Cardiovascular disease (CVD) is a major cause of morbidity and mortality in patients with chronic renal failure undergoing hemodialysis (HD) therapy, which accounts for approximately half of all deaths in this population.1 It was reported that cardiac events, such as myocardial infarction, are approximately 5 to 50 times higher than in the general population.2 The "traditional risks" for CVD in HD patients are hypertension, dyslipidemia, diabetes mellitus, and smoking. However, "non-traditional" risk factors, such as oxidative stress and inflammation, have surfaced to play an important role in excessive cardiovascular morbidity and mortality of these patients.3

Iron is essential in all organisms for key biochemical functions such as oxygen transport and oxidative phosphorylation. The benefits of intravenous iron therapy for HD patients have been well established since intravenous iron is necessary for enabling most iron-deficient HD patients to achieve normal hemoglobin (Hb) levels.4 Some studies showed that iron administration would increase the CVD risk in HD patients.5

Hereditary Hemochromatosis (HH) is an inherited disorder of iron metabolism characterized by increased intestinal iron absorption, leading to progressive iron overload.6 HH is common among populations of Northern European associated with HFE gene mutations.7 In 1996, Feder et al8 described 2 mutations in the HFE gene located on chromosome 6, C282Y and

Correlation of hemochromatosis gene mutations and cardiovascular disease in hemodialysis patients

Min Bi,a Bing Li,a Qiang Li b

From the a Department of Nephrology, The Second Affiliated Hospital of Harbin Medical University, Harbin, China; b Department of Endocrinology, The Second Affiliated Hospital of Harbin Medical University, Harbin, China

Correspondence: Prof. Qiang Li · Department of Nephrology, The Second Affiliated Hospital of Harbin Medical University, 246 Xuifu Road, Nangong District, Harbin 150081, China · T: +86-451-86605256 F: +86-451-86605256 · bimin1977@126.com

BACKGROUND AND OBJECTIVES: Cardiovascular disease (CVD) is a major cause of death in hemodialysis (HD) patients. Hemochromatosis (HFE) gene mutations are reported to be associated with CVD. The present study aims to investigate the association of HFE gene polymorphism with CVD in HD patients.

DESIGN AND SETTINGS: Cross-sectional case-control.

METHODS: C282Y/H63D mutations of HFE gene were evaluated in 560 HD patients and 480 healthy controls from 4 HD centers in North China. The results obtained from this evaluation process were correlated with biochemical parameters including iron status (serum iron, ferritin, and transferrin concentration), cardiovascular disease, and inflammation marker CRP, IL-6, TNF-α.

RESULTS: No C282Y mutations were detected in HD patients or healthy controls in this study. The genotype of H63D heterozygous mutation was similar in HD patients with CVD, HD patients without CVD, and controls. H63D homozygous mutation was 7.4% (19/257), 3.1% (9/303), and 1.0% (5/480) for the 3 groups, respectively. Compound heterozygosity was not found in this study. The relative risk for CVD in HD patients with H63D homozygous mutation was 2.59 (95% CI: 1.15-5.84). H63D homozygous mutation had significantly higher serum ferritin concentrations compared with wild-type individuals. Moreover, HD patients had significantly higher levels of inflammatory biomarkers such as CRP, IL-6, and TNF-α. The multivariate logistic regression analysis revealed that H63D mutation instead of ferritin level was an independent risk factor of CVD for HD patients.

CONCLUSIONS: Our study demonstrates for the first time that there was an association between H63D homozygous mutations and CVD in HD patients. Elevated serum CRP, IL-6, and TNF-α levels were also related to CVD in HD patients.
H63D. Patients homozygous for C282Y mutation and compound heterozygosity (C282Y and H63D) could show an increase in iron absorption. The involvement of HFE gene polymorphism in the pathogenesis of CVD has been extensively studied in various populations. But the association of HFE genotype and CVD in HD patients is still debated. Although several prospective studies have suggested an increased risk of CVD in carriers of the C282Y mutation, some researchers have showed that C282Y mutation was not significantly associated with angiographically documented coronary atherosclerosis.

We hypothesized that HFE genotypes may be related with CVD in HD patients by modulating the iron metabolism. Therefore, we undertook the cross-sectional study, which included 560 patients on HD and 480 healthy controls to examine the effect of the 2 common HFE C282Y and H63D mutations on CVD in HD patients from Northern China.

**METHODS**

**Patients**

A total of 560 HD patients from 4 HD centers in North China were enrolled in this study. Patients with active infectious disease, liver disease, inflammatory disease, or malignancies were excluded from this study. All HD patients were on conventional 4-hour HD sessions, 3 times in a week with polysulfone or polyamide hollow-fiber dialyzers and bicarbonate dialysate. The HD patients were given recombinant human erythropoietin to maintain Hb levels between 10 and 12 g/dL. Oral or intravenous iron was administered when transferrin saturation was <30% or serum ferritin <200 ng/mL. The administration of iron was temporarily suspended when ferritin was >500 ng/mL as suggested. A total of 480 healthy volunteers without major health problems matched by age (SD=3 years) and gender were included as control subjects. Informed consents were obtained from all individuals enrolled in this study. The study was conducted according to the principles contained in the Declaration of Helsinki and was approved by the Institutional Ethics Committee.

**Laboratory measurements**

Blood samples were obtained before dialysis to assess biochemical parameters using commercially available kits: serum calcium, serum phosphate, serum albumin, Hb, triglyceride, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C). Moreover, serum C-reactive protein (CRP) was determined by an immunoturbidimetric assay (Beckman, USA). Levels of IL-6 and TNF-α were determined using human enzyme-linked immunosorbent assay kits (Jingmei Biotech, Shanghai, China). Serum iron, transferrin, and ferritin were measured by immunocheminometric assay. Transferrin saturation (TS) was calculated by the formula: TS=(serum iron/total iron-binding capacity)×100%.

**CVD definition**

CVD was defined as acute myocardial infarction, angina, congestive heart failure, angioplasty, stroke, peripheral arterial disease, or abdominal aortic aneurysm during the study. Each condition was verified by biochemical parameter, radiography, and echocardiography.

**Genotyping of HFE polymorphism**

Genomic DNA was extracted from 5 mL of venous EDTA blood which was stored at –20°C using a DNA extraction kit (Tiangen Biotech Co. Ltd, Beijing, China) according to the manufacturer’s protocol. The DNA fragments of the HFE gene were amplified by polymerase chain reaction (PCR) using a PCR reaction mix kit (Takara Bio Inc, Dalian, Japan). For identification of C282Y mutation, the used primers were as follows: forward 5’-TGGCAAGGGTAAACAGATCC-3’ and reverse 5’-CTCAGGCACTCCTCTCTCAACC-3’. For identification of H63D mutation, the used primers were as follows: forward primer 5’-ACATGGTTAAGGCCTGTTGC-3’ and reverse primer 5’-GCCACATCTGGCTTGAAATT-3’. The thermocycling program for the 2 mutations was performed as follows: 40 cycles of 45 s denaturation at 95°C, 45 s annealing at 58°C, and 90 s extension at 72°C. RsaI (C282T analysis) and BclI (H63D analysis) restriction enzyme digestion was employed after PCR genomic amplification to determine the HFE genotypes.

**Statistical analysis**

SPSS, version 17.0 (Inc., Chicago, Illinois) was used for all statistical analyses. Categorical data were presented as percentages and compared by chi-square analysis or Fisher exact test when necessary. Continuous variables were presented as mean (SD) and compared by t test or analysis of variance. The relative risk associated with rare alleles was estimated as an odds ratio (OR) with a 95% confidence interval (CI). All calculated P values were 2 sided and a P value <.05 was considered statistically significant.
RESULTS

Baseline characteristics of the subjects
The relevant baseline characteristics of the patients and healthy controls were shown in **Table 1**. Among 560 patients included in the analysis, 273 (48.8%) were men and 287 (51.3%) were women. The mean age of the 560 patients was 58.75 (4.61) years. The mean duration of HD therapy was 6.5 (3.6) years (range 1-15 years). The mean value of Kt/V at the time of the investigation was 1.33 (0.36). Age, sex, and BMI were identical for the HD patients and control subjects. No significant differences between the patients and controls in biochemical parameters related to serum albumin, Hb, parathyroid hormone, serum calcium, triglycerides, LDL-C, HDL-C, serum iron, and transferrin. HD patients were more likely to have a history of hypertension and diabetes mellitus. Ferritin, CRP, IL-6, and TNF-α levels were significantly higher in the HD patients compared to the controls (P<.05).

**HFE gene mutations was related to CVD**

The genotype distribution of the 2 common *HFE* mutations C282Y and H63D among the patients and controls was shown in **Table 2**. No C282Y mutations were detected in hemodialyzed patients or healthy controls in this study. Heterozygosity for the H63D mutation was found in 18 of 257 HD patients with CVD (7.3%), in 24 of 303 (7.7%) HD patients without CVD, and in 33 of 480 controls (6.9%). Homozygosity for the H63D mutation was 7.4% (19/257), 3.1% (9/303), and 1.0% (5/480) for the 3 groups, respectively. No significant differences were observed in the prevalence of H63D heterozygous mutation between the 3 groups. However, for H63D homozygous mutation, HD patients with CVD had a significantly higher rate of mutations with respect to the other 2 groups. No significant differences were observed among the HD patients and healthy controls.

**Table 1.** Clinical and serum laboratory characteristics of hemodialysis patients compared to control subjects.

| Variable                  | HD (n=560) | Controls (n=480) |
|---------------------------|------------|------------------|
| Age (y)                   | 58.75 (4.61) | 57.34 (5.34)    |
| Sex (M/F)                 | 273/287    | 259/221          |
| Years receiving dialysis  | 6.5 (3.6)  | none             |
| BMI (kg/m²)               | 25.94 (4.22) | 25.28 (4.36)    |
| Serum albumin (g/dL)      | 3.6 (0.4)  | 3.6 (0.5)        |
| Hemoglobin (g/dL)         | 116 (12)   | 122 (11)         |
| PTH (IU/mL)               | 275.78 (56.88) | 266.68 (61.23) |
| Serum calcium (mg/dL)     | 9.5 (0.6)  | 9.9 (0.7)        |
| Serum phosphorus (mg/dL)  | 7.7 (2.6)  | 7.6 (3.3)        |
| Triglycerides (mg/dl)     | 195.46 (51.3) | 192.9 (56.7)   |
| LDL cholesterol (mg/dL)   | 121.74 (39.8) | 122.65 (39.2) |
| HDL cholesterol (mg/dL)   | 42.34 (11.3) | 43.48 (12.6)    |
| Hypertension, n (%)       | 89 (15.89) | 55 (11.46)      |
| Diabetes mellitus, n (%)  | 296 (52.86) | 217 (45.21)     |
| CRP (mg/L)°               | 5.94 (0.26) | 3.18 (0.54)     |
| IL-6 (pg/mL)°             | 29.23 (9.2) | 26.95 (1.29)    |
| TNF (pg/mL)°              | 14.15 (0.56) | 10.36 (0.78)  |
| Serum iron (µg/dL)        | 100.45 (28.44) | 91.83 (29.09) |
| Ferritin (µg/L)°          | 311.43 (103.22) | 98.81 (22.99) |
| Transferrin (mg/dL)       | 161.28 (18.63) | 201.32 (19.03) |
| Transferrin saturation %  | 49.83 (10.11) | 35.90 (10.32)  |

HD: Hemodialysis, BMI: body mass index, PTH: parathyroid hormone, CRP: C reactive protein, IL-6: interleukin-6, TNF-α: tumor necrosis factor-alpha, M/F: male/female, HDL: high-density lipoprotein, LDL: low-density lipoprotein. °P<.05.

**Table 2.** The genotype distribution of the *HFE* polymorphisms C282Y and H63D between patients and controls.*

| Genotype | HD - CVD, n (%) | HD + CVD, n (%) | Controls, n (%) | P value |
|----------|-----------------|-----------------|-----------------|---------|
| C282Y    | 303 (100%)      | 257 (100%)      | 480 (100%)      | NS      |
| H63D     | 270 (89.2%)     | 220 (85.6%)     | 442 (92.1%)     | NS      |
| +/-      | 24 (7.7%)       | 18 (7.3%)       | 33 (6.9%)       | NS      |
| ++       | 9 (3.1%)        | 19 (7.4%)       | 5 (1.0%)        | .03     |

* and –/–: Indicate the presence and absence of gene mutation;
HD – CVD: hemodialysis patients without cardiovascular disease, HD + CVD: hemodialysis patients with cardiovascular disease.

*Hemodialysis patients with cardiovascular disease had significantly higher rate for H63D homozygote mutations with respect to other two groups, no significant differences were observed among hemodialysis patients and healthy controls.
controls. In addition, compound heterozygosity was not found in this study. These data yielded an OR for CVD related to H63D homozygous mutation in HD patients (Table 3). The relative risk for CVD in HD patients with H63D homozygous mutation was 2.59 (95% CI: 1.15-5.84).

### HFE mutations and serum iron indices

H63D homozygous mutation (H63D +/+ ) in HD patients with CVD had significantly higher serum ferritin concentrations compared with wild-type (H63D –/–) individuals and HD patients without CVD (Table 4). No obvious differences in serum iron or transferring levels were observed between H63D genotype in HD patients with and without CVD. However, HD patients with CVD had higher serum ferritin and inflammation biomarkers levels of CRP, IL-6, and TNF-α. H63D genotype seems to have no relations with the increased levels of inflammatory biomarkers since no differences were found between patients with genotype H63D –/– and H63D +/+ . Taken together, these data indicated that H63D homozygous mutation (H63D +/+ ) was related with the elevated serum ferritin level for HD patients, and CRP, IL-6, and TNF-α levels were also associated with CVD in HD patients.

### Multivariate analysis

Multivariate logistic regression analysis was used to identify independent predictors of CVD. The independent predictors of CVD were presented in Table 5. The variables associated with CVD in HD patients were age, CRP, IL-6, and TNF-α. However, gender and ferritin were not independent risk factors while H63D mutation was an independent risk factor of CVD for HD patients.

### DISCUSSIONS

The present study demonstrated that HFE polymorphisms C282Y and H63D are similarly distributed in the HD patients and healthy controls except H65D homozygous mutation, which was slightly higher in HD patients with CVD. Ferritin was also elevated in CVD patients with H65D homozygous mutation. However, H63D mutation instead of ferritin was an independent risk factor of CVD for HD patients.

The HFE gene C282Y and H63D mutations are common causes of iron overload that may lead to CVD. However, up to now, few data were available from China about the prevalence of the mutations in the HD and general population. In this study, we investigated the HFE gene polymorphisms C282Y and H63D in the HD patients and healthy controls from North China. The C282Y mutation was not found in the HD patients and healthy controls. However, for H63D homozygous mutation, HD patients with CVD had a moderately higher rate of mutations.

The relative risk for CVD in HD patients with
H63D homozygous mutation was 2.59 (95% CI: 1.15-5.84). A Hemochromatosis and iron overload screening study including 12,772 Asian subjects suggested that the distribution of C282Y and H63D mutations has ethnic and environmental differences, which was in line with our data that C282Y mutations were not detected. This discrepancy may reflect a variant distribution of HFE gene polymorphisms in populations with a different ethnic background. Some studies have shown that individuals who carried HFE mutations may have a greater risk of developing coronary heart disease than those without the mutations, which was also in accordance with our results.

Pericole et al reported a trend toward higher ferritin in H63D mutation, which may play an important role in modulating iron overload in HD patients. In the present study, the serum iron level and transferrin levels were identical in the HD patients with or without CVD, which was not affected by H63D mutations. However, the ferritin level was raised in the HD patients with CVD, suggesting a role of increased iron stores in CVD, but iron overload was not an independent risk factor for patients.

Studies have shown that CRP, IL-6, TNF-α, the prototype markers of inflammation, play key roles in CVD. In the general population, CRP, IL-6, and TNF-α stayed within the normal range; in contrast, the HD patients showed a higher level even in the absence of infection complications. Since 30% to 50% of HD patients exhibit evidence of a markedly activated inflammatory response, HD can be considered a chronic systemic inflammatory state. In this study, the HD patients exhibited significant higher levels of CRP, IL-6, and TNF-α as compared with the healthy controls. These results indicated that the higher level of inflammation biomarkers in the HD patients are probably due to exposure to endotoxin and other pollutants, bioincompatible dialysis solution, and dialysis membrane. Iron therapy caused proinflammatory effects, as reflected by an increase in the production of CRP, TNF-α, and IL-6, which have been shown to play a role in CVD. Our data showed the HD patients with CVD have even higher levels of inflammation biomarkers than those patients without CVD, and inflammation is an independent predictor of CVD. It is probably that inflammation contributed to CVD in the HD patients. We postulated that H63D homozygous mutation would lead to increased body iron stores, which would promote oxidative stress and inflammatory disease, resulting in higher risk of CVD. Actually, some researchers have reported a positive correlation between H63D mutation and inflammation. However, further studies await to be carried out to confirm this hypothesis.

There are several limitations with our study. First, the relationship between the HFE gene polymorphism with CVD is described in a small group of HD patients that might lead to a lower statistical power. Second, disorders of iron metabolism include many factors, such as transferrin receptor 2, hepcidin, ferroportin, and hemosjuelin, which were omitted in this study.

In conclusion, the H63D homozygous mutations in the HFE gene slightly increased the risk for CVD in the HD patients from North China. Higher ferritin levels were found in CVD patients independent of the H63D genotype, suggesting that HFE polymorphisms were not a major determinant for the iron overload. The elevated serum CRP, IL-6, and TNF-α levels were found to be related with CVD in HD patients as well. Multivariate logistic regression analysis revealed that instead of serum ferritin levels, inflammation biomarkers (CRP, IL-6, and TNF-α) and H63D mutation were independent risk factors of CVD for HD patients.

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