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Association Between Angiotensin-Converting Enzyme 2 and Coronary Artery Calcification in Patients on Maintenance Hemodialysis Therapy

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Abstract: While all mechanisms that contribute to the pathogenesis of coronary artery calcification (CAC) are unknown, angiotensin-converting enzyme 2 (ACE2) may be involved in this process in maintenance hemodialysis (MHD) patients. The aim of this study was to investigate the association between ACE2 and CAC in patients on MHD therapy. Ninety patients on MHD therapy were involved in this prospective study. CAC was quantified by CAC score (CACs) using the Agatston method and a multi-slice CT scanner. Univariate and multivariate logistic regression were used to analyze the association between ACE2 and CACs. In the univariate analysis, CACs positively correlated with ACE2 ($r = 0.666$, $P < 0.001$). After adjusting for age, sex, smoking, hypertension, body mass index, diabetes mellitus, and hyperlipidemia, ACE2 levels continued to significantly and independently predict the presence of CAC. ROC curve analysis showed that the serum ACE2 level can predict the extent of CAC. These findings indicate that elevated serum ACE2 may be involved in vascular calcification in patients receiving MHD therapy. **Key Words:** Angiotensin-converting enzyme 2, Coronary artery calcification, Maintenance hemodialysis patients.

Patients on maintenance hemodialysis (MHD) therapy have a high prevalence of vascular calcification, especially coronary artery calcification (CAC) (1,2), its strong association with mortality has been recognized in early chronic kidney disease (CKD) (3,4), and CAC can predict mortality in MHD patients independent of traditional and nontraditional risk factors (5). The mechanisms that contribute to the pathogenesis of CAC are complex and numerous; they include both traditional and non-traditional cardiovascular risk factors such as inflammation, oxidative stress, and the renin–angiotensin system (RAS) (6,7). Angiotensin-converting enzyme 2 (ACE2) is a novel regulator of RAS that counteracts the adverse effects of angiotensin II (Ang II) (8). Altered ACE2 expression is associated with cardiac and vascular disease in experimental models of cardiovascular disease (CVD) (9,10), and ACE2 increases in failing human hearts and atherosclerotic vessels (11,12). The present study examined the association between ACE2 and CAC in patients on MHD therapy.

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PATIENTS AND METHODS

Patients

Ninety patients on MHD were asked to participate in this study from August 2012 to June 2014. Inclusion criteria were: (i) male or female >18 years of age; and (ii) patients on MHD for at least 6 months. Exclusion criteria included: (i) use of corticosteroids; (ii) presence of chronic inflammatory disease; and (iii) active malignancy. The local research ethics committee approved the study and informed consent was obtained from all participants.

CAC scoring

Patients underwent CAC quantification within 1 week after the serum and plasma were collected by a multi-slice CT scanner using a gantry rotation of 0.4 s, collimation of 2.5 mm (slice thickness), and reconstruction time of six frames per second. A calcium threshold ≥130 Hounsfield units was used. The

images were scored by a single radiologist blinded to the clinical and biochemical aspects of the patient. As described by Agatston (13), the calcium score was determined by multiplying the area of each calcified lesion by a weighting factor corresponding to the peak pixel intensity for each lesion. The sum of each lesion of all coronary arteries was used for analysis. CAC scores were categorized into five degrees according to the cutoff values commonly used in the literature: ≤10 Agatston units (AU) (minimal or none); 11–100 AU (mild calcification); 101–400 AU (moderate calcification); 401–1000 AU (severe calcification); and greater than 1000 AU (extensive calcification) (14).

ACE2 activity

Serum and plasma were collected from patients at baseline, and stored at –80°C until analysis.

Plasma ACE2 activity was measured by ELISA (Senxiong, Shanghai, China) and the procedures were as follows. ACE2 antibody was added to a microtiter well plate and incubated. Biotinylated anti-ACE2 antibody was then added and combined with streptavidin-HRP to result in a compound. After incubation and thorough washing, 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added. When the TMB substrate became blue, the reaction was terminated by the addition of a sulphuric acid solution. The color change was then measured spectrophotometrically at a wavelength of 450 nm. ACE2 concentration in the samples was then determined by comparing the OD of the samples to the standard curve. The ELISAs were performed in duplicate.

Other parameters

Other parameters included sex, age, smoking, diabetes, dialysis duration, Kt/V urea-dialysis, total cholesterol (TC), triglycerides (TG), body mass index (BMI), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), phosphorus, ionized calcium, parathyroid hormone (PTH), hypertension and hemoglobin (Hb).

Statistical analyses

Variables are described as mean and standard error, median and interquartile values, or frequency and proportion. ANOVA or χ^2 test was used to compare groups with different ACE2. Pearson's or Spearman's correlation test was used to assess the strength of the association between ACE2 and CACs, as well as with other parameters. Subsequently, multivariate logistic regression analysis was performed to determine whether ACE2 was associated with CACs. Additionally, a receiver operating characteristics (ROC) curve was plotted for serum ACE2 level with severe and extensive calcification, thus evaluating the ability of each variable to classify the severity of coronary artery calcification. The area under the curve and 95% confidence intervals were calculated for this receiver operating characteristics curve. A *P*-value of <0.05 was considered statistically significant. All statistical analyses were performed using SPSS 17 (IBM, Cary NC, USA).

RESULTS

Patient characteristics

A total of 90 consecutive patients met inclusion criteria and were enrolled in this study. Of the 90 patients, 17 patients had no evidence of CAC and the other 73 (81.1%) patients had CACs from 0.5 to 2152.2 AU, and the average was 344.36 ± 43.62 (AU). Minimal calcification (≤10 AU) was noted in six patients (6.6%), mild in 14 patients (15.6%), moderate (101–400 AU) in 27 (30%), severe (401–1000 AU) in 20 (22.2%), and extensive (>1000 AU) in six patients (6.0%). Serum ACE2 was measured two times, and the value showed a fixed tendency in that case. The average serum ACE2 level was 47.84 ± 25.61 (U/L). Among patients with higher CAC scores, ACE2 levels were also significantly increased ($P < 0.001$, Fig. S1). Clinical and laboratory data based on serum ACE2 levels are shown in Table 1. Mean age (± SE) was 60.0 years (± 14.9) and 52% of patients were male. Causes of renal failure were glomerulonephritis ($N = 37$), diabetes mellitus ($N = 24$), hypertension ($N = 23$), and other conditions ($N = 6$). Average duration of MHD therapy was 4.8 years (± 0.2) and the mean Kt/V was 1.34 (± 0.06). Gender, CAC scores, hypertension and diabetes were significantly different for the various ACE2 levels ($P < 0.05$).

Association between ACE2 and CAC

Univariate analysis showed the following: ACE2 positively correlated with CAC (r = 0.666, $P < 0.001$); hypertension (r = 0.308, $P < 0.001$); sex (r = 0.362, $P < 0.001$) and diabetes (r = 0.406, $P < 0.001$). However, ACE2 did not correlate with age; duration of dialysis; Kt/V; BMI; TG; TC; HDL-C; LDL-C; phosphorus; PTH; and Hb (Table 2). Using the lowest ACE2 tertile as a reference, the unadjusted odds ratio for the presence of CAC in the highest ACE2 tertile was 1.45 (95% confidence interval [CI], 1.04–2.37). The age-adjusted odds ratio was 1.48 (95% CI, 1.10–2.32) for the second ACE2 tertile and 2.21 (95% CI, 1.37–3.04) for the highest ACE2 tertile. After

TABLE 1. *Clinical and laboratory data for maintenance hemodialysis (MHD) patients divided into tertiles of serum angiotensin-converting enzyme 2 (ACE2) levels (U/L)*

	All patients ($N = 90$)	ACE2 ≤ 30 ($N = 17$)	30 < ACE2 < 60 ($N = 52$)	ACE2 ≥ 60 ($N = 21$)	*P*-value
Male (n, %)	47 (52.0)	5 (29.4)	24 (46.1)	18 (85.7)	<0.001*
Age (years)	60.0 ± 14.9	53.0 ± 17.7	60.6 ± 14.3	64.9 ± 12.3	0.067
Smokers (n, %)	24 (26.6)	5 (29.4)	14 (26.9)	5 (23.8)	0.925
Diabetes (n, %)	30 (33.3)	3 (17.6)	13 (25.0)	14 (66.7)	<0.001*
Dialysis duration (y)	4.8 ± 0.2	5.3 ± 0.4	4.9 ± 0.3	4.1 ± 0.4	0.175
Kt/V urea-dialysis	1.34 ± 0.06	1.36 ± 0.16	1.31 ± 0.08	1.39 ± 0.07	0.534
BMI (kg/m^2)	22.3 ± 0.4	21.6 ± 1.1	22.5 ± 0.4	22.4 ± 0.5	0.691
TC (mmol/L)	4.33 ± 0.67	4.35 ± 0.71	4.25 ± 0.66	4.49 ± 0.69	0.387
TG (mmol/L)	1.8 ± 0.2	1.7 ± 0.4	1.8 ± 0.1	1.9 ± 0.7	0.188
LDL-C (mmol/L)	2.45 ± 0.58	2.63 ± 0.56	2.47 ± 0.60	2.26 ± 0.54	0.145
HDL-C (mmol/L)	1.24 ± 0.24	1.20 ± 0.16	1.27 ± 0.26	1.20 ± 0.24	0.407
Phosphorus (mmol/L)	1.56 ± 0.38	1.41 ± 0.29	1.57 ± 0.39	1.64 ± 0.42	0.169
Calcium (mmol/L)	2.29 ± 0.02	2.34 ± 0.21	2.38 ± 0.03	2.02 ± 0.04	0.543
PTH (pg/mL)	223.5 ± 20.8	204.12 ± 38.3	243.3 ± 30.5	190.0 ± 36.7	0.533
Hypertension(n, %)	72 (80.0)	10 (58.9)	42 (80.7)	20 (95.2)	0.020*
Hb (g/dL)	10.0 ± 2.1	9.64 ± 2.01	9.94 ± 2.23	10.02 ± 2.30	0.852
CAC (AU)	344.36 ± 43.62	59.33 ± 18.25	284.59 ± 37.89	723.18 ± 126.88	<0.001*

Results were expressed as mean ± SE or number (percentage), and were analyzed by ANOVA or Chi-square test; *$P < 0.05$. BMI, body mass index; CAC, coronary artery calcification; Hb, hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PTH, parathyroid hormone; TC, total cholesterol; TG, triglycerides.

further adjustment for age, sex, smoking, hypertension, body mass index, diabetes mellitus, hyperlipidemia, ACE2 levels were still associated with the presence of CAC, with an odds ratio of 1.58 (95% CI, 1.10–2.84) in the highest ACE2 tertile (Table 3). Receiver-operating characteristic curve analysis (Fig. S2) showed that serum ACE2 level can predict the extent of CAC (area under the curve values were 0.752, ($P < 0.001$)). The sum of sensitivity and specificity for prediction of the extent of CAC was maximal at an ACE2 level of ≥32.55 U/L (sensitivity = 96.7% specificity = 60%).

TABLE 2. *Bivariate correlation between serum angiotensin-converting enzyme 2 (ACE2) levels and cardiovascular risk factors*

Variable	Correlation coefficient	*P*-value
Male (*N*, %)	0.362	<0.001*
Age (years)	0.215	0.052
Smokers (*N*, %)	0.073	0.492
Diabetes (*N*, %)	0.406	<0.001*
Dialysis duration (y)	–0.207	0.050
Kt/V urea-dialysis	–0.112	0.293
BMI (kg/m^2)	–0.056	0.599
TC (mmol/L)	0.022	0.839
TG (mmol/L)	0.042	0.692
LDL-C (mmol/L)	0.126	0.235
HDL-C (mmol/L)	–0.075	0.939
Phosphorus (mmol/L)	0.086	0.423
Calcium (mmol/L)	–0.156	0.143
PTH (pg/mL)	–0.052	0.626
Hypertension(n, %)	0.308	<0.001*
Hb (g/dL)	0.023	0.826
CAC (AU)	0.666	<0.001*

*$P < 0.05$.

ACE2, angiotensin-converting enzyme 2; BMI, body mass index; CAC, coronary artery calcification; Hb, hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MHD, maintenance hemodialysis; PTH, parathyroid hormone; TC, total cholesterol; TG, triglycerides.

DISCUSSION

This cross-sectional study of MHD subjects evaluated the association between serum ACE2 level and CAC, which is a good biomarker for the presence and extent of coronary atherosclerosis. The major finding of the study was that ACE2 positively correlated with CAC and that ACE2 was an independent risk factor for the presence of CAC.

Hemodialysis patients were found to have significantly more CACs than non-HD patients of the same age and sex (15). Many studies have shown the prevalence of CAC in HD patients to be 72 to 83% (1,16). Results of the current study indicated that 81.1% of MHD patients had CACs >0, thereby supporting the high prevalence of coronary calcification in this population. In agreement with previous studies, the current study observed that gender, hypertension and diabetes were significantly different for the various ACE2 levels (17). To the author's knowledge, the present study is the first to report a significant and independent association between serum ACE2 and CAC in MHD patients.

TABLE 3. *Serum angiotensin-converting enzyme 2(ACE2) levels predict the presence of coronary artery calcium for multivariate logistic regression analysis model*

	ACE2			
Variable	Tertile 1	Tertile 2	Tertile 3	*P*-value
Unadjusted OR	1.0	1.21 (1.02–1.84)	1.45 (1.04–2.37)	0.020
Age adjusted OR	1.0	1.48 (1.10–2.32)	2.21 (1.37–3.04)	0.016
Multivariate model 1† OR	1.0	1.12 (0.82–1.75)	1.32 (0.95–2.21)	0.034
Multivariate model 2‡ OR	1.0	1.13 (0.82–1.86)	1.58 (1.10–2.84)	0.021

†Adjusted for age, sex, smoking. ‡Adjusted for age, sex, smoking, hypertension, body mass index, diabetes mellitus, hyperlipidemia. Values in parentheses are 95% confidence intervals. OR, odds ratio.

ACE2 is a negative regulator of the renin-angiotensin system, which catalyzes the conversion of angiotensin II to angiotensin-(1–7), thereby counterbalancing ACE activity (18,19). Animal studies have shown that ACE2 inhibits vascular calcification in rats (9), and that oral administration of an ACE2 activator improves diabetes-induced cardiac dysfunction (20). Several studies have shown that the enzymatic activity of ACE2 has a protective role in cardiovascular diseases in humans (21), and loss of ACE2 can be detrimental, as it leads to functional deterioration of the heart and progression of cardiac, renal, and vascular pathologies (22,23). So ACE2 is supposed to act protectively against the progression of cardiovascular tissue lesions including arterial calcification. However, our study shows the positive correlation between CAC and ACE2 activity, and this is actually in agreement with previous studies. Plasma ACE2 levels are low in normal healthy volunteers (24), and are upregulated in cardiovascular diseases (25). That is to say, increased ACE2 is the result of CAC, and this may partly interpret the positive correlation between CAC and ACE2 in our research.

ACE2 is predominantly expressed in the kidneys, heart, and testes, and at lower levels in a wide variety of tissues, particularly the colon and lung (19). The source of circulating ACE2 in healthy individuals and CKD patients is not clear but release from the kidneys is a possibility. The levels of ACE2 activity have been found to be 10- to 30-fold higher in mouse kidney cortex than in the heart (26). The mechanism of how ACE2 reaches the circulation and is then released into plasma is not well understood and requires further examination. But in our study, we think that the heart was the most important origin of higher ACE2 levels, because the kidneys are quite atrophied in ESRD so they do not generate much ACE2. Roberts et al. (17). showed that among patients with CKD, plasma ACE2 activity was lower in those undergoing hemodialysis for end stage renal disease (ESRD) when compared with predialysis patients with CKD or renal transplant patients. That can be easy understood: ACE2 was distributed to the luminal surface of tubular epithelial cells in the kidney (8), and that plasma ACE2 activity increased early in the course of CKD followed by a relative decrease as CKD became established, with a further decrease in end-stage kidney disease (ESRD) (27).

When CAC occurred in the MHD patients, as a form of coronary atherosclerosis lead to endothelial damage, and as a regulator of heart disease, ACE2 expression was upregulated in calcified arteries, and prevented the phenotypic transition of vascular smooth muscle cell (VSMC) into osteoblasts. Thus, CAC development was attenuated (9), at least in part by decreasing the levels of the endogenous ACE/Ang II/AT1 axis, the inhibition of which has been shown to attenuate the progression of vascular calcification (28,29). Meanwhile, in another animal study, Zhang et al. demonstrated that ACE2 overexpression significantly inhibited early atherosclerotic lesions by suppressing VSMC proliferation and migration and improving endothelial cell function (30).

CONCLUSION

In summary, we provide the first evidence that elevated serum angiotensin-converting enzyme 2 may be involved in vascular calcification in patients receiving maintenance hemodialysis therapy. This study was a preliminary study, further investigations using larger patient cohorts are required to better understand the contribution of ACE2 to the high prevalence of vascular calcification in MHD patients.

Conflict of interest: The authors declare that they have no conflict of interests.

Disclosures: none.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. The mean and intertertile ranges of ACE2 in patients with minimal, mild, moderate, severe, and extensive coronary calcification respectively.

Fig. S2. Receiver-operating characteristic curve analysis showing the prognostic value of ACE2 levels in predicting severe and extensive calcification (CAC score >400).