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Relation between development of cardiovascular disease and the C242T CYBA polymorphism of the NADPH oxidase in ESRD patients

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Abstract. *Background*: The development of cardiovascular disease in ESRD patients is considered to be associated with oxidative stress. NAD(P)H oxidase has attracted attention as mechanisms of generating oxidative stress. We investigated the relation between the genotype of the C242T CYBA polymorphism of the NADPH oxidase and the development of cardiovascular disease in ESRD patients.
Methods: A total of 289 ESRD patients were recruited and allocated to one of the two groups: patients without cardiovascular disease (group N; $n = 192$) and patients developing cardiovascular disease (group D; $n = 97$). The C242T CYBA polymorphism was determined by RFLP-PCR methods.
Results: The frequency of the C242T CT+TT genotype was significantly lower in group D than in group N (9.1 vs. 20.2%). In multiple Logistic regression analysis, systolic blood pressure, smoking history and this gene polymorphism were shown to be independent variables for the development of cardiovascular disease in ESRD patients.
Conclusions: These results suggest that assessment of the C242T CYBA polymorphism of the NADPH oxidase may be useful in identifying the risk for developing cardiovascular disease in ESRD patients.

Keywords: NAD(P)H Oxidase, gene polymorphism, cardiovascular disease

1. Introduction

Despite significant progress in renal replacement therapy, the mortality from cardiovascular disease (CVD) in patients with end-stage renal failure (ESRD) is many times higher than in the general population. Although the traditional risk factors are frequently present in ESRD patients, these factors do not fully explain the extraordinary increase in morbidity and mortality in CVD among patients with ESRD. Recent studies have highlighted the accumulation of reactive oxygen species (ROS) as one of the mechanism involved in the development of CVD of ESRD patients [1], via the following pathological pathways: ROS may injure the endothelial cell membrane, inactivate NO, and cause oxidation of an essential cofactor of nitric oxide synthase (NOS) [2,3]. It has been proposed that genes associated with oxidative stress contribute to the risk of CVD in ESRD patients. Recently, NADPH oxidase has received attention as mechanisms of generating oxidative stress. Catalyzing the l-electron reduction of oxygen using NAD(P)H as the electron donor, NADH/NADPH

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oxidase is a membrane-associated enzyme for superoxide production in vascular smooth muscle cells and endothelial cells [4]. Studies indicate that NAD(P)H oxidase is the most important source of ROS in intact arteries [5–8]. In addition, it has reported that the C242T CYBA polymorphism of the NADPH oxidase is associated with vascular superoxide production in human blood vessels from CVD patients [9]. Therefore, it is possible that this polymorphism may contribute to the imbalance of ROS found in CVD in ESRD patients. In this study, we decide to investigate the association of genotype of the susceptibility to developing CVD in Chinese ESRD patients.

2. Materials and methods

2.1. Subjects

The subjects were 289 unrelated Chinese patients diagnosed with ESRD who were recruited in the outpatient clinic and inpatient department of the second Xiangya Hospital of Central South University since 2005. From these subjects, we allocated them to one of the two groups: group N, comprised of 192 patients without CVD, and group D, comprised of 97 patients with CVD. Of the 97 ESRD patients with CVD, 26 patients had experienced a myocardial infarction, and 71 patients had angina pectoris diagnosed by an exercise test or by scintigraphy. Group N was defined using the following criteria: 1) all participants had creatinine clearance of $<$ 10 ml/min for 5 years and 2) subjects with diagnosis of CVD according to the following criteria were excluded. Group D was defined according to the following criteria: 1) all participants had creatinine clearance of $<$ 10 ml/min for 5 years and 2) a previous myocardial infarction was defined as a heart attack diagnosed by a physician based on chest pain, electrocardiographic changes, and enzyme determinations or the results of coronary angiography. In patients with chest pain and ischemic electrocardiographic abnormalities, an exercise test or dipyridamole thallium scintigraphy was performed. Patients with a history of myocardial infarction or a positive result in one of these tests were diagnosed with coronary artery disease [10].

Clinical data, including onset age, BMI at the diagnosis of ESRD, history of hypertension, blood pressure, history of smoking and lipid profiles were obtained from the medical records of the patients. BMI was calculated as body weight in kilograms divided by the square of body height in meters. Body weight and body height were measured with light clothes and bare feet. The systolic and diastolic blood pressures used in the analysis were the average of three sitting blood pressure readings. Smoking history was defined on the basis of current cigarette smoking or previous habitual smoking. After at least an 8-h fast, blood samples were collected from the subjects, and serum total cholesterol, LDL cholesterol, HDL cholesterol, creatinine, and triglyceride were determined by at the Nephrology Laboratories of the second Xiangya Hospital of Central South University following standard laboratory protocols. LDL cholesterol was calculated using the Friedewald formula. Serum creatinine was performed with a Beckman CXR rate method using the Jaffe reaction. Creatinine clearance calculated according to the Cockcroft-Gault formula.

Each patient was diagnosed by at least two physicians on the basis of unstructured interviews and information from medical records, and the Kappa coefficients of agreement between raters were 0.91. All participants belong to the Han ethnic group in Hunan Province. The local ethical committee approved the experimental design, and all participants gave their informed consent.

2.2. Genotyping

Genomic DNA was extracted from peripheral blood cells using a DNA extraction kit (PUREGENE$^{\mathrm{TM}}$ Blood Kit; Gentra) and the DNA concentration was then determined by spectrophotometry. The C242T polymorphism was determined by PCR and *Rsa*I digestion.

The DNA fragment containing the polymorphic site was amplified from genomic DNA by PCR with sense oligonucleotide primer 5'- CTC TGT GTT GTC TTC AGT AAA GG-3' and antisense oligonucleotide primer 5'-ACT CAC AGG AGA TGC AGG ACG-3' [11]. The 50 μl reaction mixture contained 0.1 μg of genomic DNA; 5 μl of 10$\times$Taq buffer with Kcl (MBI, fermentas); 4 μl of 25 mM $MgCl_2$ solution; 2 μl each of the 10 μM sense and antisense primers; 1 μl of 10 mM deoxy-nucleotide triphosphates, specifically deoxy-adenosine triphosphate, guanosine triphosphate, cytosine triphosphate, and thymidine triphosphate; and 1.25U of Taq DNA polymerase. The PCR consisted of the initial denaturation step for 5 min at 94°C, 32 cycles of denaturation for 30 s at 92°C, primer annealing for 45 s at 62°C, and primer extension for 90 s at 72°C, followed by a final extension step for 10 min at 72°C. After digestion with *Rsa*I (MBI, Fermentas), the

Table 1
Clinical characteristics of ESRD patients with CVD and without

Variables	Group D($n = 97$)	Group N($n = 192$)
Age(yr)	54.4 ± 11.5	53.3 ± 12.6
sex(M/F)	50/47	99/93
BMI	23.2 ± 2.1	24.1 ± 3.0
Hypertension(yes/no)	57/40	110/82
SBp(mmHg)	160.1 ± 26.42[a]	115.2 ± 21.21
DBp(mmHg)	81.23 ± 17.1	80.98 ± 15.9
Smoking history(yes/no)	60/37[a]	90/102
Total cholesterol(mmol/L)	5.20 ± 0.66	5.12 ± 1.01
Triglycerides(mmol/L)	1.58 ± 0.99	1.57 ± 1.09
HDL cholesterol(mmol/L)	1.01 ± 0.32	1.11 ± 0.23
LDL cholesterol(mmol/L)	3.12 ± 1.31	3.11 ± 1.03

[a]$p < 0.05$ *versus* controls.

Table 2
Frequencies of genotypes of p22phox C242T polymorphism

Genotype	Group D($n = 97$)	Group N($n = 192$)	χ^2	p
CC	87 (89.7)	152 (79.2)		
CT+TT	10 (10.3)	40 (20.8)	4.988	0.026

Numbers in parentheses indicate percentages.

samples were separated on 1.5% agarose gel and visualized with ethidium bromide. Digestion of the PCR products yielded bands of 348 bp in CC homozygotes, 188 and 160 bp in TT homozygotes, and all three bands in heterozygotes. Each genotype was read by two individuals independently; if in conflict, genotyping was repeated.

2.3. Statistical analysis

Hardy-Weinberg Simulator software (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwal.pl) was used to test the genotype distribution for Hardy-Weinberg equilibrium. Data were expressed as means $\pm$ SD. Student's t test and chi-square test were used to compare the differences of the continuous and categorical variables between groups. χ^2 analysis was used to compare the genotype distributions of genotypes and alleles between groups. Logistic regression analyses were used to adjust for possible confounders including age, sex, BMI, smoking habit, history of hypertension, SBP and DBP and evaluate the OR of the genotypes and alleles for ESRD patients with CVD and their 95% confidence interval (CI) with two-tailed p values. All statistical analysis was performed using SPSS 13.0.

3. Results

Table 1 shows the clinical characteristic of the enrolled patients. Clinical data, including age, sex, BMI, history of hypertension, blood pressure, history of smoking, and lipid profiles were obtained from the medical records. There were no significant differences in sex, age, BMI, history of hypertension, diastolic blood pressures, serum total cholesterol, HDL, LDL cholesterol, and triglyceride between group N and D, and the systolic blood pressure and smoking history of group D were significantly higher than that of group N.

The genotype distribution of this gene in each group is shown in Table 2. The genotype frequencies in both groups were in the Hardy-Weinberg equilibrium. Because the TT genotype frequency was low, we divided the enrolled subjects into two groups: CC and CT+TT. The frequency of the CC, CT+TT genotypes of the p22phox gene were 79.2 and 20.8% in group N compared with 89.7 and 10.3% in group D, respectively. The frequency of the CT+TT genotypes was significantly higher in group N than in group D ($\chi^2 = 4.988$, $p = 0.026$).

The risk factors for the development of CVD in the ESRD patients are shown in Table 3. Logistic regression analysis identified systolic blood pressure and smoking history as independent risk factors for CVD of ESRD within the following variables: age, sex, body mass index (BMI), diastolic blood pressure (DBP), history of hypertension, smoking history, serum total cholesterol, HDL, LDL cholesterol, and triglyceride levels. When the CT+TT genotypes were introduced into this model, the CT+TT genotypes were an independent protective factor for CVD in the ESRD patients: adjusted odds ratio 0.423 (95% CI 0.239-0.711,

Table 3
Risk of CVD in ESRD patients

Variables	Adjusted odds ratio	95% CI	*P*value
Age(yr)	0.912	0.318–1.476	0.721
Gender(M/F)	1.001	0.880–1.234	0.113
BMI	1.002	0.894–1.345	0.132
Hypertension(%)	1.002	0.768–1.612	0.881
SBp(mmHg)	1.997	1.038–3.711	0.021
DBp(mmHg)	1.107	0.499–2.411	0.317
Smoking history(%)	2.081	1.413–4.478	0.004
Total cholesterol(mmol/L)	1.043	0.472–1.898	0.612
Triglycerides(mmol/L)	1.004	0.521–1.988	0.781
HDL cholesterol(mmol/L)	0.871	0.234–1.891	0.478
LDL cholesterol(mmol/L)	1.011	0.201–1.991	0.572
CC	2.391	1.101–4.289	0.004
CT+TT	0.423	0.239–0.711	0.011

Logistic regression analysis taking into account of age, gender, BMI, Hypertension, SBp, DBp, Total cholesterol, Triglycerides, HDL cholesterol and LDL cholesterol. The concordance between the observed and predicted risk of CVD in ESRD patients was 57.9%.

$P = 0.011$). These analyses revealed that this polymorphism is a significant factor in the development of CVD.

4. Discussion

In this study, we investigated the relationship between CVD in ESRD patients and the C242T polymorphism and found that the 242T allele is associated with significantly lower risk of CVD in ESRD patients. These findings provide direct data relating CVD in ESRD patients to the C242T polymorphism.

NAD(P)H oxidase is a critical enzyme for superoxide production in phagocytes, vascular smooth muscle cells, and mesangial cells [4]. P22phox is an essential component of NADPH oxidase. The C242T polymorphism results in an amino acid polymorphism (His/Tyr) at residue 72 of p22phox involving a potential heme-binding site [12]. Because the histidine residue is considered to be a candidate for the ligand of the heme prosthetic group of cytochrome b, it has been revealed that this polymorphism is associated with the function of p22phox and significantly lower basal and NADPH-stimulated vascular superoxidase production in human blood vessels from patients with atherosclerosis [13, 14]. Individuals with the 242T genotype might have lower oxidative stress as a result of lower O_2^- production compared with individuals bearing the 242C genotype.

Previous studies have examined this polymorphism in association with CVD. Inoue et al. found that the 242T allele was associated with a reduced risk of coronary artery disease [9], whereas other studies showed conflicting findings in patients with CVD and cerebrovascular disease [15–20]. However, the genotyping of patients in the prospective Lipoprotein and Coronary Artery Study (LCAS) suggested a significant association between the 242T allele and the progression of coronary atherosclerosis [17]. At the same time, studies on this polymorphism in association with CKD have produced conflicting results. Matsunaga-Irie et al. found a significant association with diabetic nephropathy in type 2 diabetes [21]. Hodgkinson et al. showed an association of the C242T polymorphism with diabetic nephropathy in type 1 diabetes [22]. However, Wolf et al. could not demonstrate any significant association of the C242T polymorphism with IgA nephropathy and lupus nephritis [23]. Doi et al. found that the T allele of the C242T polymorphism showed a protective effect against ESRD only in the nondiabetic (non-DM) group [24]. We performed a case-control association study consisting of a total of 97 cases and 192 control samples to elucidate the polymorphism in the CYBA gene encoding NAD(P)H p22 phox and identified a strong association of the CT+TT genotype of the C242T polymorphism with CVD in ESRD even after adjusting for confounding factors.

The conflicting results may reflect the ethnic backgrounds of the study populations. Inadequacy in the sample size, genetic heterogeneity, environmental background, and different definition of renal disease phenotype are additional limited factors. We have attempted to minimize these pitfalls by selecting well-matched samples from one single center. Moreover, our population can be considered fairly homogeneous, with all subjects originating from the same geographically well-defined catchment area (city of Hunan Province

of China). ESRD with CVD phenotype was also defined strictly. In our study, adhering to a case-control association strategy, we found that patients with the CT+TT genotype have a lower risk of developing CVD in ESRD. After adjusting for the confounding factors in Logistic regression model, the CT+TT genotypes were an independent protective factor for CVD in the ESRD patients.

In conclusion, we propose that the C242T CYBA polymorphism of the NADPH oxidase is associated with the development of CVD in ESRD. To further elucidate the potential association between the C242T CYBA polymorphism of the NADPH oxidase and CVD in ESRD patients, investigations with larger sample size are needed to confirm these associations.

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