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Effect of blood pressure and glycemic control on the plasma cell-free DNA in hemodialysis patients

Da Wun Jeong, Ju-Young Moon, Young-Wook Choi, Haena Moon, Kipyo Kim, Yu-Ho Lee, Se-Yeun Kim, Yang-Gyun Kim, Kyung-Hwan Jeong, Sang-Ho Lee*

Division of Nephrology, Department of Internal Medicine, College of Medicine, Kyung Hee University, Seoul, Korea

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Abstract

Background: The plasma levels of cell-free DNA (cfDNA) are known to be elevated under inflammatory or apoptotic conditions. Increased cfDNA levels have been reported in hemodialysis (HD) patients. The aim of this study was to investigate the clinical significance of cfDNA in HD patients.

Methods: A total of 95 patients on HD were enrolled. We measured their predialysis cfDNA levels using real-time *EIF2C1* gene sequence amplification and analyzed its association with certain clinical parameters.

Results: The mean plasma cfDNA level in the HD patients was 3,884 ± 407 GE/mL, and the mean plasma cfDNA level in the control group was 1,420 ± 121 GE/mL ($P < 0.05$). Diabetic patients showed higher plasma cfDNA levels compared with nondiabetic patients ($P < 0.01$). Patients with cardiovascular complications also showed higher plasma cfDNA levels compared with those without cardiovascular complication ($P < 0.05$). In univariable analysis, the cfDNA level was associated with 3-month mean systolic blood pressure (SBP), white blood cell, serum albumin, creatinine (Cr), normalized protein catabolic rate in HD patients. In diabetic patients, it was significantly correlated with SBP, hemoglobin A1c, and serum albumin. In multivariate analysis, SBP was the independent determinant for the cfDNA level. In diabetic patients, cfDNA level was independently associated with hemoglobin A1c and SBP.

Conclusions: In patients with HD, cfDNA is elevated in diabetic patients and patients with cardiovascular diseases. Uncontrolled hypertension and poor glycemic control are independent determinants for the elevated cfDNA. Our data suggest that cfDNA might be a marker of vascular injury rather than proinflammatory condition in HD patients.

Introduction

First described in 1948 by Mandel and Metais, circulating plasma cell-free DNA (cfDNA) is nucleic acids in peripheral blood that originate from cell death caused by injury, apoptosis, and necrosis [1,2]. cfDNA is normally found in small amounts in

* Corresponding author. Division of Nephrology, Department of Internal Medicine, Kyung Hee University Hospital at Gangdong, College of Medicine, Kyung Hee University, Sangil-dong 149, Gangdong-gu, Seoul 134-727, Korea.
E-mail address: lshkidney@khu.ac.kr (S-H Lee).

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the blood of healthy individuals, although increased cfDNA levels have been reported in patients with various clinical conditions including infection, inflammation, malignancy, connective tissue diseases, ischemic stroke, myocardial infarction, pregnancy-associated disorders, and hemodialysis (HD) [3–12]. cfDNA has been the focus of research because of its potential use as a tool to diagnose malignant disease and to monitor the severity of injury caused by several diseases. Recently, Tovbin et al [13] have reported that postdialysis cfDNA levels are an independent predictor of all-cause mortality in patients undergoing HD.

End-stage renal disease (ESRD) patients undergoing HD are characterized by proinflammatory conditions. In dialysis patients, chronic sterile inflammation is related to innate and adaptive immune system activations, which result in an increased level of inflammatory cytokines. In previous studies, increased cfDNA levels were reported in HD patients, and cfDNA is able to selectively induce the production of interleukin 6 (IL-6) in human monocytes [14]. However, limited data are available concerning the clinical relevance of cfDNA in HD patients. The aim of this study was to investigate the clinical significance of cfDNA in patients undergoing maintenance HD.

Methods

Patients

The 95 ESRD patients undergoing maintenance HD and the 15 healthy controls were enrolled in the study in April 2015. The ESRD patients underwent 4 hours of HD per session, 3 times per week. Patient medical record was collected retrospectively, and it composed of the underlying causes of ESRD, comorbidities, and clinical data including age, body mass index (BMI), duration of HD, systolic blood pressure (SBP), diastolic blood pressure, and laboratory findings such as mean level of white blood cell (WBC), high sensitivity C-reactive protein (hsCRP), hemoglobin A1c (HbA1c), marker of dialysis adequacy Kt/V, normalized protein catabolic rate (nPCR), low density lipoprotein (LDL), total cholesterol, triglyceride (TG), blood urea nitrogen (BUN), and creatinine (Cr) during last 3 months. The cause of ESRD was categorized as diabetes mellitus (DM), hypertension, glomerulonephritis (GN), other, or unknown. All of blood samples were done before HD session. As proinflammatory marker, cfDNA level might be affected by any infection. Therefore, among HD patients, we excluded patients with signs of current infection such as WBC >10,000, fever, and symptoms for urinary tract infection and respiratory tract infection. In 95 HD patients, the mean value of hsCRP was 3.340 ± 0.9. Patients with recent (less than 1 month) history of cardiovascular (CV) complications including cerebrovascular disease and myocardial infarction and history of an intervention for vascular access were also excluded.

The study was approved by the local ethics committee, and patients provided signed informed consent before entering the study.

cfDNA analysis

We extracted cfDNA from a 1-mL plasma sample using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, USA). As described in the manufacturer's protocol, the samples were treated with proteinase K to inactivate DNases and RNases

Table 1. Baseline clinical characteristics of 95 hemodialysis patients

	Total ($n = 95$)	Non-DM ($n = 45$)	DM ($n = 50$)	P
Cause of ESRD (%)				<0.001
Diabetic nephropathy	46 (48.4)	0	46 (92)	
Hypertension	21 (22.1)	18 (40)	3 (6)	
GN	15 (15.7)	15 (33.3)	0	
Others	3 (3.1)	2 (4.4)	1 (2)	
Unknown	10 (10.5)	10 (22.2)	0	
Comorbidities (%)				
CVA	21 (21.9)	7 (15.5)	14 (28)	0.14
CAD	13 (13.5)	4 (8.9)	9 (18)	0.20
CHF	21 (21.9)	8 (17.8)	13 (26)	0.34
Total CV complication	43 (45.3)	17 (37.8)	26 (52)	0.03
History of malignancy	7 (7.3)	2 (4.4)	5 (10)	0.30
Age (y)	58.0 ± 1.5	55.4 ± 2.4	66.4 ± 1.8	0.09
BMI (kg/m^2)	23.4 ± 0.4	21.8 ± 0.5	24.8 ± 0.6	0.18
Mean dialysis duration (mo)	48.8 ± 5.3	56.9 ± 7.9	42.5 ± 6.9	0.18
WBC ($\times 10^3/\mu L$)	5,883.4 ± 157.2	5,447.6 ± 212.6	6,255.6 ± 215.7	0.98
hsCRP (mg/L)	3.3 ± 0.9	2.9 ± 0.7	3.8 ± 1.5	0.59
SBP (mmHg)	141.0 ± 1.7	136.3 ± 2.4	145.3 ± 2.3	0.68
DBP (mmHg)	80.8 ± 0.7	80.1 ± 0.9	81.4 ± 0.9	0.68
HbA1c (%)	6.8 ± 0.2	5.5 ± 0.2	7.3 ± 0.3	0.11
Kt/V	1.5 ± 0.03	1.6 ± 0.04	1.5 ± 0.04	0.01
LDL (mg/dL)	78.2 ± 2.1	81.9 ± 3.3	74.7 ± 2.7	0.09
Total cholesterol (mg/dL)	139.6 ± 3.3	144.7 ± 5.4	135.0 ± 4.0	0.15
TG (mg/dL)	112.2 ± 6.1	97.2 ± 5.4	125.0 ± 10.0	0.02
HDL (mg/dL)	43.2 ± 1.2	45.1 ± 1.8	41.5 ± 1.6	0.15
Albumin (g/dL)	3.9 ± 0.03	3.9 ± 0.1	3.9 ± 0.04	0.73
nPCR (g/kg/d)	0.95 ± 0.04	0.91 ± 0.06	0.98 ± 0.05	0.37
BUN (mg/dL)	64.0 ± 2.1	64.0 ± 3.0	62.9 ± 2.9	0.79
Cr (mg/dL)	9.6 ± 0.3	10.3 ± 0.5	8.9 ± 0.4	0.03

BMI, body mass index; BUN, blood urea nitrogen; CAD, coronary artery disease; CHF, congestive heart failure; Cr, creatinine; CV, cardiovascular; CVA, cerebrovascular accident; DBP, diastolic blood pressure; DM, diabetes mellitus; ESRD, end-stage renal disease; GN, glomerulonephritis; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; nPCR, normalized protein catabolic rate; SBP, systolic blood pressure; TG, triglyceride; WBC, white blood cell.

and to degrade cellular debris. The samples were then buffered and carrier RNA was added to enhance the binding of nucleic acids to the membrane. The lysate was run through a DNA binding column and washed multiple times with buffers and 100% ethanol using the QIavac 24 Plus. The DNA was eluted in 50 μL of the supplied elution buffer. Circulating cfDNA was quantified using real-time (RT) polymerase chain reaction (PCR) with Chromo 4 RT PCR system (Bio-Rad, Hercules, CA, USA) using custom Taqman gene expression assays for *EIF2C1* and Taqman master mix (Applied Biosystems, Foster City, CA, USA).The following primer sequences were used: forward 5′-CCT GCA GCA GGT GTT CCA -3′; reverse 5′- GCC AGG AGC TTG ATT GGT TTC -3′; and probe 5′- FAM-CAC AGT GCC AAT GCC A -NFQ-3′ or forward 5′- GCC GGC CTG GCA TTG -3′; reverse 5′-GAT CTT AGG GAT GTC CAC CTC AAA -3′; and probe 5′- FAM-CTC CTG GCC AAT TAC -NFQ-3′. A standard curve was created using serial diluted human genomic DNA: Male (Promega, Madison, WI, USA). The DNA concentration was expressed and calculated as genome equivalents/mL (GE/mL).

Statistical analysis

The baseline characteristics were presented as the mean, standard deviation, and frequency and then compared using an independent sample test. In case of continuous variables, correlations between variables were analyzed using the Pearson's correlation coefficient. We used a multiple regression analysis to evaluate the laboratory and clinical variables independently associated with cfDNA level by dividing 2 groups in chronic HD patients and in diabetic HD patients, as well as the factors that were significantly associated with cfDNA ($P < 0.05$). Statistical analyses were performed using SPSS software, version 18 (PASW, Chicago, IL, USA).

Results

The baseline characteristics of the studied patients

Table 1 lists the baseline characteristics of the 95 HD patients (mean overall patient age, 58 ± 1.5 years; mean duration of dialysis, 48.8 ± 5.3 months). The mean age of the healthy controls was 32.8 ± 1.2 years. The main causes of ESRD were diabetic nephropathy (48.4%), hypertension (22.1%), and GN (15.7%). We defined CV complication (total CV complication) as cerebrovascular accidents, coronary artery disease, or congestive heart failure, and Table 1 shows that total CV complication was significantly higher in diabetic HD groups than nondiabetic HD patients ($P = 0.03$). In other parameters, demographic factors such as age, BMI, and mean dialysis duration were not statistically different. But Kt/V and Cr were higher in nondiabetic HD group than those in diabetic HD group ($P < 0.05$, respectively) and TG was higher in diabetic group than that in nondiabetic group.

cfDNA levels and comorbid condition in HD patients

The mean plasma cfDNA level was 3,884 ± 407 GE/mL in the HD patients, and it was significantly higher than that of healthy controls (1,420 ± 121 GE/mL). Diabetes is the most common cause of ESRD and is a well-known sterile inflammatory disease. We evaluated the difference in cfDNA levels between the diabetic and nondiabetic HD patients. The cfDNA levels were significantly higher in the diabetic HD patients compared with those in the nondiabetic HD patients (4,612 ± 640 vs. 2,858 ± 385 GE/mL, respectively, $P < 0.01$, Fig. 1A). The cfDNA levels were also correlated with HbA1c levels in the diabetic HD patients ($r = 0.37$, $P = 0.003$, Fig. 1B). In addition, we also evaluated the difference in cfDNA levels between the 43 patients who had total CV complication and 53 patients without total CV complication. The cfDNA levels were significantly higher in the total CV complication group than in those without CV complication (2,193 ± 322 GE/mL vs. 1,573 ± 206 GE/mL, respectively, $P < 0.05$).

cfDNA levels and clinical parameters in HD patients

We evaluated the clinical factors associated with log value of cfDNA (log cfDNA) in the HD patients through correlation analysis. Three-month mean WBC counts ($r = 0.32$, $P = 0.002$, Fig. 2A), SBP ($r = 0.36$, $P < 0.001$, Fig. 2B), Cr ($r = 0.06$, $P = 0.02$, Fig. 2C), albumin ($r = 0.75$, $P = 0.2$, Fig. 2D), and nPCR ($r = 0.26$, $P = 0.02$, Fig. 2E) were significantly correlated with log cfDNA. However, there was no association with age, sex, hemoglobin, uric acid, Ca, Ca × P, low-density lipoprotein. In addition, hsCRP was not significantly associated with log cfDNA. Because cfDNA was significantly increased in diabetic patients, we additionally evaluated the association of clinical parameters and log cfDNA in diabetic HD patients. The SBP ($r = 0.35$, $P = 0.12$), HbA1c

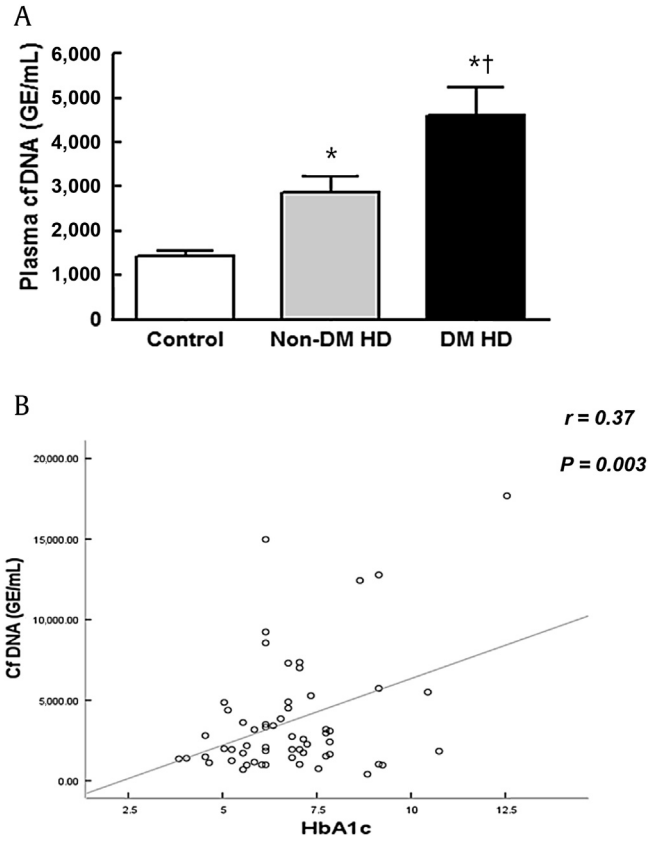

Figure 1. Plasma level of cell-free DNA in HD patients. (A) Cell-free DNA levels in the controls and DM and non-DM HD patients. (B) Correlation between the cell-free DNA and HbA1c levels in diabetic HD patients.
*$P < 0.05$ compared with the controls.
†$P < 0.05$ compared with the non-DM HD patients.
cfDNA, cell-free DNA; DM, diabetes mellitus; HbA1c, hemoglobin A1c; HD, hemodialysis.

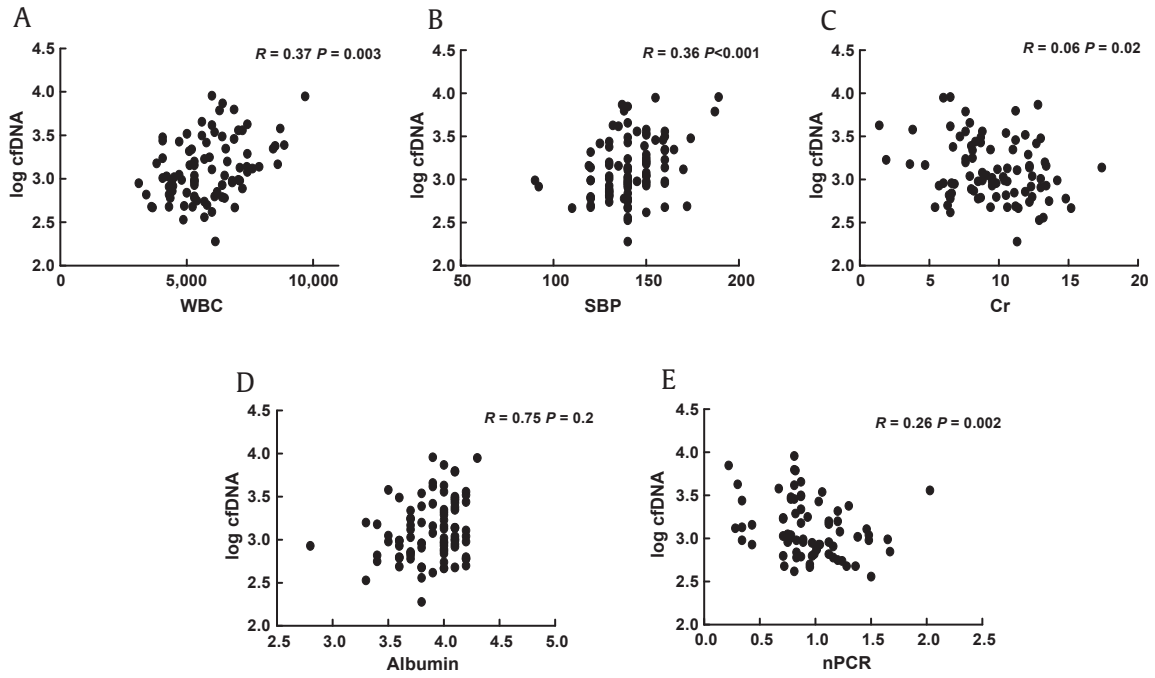

Figure 2. Correlation between clinical parameters and cfDNA (log). (A) WBC counts ($r = 0.32$, $P = 0.002$), (B) SBP ($r = 0.36$, $P < 0.001$), (C) Cr ($r = 0.06$, $P = 0.02$), (D) Albumin ($r = 0.75$, $P = 0.2$), and (E) nPCR ($r = 0.26$, $P = 0.02$).
cfDNA, cell-free DNA; Cr, creatinine; nPCR, normalized protein catabolic rate; SBP, systolic blood pressure; WBC, white blood cell.

($r = 0.37$, $P = 0.003$), and albumin ($r = 0.29$, $P = 0.05$) were also significantly correlated.

Multivariate analysis for the determinants of cfDNA in HD patients

Table 2 presents multivariable regression analysis in total HD patients, for the associations between log cfDNA levels and the selected parameters that were associated with log cfDNA in univariable analysis. Three-month mean SBP was the only independent determinant for the plasma level of cfDNA .We also evaluated the independent parameters, which affect cfDNA in diabetic patients using multivariable regression analysis. Table 3 shows 3-month mean SBP and HbA1c were independently associated with the plasma level of cfDNA in diabetic patients.

Table 2. Multivariate regression analysis of factors associated with cfDNA level in hemodialysis patients

Parameters	Beta coefficient	*P*
Sex	0.179	0.20
Age (y)	0.039	0.80
WBC ($\times 10^3/\mu L$)	0.056	0.69
SBP (mmHg)	0.523	0.001
Total CV complication	0.169	0.24
Albumin (g/dL)	0.058	0.67
hsCRP (mg/L)	0.179	0.26
Cr (mg/dL)	0.149	0.34
nPCR (g/kg/d)	−0.175	0.22

cfDNA, cell-free DNA; Cr, creatinine; CV, cardiovascular; hsCRP, high-sensitivity C-reactive protein; nPCR, normalized protein catabolic rate; SBP, systolic blood pressure; WBC, white blood cell.

Discussion

In this study, the mean plasma cfDNA level in the HD patients was higher than that of the control group. Diabetic patients showed higher plasma cfDNA levels as compared to nondiabetic HD patients. In addition, the cfDNA level was significantly correlated with current HbA1c levels in the diabetic HD patients. Patients with CV complications also showed higher plasma cfDNA levels compared with those without CV complication. A previous study suggested that the state of uremia is associated with accelerated apoptosis of lymphocytes, monocytes, and neutrophils [15]. In dialysis patients, chronic sterile inflammation is related to innate and adaptive immune system activations, which result in an increased level of inflammatory cytokines. In previous studies, increased cfDNA levels were reported in HD patients, and cfDNA is able to selectively induce the production of IL-6 in human monocytes [14]. ESRD patients undergoing HD are characterized by proinflammatory conditions. Various pathologic processes occur in HD patients leading to their impaired function and

Table 3. Multivariate regression analysis of factors associated with cfDNA level in diabetic HD patients

Parameters	Beta coefficient	*P*
Age (y)	−0.019	0.93
Sex	0.270	0.22
SBP (mmHg)	0.525	0.02
WBC ($\times 10^3/\mu L$)	0.265	0.19
HbA1c (%)	0.597	0.01
nPCR (g/kg/d)	−0.013	0.59
hsCRP (mg/L)	−0.144	0.57
Albumin (g/dL)	0.013	0.95

cfDNA, cell-free DNA; HbA1c, hemoglobin A1c; HD, hemodialysis; hsCRP, high-sensitivity C-reactive protein; nPCR, normalized protein catabolic rate; SBP, systolic blood pressure; WBC, white blood cell.

tissue damage; these processes are associated with their deteriorating clinical conditions and with diabetes, CV disease, infection, and malnutrition.

El Tarhouny et al [16] have reported that high levels of cfDNA were present not only in the complicated group but also in the diabetic patients without complications, although to a lesser extent. Tovbin et al [13] have found that cfDNA levels correlate with IL-6 and are particularly elevated in patients with proinflammatory conditions, such as DM. The association among cfDNA, IL-6, and diabetes is not surprising because both inflammation and diabetes are destructive conditions that increase cell death. Previous studies have reported that increased cfDNA levels after acute ischemic stroke reflected the clinical severity of ischemic stroke (in the acute stage) in patients, and a study measuring cfDNA in rat models of focal brain ischemia found correlations with the extent of ischemic injury, brain edema, and neurologic outcomes in rats 24 hours after middle cerebral artery occlusion. cfDNA levels have also been the focus of extensive research diagnosing and monitoring malignancy, acute myocardial infarct, and infection.

Among the clinical parameters, 3-month mean SBP was independently correlated with the cfDNA level in HD patients and diabetic HD patients. But there were few studies about elevation of cfDNA and hypertension directly even if Levine et al [17] have found elevation in cell-free fetal DNA in maternal plasma by pre-eclampsia, which is a hypertensive syndrome that occurs only during pregnancy. Jylhava et al [18] demonstrated that high levels of plasma cfDNA are closely linked with the presence of cardiometabolic risks, including impaired glucose tolerance (higher homeostasis assessment of insulin resistance and insulin levels) and elevated blood pressure and inflammatory cytokines [CRP, IL-6 and tumor necrosis factor (TNF) α] in clinically healthy subjects. Therefore, this result that cfDNAs are related with SBP in HD patients and diabetic HD patients suggested that cfDNA elevation can be influenced by hypertension like other diseases mentioned before. Recently, the presence of free nucleic acids in the peripheral circulation, referred to as cfDNA, has been proposed as a novel biomarker of CV risk [19]. Amanda et al [20] demonstrated that cfDNA levels are not influenced by renal impairment but do reflect endothelial dysfunction in patients with chronic kidney disease (CKD). Our data suggest that cfDNA could be an indicator of vascular injury rather than proinflammatory marker. Our data could not show the enough evidence of cfDNA enough as proinflammatory marker (such as hsCRP) in HD patients. Tovbin et al [13] showed that the postdialysis cfDNA level was an independent predictor of all-cause mortality in patients undergoing HD.

There were limitations in this study. We did not show the correlation with cfDNA in various inflammatory markers because of the limitation of the study design and the small number of patients. There is a need for large prospective studies in different types of other inflammatory markers such as IL-6, erythrocyte sedimentation rate, TNF α, and biomarker of endothelial marker such as von Willebrand factor. In this study, age between HD patients and the control group shows a considerable gap, and there was a concern that the gap might affect the distinction of cfDNA level. In order to solve this dispute, we compared 17 patients with HD (age, 34.9 ± 2.7 years) with 15 controls (age, 32.8 ± 1.2 years). And mean of cfDNA levels of 17 patients with HD was still higher than 15 controls (3,558.7 ± 45.7 GE/mL vs 1,420.0 ± 121.0 GE/mL, $P < 0.05$). In both univariable regression analysis and multivariable regression analysis, age was not closely linked with plasma cfDNA levels. Several other important clinical issues need to consider whether physical activity, common vascular disease risk factors (age, gender, smoking, and so forth), other confounding factors, and pharmacologic treatments affect the quantity and quality of circulating cfDNA levels or not.

In conclusion, cfDNA was associated with the presence of DM, comorbid condition of CV diseases, and SBP in HD patients. It is the first report that showed that uncontrolled hypertension and poor glycemic control are independent determinants for the elevated cfDNA in dialysis patients. Our data suggest that cfDNA could be an indicator of vascular injury, especially in diabetic HD patients.

Conflicts of interest

All authors have no conflicts of interest to declare.

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