creatinine clearance, PRA: plasma renin activity, PAC: plasma aldosterone concentration, s-ACE: serum angiotensin converting enzyme. To normalize the data distribution for statistical analysis, square root transformation was performed for PRA and logarithmic transformation was performed for UNaE, UPE, and ACE and ACE2 gene expressions (after adjustment with GAPDH)square root transformation was performed for PRA and logarithmic transformation was performed for UNaE, UPE, ACE/GAPDH gene expressions and ACE2/GAPDH gene expression. \*p <0.05.

|                                   | ACE/ACE2       |          |             |      |
|-----------------------------------|----------------|----------|-------------|------|
| Variables (n)                     | mean±SD        | р        |             | _    |
| Gender                            |                |          |             | _    |
| male (38)                         | 0.76±0.22      | 0.07     |             |      |
| female (40)                       | 0.67±0.21      |          |             |      |
| HT                                |                |          |             |      |
| - (58)                            | 0.69±0.20      | 0.05049  | )*          |      |
| +(20)                             | 0.80±0.25      |          |             |      |
|                                   |                |          |             |      |
| Glomerular sclerosis scor         | re             |          |             |      |
| l (36)                            | 0.74±0.24      | 0.38 (vs | 5. II)      |      |
| II (29)                           | 0.69±0.20      | 0.96 (vs | 5. IIÍ, IV) |      |
| III and IV (12)                   | 0.70±0.20      | 0.54 (vs | s. I)       |      |
| <b>-</b> 1 1 1 <i>1 2 2 2 2 1</i> |                |          |             |      |
| I ubulo-interstitial score        |                |          |             | ,    |
| l (25)                            | 3.430.68±4.980 | .26      | 0.94 32     | (vs. |
| II)                               |                |          |             |      |
| II (39)                           | 3.560.74±6.420 | .19      | 0.82 88     | (vs. |
| III)                              |                |          |             |      |
| <u> </u>                          | 4.010.73±8.380 | .21      | 0.79 55     | (vs. |
| 1)                                |                |          |             |      |

Table 4. Clinico-pathological variables and the renal ACE /ACE2 expression ratio

HT: hypertension, \*\*Glomerular injury was classified to 4 grades (I, IV) according to Jackson's method  $(25)^{23}$ . II, III and Tubulo-interstitial injury was classified to 3 grades (I, II and III) according to Bertani's method (26)<sup>24</sup>{Bertani T, 1986 #31}(24). One of samples was not adequate for glomerular assessment and the not adequate for tubulo-interstitial other one was assessment.{Jackson B, 1988 #30}(23) To normalize the data distribution for statistical analysis, logarithmic transformation was performed for ACE and ACE2 gene expressions (after adjustment with GAPDH).logarithmic transformation was performed for ACE/GAPDH gene expressions and ACE2/GAPDH gene expression. \*p <0.05.

Table 5. Stepwise regression analysis for the renal ACE /ACE2 expression ratio

| Variable | r     | р      |
|----------|-------|--------|
| HT       | 0.239 | 0.045* |

| Gender                     | 0.226 |
|----------------------------|-------|
| UPE                        | 0.466 |
| Ccr                        | 0.831 |
| Glomerular sclerosis score | 0.281 |
| Tubulo-interstitial score  | 0.872 |

HT: hypertension, UPE: urinary protein excretion, Ccr: creatinine clearance. To normalize the data distribution for statistical analysis, logarithmic transformation was performed for ACE/GAPDH gene expressions and ACE2/GAPDH gene expression. HT was coded 0 or 1 with reference to - or +. Gender was coded 0 or 1 with reference to female or male. Glomerular sclerosis score and tubulo-interstitial score was coded 1, 2 or 3 with reference to the grades. \*p <0.05.