

ELEVATED CONCENTRATION OF SERUM CYCLOPHILIN A IN PATIENTS WITH CHRONIC KIDNEY DISEASE

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Abstract

Cyclophilin A (CypA) is a secreted protein which is produced from various kinds of cell types in response to oxidative stress. The elevation of CypA is related to pathogenesis of chronic inflammatory diseases, such as chronic kidney disease (CKD), and may be used as a prognosis marker for the advanced stage of the disease. This study aimed to investigate the associated factors which affect the serum CypA levels and measure those levels among non-CKD and CKD groups. We examined the levels of serum CypA from 358 cases (137, 105, and 116 cases from the non-CKD group, early stage of CKD group, and late stage of CKD group, respectively) using an indirect ELISA technique. The associated factors were analyzed by multiple linear regression analysis. The levels of the CypA concentration were statistically significantly higher in developing renal inflammation patients (the late stage of CKD group) than in the non-CKD group ($p < 0.001$). Fasting blood sugar (FBS), HbA1c, systolic blood pressure (SBP), and estimated glomerular filtration rate (eGFR) were associated with the elevation of serum CypA levels. The multivariate analysis revealed that the early (adjusted OR 1.350, 95%CI: 1.093, 1.667) and late (adjusted OR 1.415, 95%CI: 1.103, 1.811) stages of CKD had higher serum CypA levels than the non-CKD group. Therefore, the levels of CypA in a serum could be associated with a progression of CKD and be related to other

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risk factors (FBS, HbA1c, and SBP) of CKD which have an effect on the development of renal inflammation. Our results indicate that the serum CypA level may represent a screening factor for predicting the severity of the early and late stages of CKD.

Keywords: Cyclophilin A, serum, chronic kidney disease, biomarker

Introduction

Chronic kidney disease (CKD) is an inflammatory disease which leads to end-stage renal disease (Liu *et al.*, 2015) and becomes an enormous global public health problem (Levey *et al.*, 2007). The prevalence of CKD is associated with an increase of mortality. In 2015, the Global Burden of Disease Study reported that the CKD mortality rate had increased by 31.7% over the previous 10 years and it was the twelfth most common cause of death worldwide (GBD 2015 Mortality and Causes of Death Collaborators, 2016; Neuen *et al.*, 2017). However, a screening program for diagnosis of the early stage of CKD is negligible (Mihai *et al.*, 2018). The CKD definition is abnormalities of kidney structure or function which increase urinary albumin excretion, while a glomerular filtration rate is reduced (Kidney Disease Improving Global Outcomes, 2013; Jha *et al.*, 2013). Risk factors of CKD are classified into 2 categories, which are the personal factor (such as race, gender, age, and family history), and behavioral factor (such as alcohol consumption, smoking, hypertension, and diabetes mellitus) (Jha *et al.*, 2013; Kazancioglu, 2013). In Thailand, diabetes mellitus (DM) and hypertension (HT), which are caused by oxidative stress production (Dang *et al.*, 2010; Thanakiggaru, 2015), are the main risk factors of CKD (Ongajyooth *et al.*, 2009). Due to oxidative stress which is generated during renal cell damage (Daenen *et al.*, 2019), many biomolecules related to these processes have been proposed for CKD development (Duni *et al.*, 2017), such as Cyclophilin A (CypA) (Nigro *et al.*, 2013).

CypA is an 18-kDa protein, discovered as a cellular receptor for cyclosporine A (immunosuppressive drug) (Takahashi *et al.*, 1989) which is expressed in human endothelial cells, vascular smooth muscle cells, and monocytes (Hohman and Hultsch, 1990; Nigro

et al., 2013). Interestingly, CypA is secreted in response to oxidative stress and promotes an inflammatory process (Xue *et al.*, 2017) which plays a crucial role in many kinds of diseases, such as coronary disease, rheumatoid arthritis, sepsis, and DM (Nigro *et al.*, 2013; Lu *et al.*, 2018). The expression of CypA is related to a stage of CKD in diabetes nephropathy patients (Tsai *et al.*, 2015), indicating that the elevation of CypA might be related to impaired renal function (Liu *et al.*, 2015). However, the elevation of CypA levels in inflammatory diseases may benefit from treatment with a targeted molecule (Zhu *et al.*, 2014) by designed therapeutic agents with the capacity to block specific functions of CypA. Daum and colleagues reported the development of a small-molecule compound with the capacity to specifically inhibit the function of CypA without any effect on other Cyp families (Daum *et al.*, 2009).

At present, a screening biomarker for CKD is important. In addition, if a biomarker can be used to differentiate any stage of CKD, it may be helpful for monitoring and planning the therapeutic treatment for CKD patients. The stage of CKD is defined by the estimated glomerular filtration rate (eGFR) value, which is classified as CKD when the eGFR <60 ml/min/1.73 m² (Hemmelgarn *et al.*, 2010). In our study, we categorized the CKD groups into early (Stage I and II; eGFR ≥60 ml/min/1.73 m²) and late (stage III, IV, and V; eGFR <60 ml/min/1.73 m²) stages based on their eGFR values and measured the levels of serum CypA among them and the non-CKD group. The CypA levels were significantly elevated in the progression of CKD. Therefore, the levels of serum CypA may be appropriate for both a screening and monitoring biomarker in CKD patients. Moreover, CypA levels were

significantly inversely correlated with eGFR levels. In contrast, fasting blood sugar (FBS), HbA1c, and systolic blood pressure (SBP) were correlated with serum CypA levels.

Materials and Methods

Subjects

In this cross-sectional study, all 358 subjects (from October 2017 through September 2018) were recruited from the Chronic Kidney Disease Prevention in the Northeast Thailand (CKDNET) Research Group, with informed consent and all the protocols were approved by the Human Ethics Committee of Khon Kaen University (coded H.E 611152). All the subjects were divided into 3 groups which were identified as group I: non-CKD group ($n = 137$), group II: early stage of CKD (CKD stage 1 and 2; $n = 105$) patients, and group III: late stage of CKD (CKD stage 3, 4 and 5; $n = 116$) patients. In both groups of CKD patients, DM and HT was included, while other diseases or symptoms, such as, recent inflammation, infection, liver disease, malignancy, and autoimmune diseases, were excluded.

Baseline Measurements

A questionnaire was used to collect the demographic and behavioral factors from each subject. All subjects were measured for blood pressure before a blood examination was taken. Laboratory parameters including FBS, HbA1c, LDL cholesterol, alanine transaminase (ALT), uric acid, serum creatinine (SCr), and eGFR (using the CKD-Epidemiology formula), were determined at Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand. In addition, the serum albumin to creatinine ratio (UACR) was also calculated for use as another marker of CKD. All data including laboratory and clinical parameters were collected twice within three months.

Enzyme Linked Immunosorbent Assay (ELISA)

A leftover serum from the laboratory parameters' measurement was aliquoted and kept at -20°C for serum CypA investigation.

Serum CypA analysis was performed by using an indirect ELISA technique. All serum samples were used as an antigen by 1:1,000 diluted in 1.5 mM carbonate buffer (sodium bicarbonate and sodium carbonate) pH 9.5. Each diluted serum sample was coated into each well of a microplate (Thermo Scientific, Inc., Waltham, MA, USA) and then incubated at 4°C overnight. The unbound diluted serum was washed 3 times with PBST (phosphate buffer solution and 0.05% Tween-20) pH 7.4. A blocking solution (3% skim milk in PBST) was added and incubated at 37°C for 2 h, and then washed 3 times with PBST. Each microplate well was treated with 1:250 mouse monoclonal anti-human CypA (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 37°C for 2 h. The microplate was washed with PBST 3 times, and then 100 μl of 1:2,500 diluted anti-mouse IgG conjugated with alkaline phosphatase (Thermo Scientific, Inc., MA, Waltham, USA) in PBST was added and incubated at 37°C for 2 h. After that, unbound complexes were washed 3 times with PBST and a phosphate-buffered saline solution, respectively. A substrate, 50 mM *p*-nitrophenyl phosphate, was added and incubated at 37°C for 2 h. The reaction was measured with an ELISA reader (PerkinElmer, Inc., Waltham, MA, USA) at 405 nm.

Statistical Analyses

The data of serum CypA levels among the study groups were compared and represented as mean $\pm$ SD by ANOVA. The association factors with serum CypA levels were examined by multiple linear regression and described as risks of the levels with the progression of CKD via an adjusted odd ratio (aOR) by multinomial logistic regression analysis. All statistical analysis was analyzed by STATA version 13.0 (Stata Corporation, College Station, TX, USA).

Result and Discussion

The concentrations of serum CypA among the non-CKD and CKD groups were investigated by indirect ELISA. The baseline characteristics

from each group were analyzed. The significant parameters were selected and then characterized with the associated factors which have an effect on serum CypA secretion.

Baseline Characteristics

Regarding the baseline characteristics' (Table 1) data among the non-CKD, early stage, and late stage of CKD groups, we found that factors such as age, FBS, HbA1c, SBP, DBP, LDL, ALT, uric acid, eGFR, UACR, and serum CypA, were significantly different ($p<0.001$) among those 3 groups. The expression of serum

CypA from all subjects was measured as 0.8 $\mu\text{g/mL}$ to 1008 $\mu\text{g/mL}$ and the median value was 174.02 $\mu\text{g/mL}$. To compare the levels of serum CypA among each group, we found that serum CypA levels were significantly elevated in the early stage (264.68 ± 143.98 $\mu\text{g/mL}$) and late stage of CKD groups (532.10 ± 455.71 $\mu\text{g/mL}$) compared with that of the non-CKD group (Figure 1). In addition, the levels of serum CypA also significantly increased in the late stage of CKD group compared with that of the early stage of CKD group ($p<0.001$) (Figure 1).

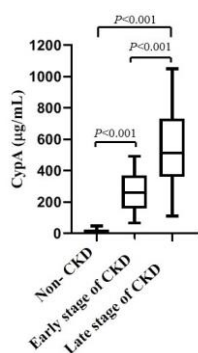


Figure 1. Serum CypA levels among the non-CKD, early stage of CKD, and late stage of CKD groups

Elevation of CypA and Its Associated Factors

The associated factors with elevated serum CypA were retrieved by using linear regression analysis. The result from simple linear regression analysis (Table 2) showed the significant correlation between serum CypA levels and other factors, including age, FBS, HbA1c, SBP, uric acid, UACR, eGFR, and exercise. To control other confounding factors, those correlated factors were input into a multiple linear regression model. We found that the FBS, HbA1c, SBP, and eGFR (Table 3) were associated with an increase of serum CypA. Serum CypA was increased by 0.30 and 1.69 $\mu\text{g/mL}$, for each increase of 1 mg/dL FBS

Table 1. The baseline characteristics among the non-CKD and CKD groups

Factor	Non-CKD gr.	Early stage of CKD gr.	Late stage of CKD gr.	p-value
Number	137	105	116	
Sex				0.470
Male	51(37.22)	42(40.00)	52(44.83)	
Female	86(62.77)	63(60.00)	64(55.17)	
Age	54.53 $\pm$ 13.56	59.93 $\pm$ 10.84	70.03 $\pm$ 8.60	<0.001
FBS (mg/dL)	87.27 $\pm$ 11.07	142.39 $\pm$ 76.41	137.47 $\pm$ 91.40	<0.001
HbA1c (%)	5.57 $\pm$ 0.63	7.33 $\pm$ 2.62	6.76 $\pm$ 1.81	<0.001
SBP (mm Hg)	124.47 $\pm$ 16.59	136.14 $\pm$ 17.87	139.03 $\pm$ 16.94	<0.001
DBP (mm Hg)	80.46 $\pm$ 11.95	83.01 $\pm$ 9.67	80.43 $\pm$ 10.73	0.131
LDL (mg/dL)	124.87 $\pm$ 30.84	122.34 $\pm$ 32.83	117.78 $\pm$ 58.26	0.408
ALT (U/L)	21.80 $\pm$ 10.04	24.99 $\pm$ 13.92	28.37 $\pm$ 9.46	<0.001
Uric acid (mg/dL)	5.28 $\pm$ 1.45	5.34 $\pm$ 1.34	7.29 $\pm$ 1.66	<0.001
eGFR (mL/min/1.73m ²)	98.2 $\pm$ 5.84	86.51 $\pm$ 15.22	36.41 $\pm$ 16.63	<0.001
UACR (mg/g)	34.22 $\pm$ 248.33	110.94 $\pm$ 143.20	301.43 $\pm$ 475.46	<0.001
Serum CypA ($\mu\text{g/mL}$)	11.02 $\pm$ 6.17	264.68 $\pm$ 143.98	532.10 $\pm$ 455.71	<0.001
Exercise				0.026
No	58(42.34)	60(57.14)	66(56.90)	
Yes	79(57.66)	45(42.86)	50(43.10)	
Smoking				0.565
No	99(72.26)	71(67.62)	77(66.38)	
Yes	38(27.74)	34(32.38)	39(33.62)	

Data presented in numbers (percentages) or mean $\pm$ SD

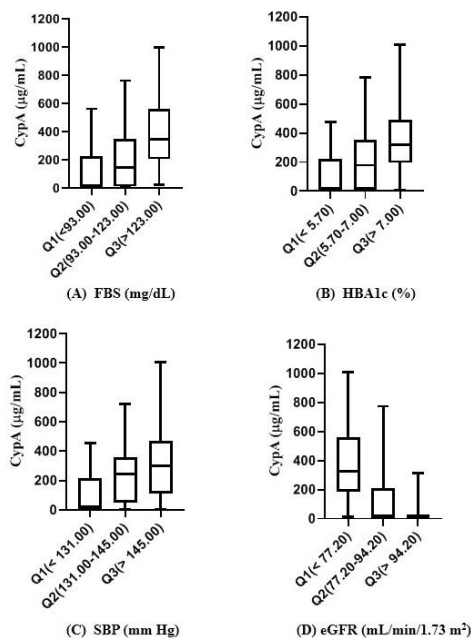


Figure 2. The associated factors and serum CypA levels: (A) FBS, (B) HbA1c, (C) SBP, and (D) eGFR

(95%CI 0.04, 0.60; $p = 0.023$) and 1% HbA1c (95% CI 0.64, 2.74; $p = 0.002$), respectively. Likewise, serum CypA was elevated by 0.16 and 0.31 $\mu\text{g/mL}$, for each increase of 1 mmHg SBP (95% CI 0.08, 0.24; $p < 0.001$) and decrease of 1 mL/min eGFR (95%CI -0.36, -0.26; $p < 0.001$), respectively. The levels of serum CypA and each associated value are presented in Figure 2, which categorized them as tertile values (Q1, Q2, and Q3).

Serum CypA Levels and Progression of CKD

Serum CypA was significantly increased in both the early and late stages of CKD groups compared with the non-CKD group (Figure 1). Moreover, the result from multinomial logistic regression indicated that serum CypA was associated with the progression of CKD by aOR values. We found the elevation of each 1 $\mu\text{g/mL}$ serum CypA increased the risk for transformation from non-CKD to early stage of CKD by 1.350 times (aOR 1.350, 95%CI 1.093, 1.667) (Table 4). Furthermore, the risk for transformation from non-CKD to late stage of CKD was increased by 1.413 times (aOR

Table 2. Univariate analysis for correlated factors with serum CypA levels

Factor	Mean difference	95%CI	p-value
Sex	-0.254	-0.640, 0.131	0.196
Age	0.056	0.043, 0.070	< 0.001
FBS	0.009	0.006, 0.011	< 0.001
Hb1Ac	0.316	0.223, 0.408	< 0.001
SBP	0.034	0.024, 0.044	< 0.001
DBP	0.008	-0.008, 0.25	0.339
LDL	-0.002	-0.006, 0.003	0.447
ALT	-0.003	-0.020, 0.013	0.680
Uric acid	0.389	0.288, 0.489	< 0.001
eGFR	-0.035	-0.041, -0.030	< 0.001
UACR	0.001	0.0008, 0.002	< 0.001
Exercise	-0.630	-1.003, -0.256	0.001
Smoking	0.142	-0.268, 0.552	0.495

*The univariate linear regression model included variables with p value < 0.05

Table 3. Multivariate analysis for associated factors with serum CypA levels

Factor	Adjusted mean difference (95%CI)	p-value
FBS	0.003 (0.0004, 0.006)	0.023
HbA1c	0.169 (0.064, 0.274)	0.002
SBP	0.016 (0.008, 0.024)	< 0.001
eGFR	-0.031 (-0.036, -0.026)	< 0.001

*The multiple linear regression model included variables with p value < 0.05

1.413, 95%CI 1.103, 1.811) when serum CypA was elevated by every 1 µg/mL (Table 4).

CKD is a chronic inflammatory disease which is a public health problem worldwide (Weiner, 2009). Early screening could be a benefit for planning treatment. A biomarker for CKD screening with both high sensitivity and specificity is obligatory, especially the markers used to monitor the stage transformations of CKD. In clinical medicine, eGFR is recognized as a gold standard marker for determining CKD and kidney function (Stevens and Levey, 2009). However, eGFR is not responsible for any specific physiological changes inside renal cells. Several methods and biomarkers, such as creatinine (Earley *et al.*, 2012), cystatin C (Shlipak *et al.*, 2006), blood urea nitrogen (Levey, 1990), and albuminuria or microalbuminuria (Bidin *et al.*, 2019), have been established but cannot be used for early CKD detection (Bidin *et al.*, 2019).

In this study, we measured the levels of serum CypA among non-CKD and CKD patients using an indirect ELISA technique. We found that the levels of CypA were significantly raised by a degree of renal dysfunction (Figure 1). Moreover, our results indicated that in patients who had hyperglycemia (FBS and HbA1c), high blood pressure (SBP), and renal function deficiency (eGFR low levels), there were elevated levels of serum CypA (Tables 2 and 3). Furthermore, an increase of serum CypA by every 1 µg/mL indicated a risk for transformation from non-CKD to early and late stages of CKD by 1.350 and 1.413 times, respectively (Table 4).

Chronic hyperglycemia (uncontrolled FBS and HbA1c) increased reactive oxygen species cause chronic inflammation (Mitsuo *et al.*, 1980). Under high glucose conditions, monocytes were activated to produce various

kinds of proteins which increased the risk of vascular lesion formation (Ramachandran *et al.*, 2012) and ultimately promoted atherogenesis (Nagareddy *et al.*, 2013). One such protein is CypA which was discovered to be elevated in the serum of type 2 DM patients (Satoh *et al.*, 2010; Ramachandran *et al.*, 2014). CypA is present in monocytes (Ramachandran *et al.*, 2014), endothelial cells (Jin *et al.*, 2000), and vascular smooth muscle cells (Suzuki *et al.*, 2006), resulting in a response to inflammatory stimuli such as hyperglycemia, hypoxia, infection, and oxidative stress (Jin *et al.*, 2000; Suzuki *et al.*, 2006). CypA in high glucose conditions can function as a chemoattractant increasing monocyte adhesion to the endothelium and enhancing transmigration (Ramachandran *et al.*, 2014) which increases endothelial cell activation and inflammation in vascular wall cells (Soe *et al.*, 2013). Hyperglycemia could be a critical factor in the development of renal nephropathy especially in DM patients because of its effects on glomerular and mesangial cells (Dronavalli *et al.*, 2008). Mesangial cells are importance for glomerular structural maintenance and for the regulation of glomerular filtration (Schlondorff, 1987). From *in vitro* studies, it has been demonstrated that hyperglycemia should be involved with increased mesangial cell matrix production (Harris *et al.*, 1991; Heilig *et al.*, 1995) and mesangial cell apoptosis (Mishra *et al.*, 2005). In addition, under high glucose concentration or a free radicals condition it was revealed that CypA had been produced from mesangial cells (Tsai *et al.*, 2015).

The correlation of hypertension and the elevation of serum CypA had been proposed both in animal models and clinical data. The study from a rat model demonstrated that CypA levels gradually were raised in serum

Table 4. Multinomial logistic regression analysis for associated factors with serum CypA levels

Group	Number of subjects	aOR* (95%CI)	p-value
Non-CKD gr.	137	1.000	1.00
Early stage of CKD gr.	105	1.350 (1.093,1.667)	< 0.001
Late stage of CKD gr.	116	1.413 (1.103,1.811)	< 0.001

*Adjusted Odds Ratios for FBS, HbA1c, SBP, and eGFR

with the development of hypertension (Tang *et al.*, 2015). In clinical data, serum CypA was definitely correlated with both systolic and diastolic blood pressure in untreated hypertensive patients (Chang *et al.*, 2013). CypA could increase high blood pressure by mediating the production of reactive oxygen species through the ERK1/2, AKT, and JAK signaling pathways causing the endothelial nitric oxide synthase levels to decrease, which can inhibit nitric oxide production resulting in vasoconstriction and then increased blood pressure (Sato *et al.*, 2008).

In Thailand, CKD is a major complicated disease which occurs in patients who cannot either control their blood sugar or blood pressure (Thanakiggaru, 2015) for a long time. The present studies found evidence that revealed that the levels of serum CypA, which is induced by a product of oxidative stress and a atherogenesis processes, is associated with hyperglycemia, hypertension, and eGFR. CypA might affect the vasculature via either enriched serum levels from several cell types' stimulation, or by increased secretion from the kidney directly due to the pathology of CKD (Liu *et al.*, 2015).

From a previous study, the elevation of serum CypA levels was detected in peripheral arterial occlusion disease, especially with CKD (Liu *et al.*, 2015). Therefore, production of CypA might be related to a progression of renal function in those patients. In the case of systemic inflammation in hemodialysis and peritoneal dialysis patients, plasma CypA was significantly elevated and correlated with that pathophysiology (Jin and Vaziri, 2017). The concentration of CypA can be detected in urine from diabetic nephropathy patients. The levels of CypA were correlated with the progression of renal function which significantly increased in urinary CypA with stage II of CKD and persisted in later stages (Tsai *et al.*, 2015). Thus, CypA levels either from serum or urine are used as markers to differentiate the severity of kidney dysfunction (Ramachandran *et al.*, 2014; Tsai *et al.*, 2015).

In our study, the levels of serum CypA were significantly different between non-CKD and CKD patients. In addition, the markedly

different serum CypA levels can be distinguished between the non-CKD and the early stage of CKD groups. Therefore, serum CypA should be used as a screening biomarker which can be developed as a lateral flow screening kit in the community. However, we did not measure the levels of urine CypA in this study; the correlation of serum and urine CypA should be explored in further studies. The role of CypA in the pathophysiology of CKD should be further investigated.

Conclusions

This study demonstrated that the serum CypA levels were significantly elevated in CKD patients compared to non-CKD patients and these levels were associated with FBS, HbA1c, SBP, and eGFR. In addition, the serum CypA level may represent a screening marker for predicting the severity of the early and late stages of CKD.

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Conflict of Interests

The authors declare that there are no conflict of interests regarding the publication of our study.

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