IL-6 Haplotypes, Inflammation, and Risk for Cardiovascular Disease in a Multiethnic Dialysis Cohort

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It is unknown whether IL-6, a central regulator of inflammation, is a cause of or just a marker of atherosclerosis. Studies of genetic susceptibility to inflammation, however, avoid the potential for reverse causality. Variation in IL6 gene was studied as a predictor of cardiovascular disease (CVD) risk in a cohort of 775 incident dialysis patients, in whom IL-6 levels are elevated. On the basis of published resequencing data on the IL6 gene, a phylogenetic tree with three main branches (clades 1 to 3) was constructed. Two “clade tag” polymorphisms, −174G/C and 1888G/T, and two missense variants, Pro32Ser and Asp162Val, were genotyped. Circulating IL-6 and albumin were measured a median of 5 mo after the start of dialysis. CVD events were ascertained from medical records. During a median follow-up of 2.5 yr, 294 CVD events occurred. The two coding variants, Pro32Ser (present only in black patients, 10% Ser allele) and Asp162Val (present only in white patients, 1% Val), were associated with lower levels of IL-6 and higher levels of albumin. The common variant in the promoter region, −174G/C, was strongly associated with higher CVD risk and weakly with IL-6 levels. Clade 3 (−174C carriers in the absence of 162 Val allele) was associated with higher IL-6 levels (P = 0.03) and higher CVD risk (hazard ratio 1.44, P = 0.006) after adjustment for covariates. The IL6 gene has functional variants that affect inflammation and risk for CVD among dialysis patients, supporting a causal role for IL6 in CVD.


Inflammation is strongly and independently associated with cardiovascular disease (CVD) risk. It may explain the excess CVD risk in dialysis patients, in whom inflammation is common (1). Interleukin-6 (IL-6) is a major proinflammatory cytokine that is central to the inflammatory response, regulating the hepatic synthesis of acute-phase proteins, such as fibrinogen, C-reactive protein, and albumin. IL-6 mRNA is present in atherosclerotic arteries at a 10- to 40-fold higher level than in nonatherosclerotic vessels, and elevated levels of IL-6 are associated with increased risk for CVD, suggesting that IL6 has a role in the pathogenesis of atherosclerosis (2,3). Whether the relationship of IL-6 with CVD is causal, however, is difficult to prove because atherosclerosis also may induce the synthesis of inflammatory markers.

Establishing a role of genetic variants in susceptibility to CVD would bolster the inference that high levels of the inflammatory response to environmental stimuli can lead to CVD, because genetic variants cannot be a consequence of CVD. More light has been shed on a common G/C polymorphism at position −174 in the promoter region of the IL6 gene. The −174C allele has been associated with higher risk for CVD incidence and mortality in some studies of white patients (4–7) but not in others (8,9). Circulating concentrations of IL-6 are thought to be largely regulated at the level of expression; however, the role of the −174G/C variant is uncertain (10–12). Moreover, two missense variants in the coding region have been reported (13) but have not yet been examined in an association study.

The high variability in levels of inflammatory markers along with the very high rate of CVD in dialysis patients may allow the determination of subtle effects of the IL6 gene. Also, allelic heterogeneity should be considered because individual haplotypes may have differential effects (12). We investigated the influence of potential functional variants and common haplotypes in the IL6 gene on levels of gene expression, inflammatory marker, and CVD risk in a prospective study of dialysis patients.

Materials and Methods
Study Design and Population
The CHOICE study is a national prospective cohort study of 1041 incident dialysis patients who are aged 19 to 95 yr and from 81 dialysis clinics. The study was approved by the Institutional Review Boards, and participants provided written informed consent. The study design and enrollment criteria have been described elsewhere (14). Particip-
pants were enrolled from October 1995 to June 1998, median of 45 d after initiation of dialysis (95% within 3.5 mo), and were followed up through November 2000. Genotype information was available on 775 of 898 participants for whom blood was drawn before a dialysis session at a median of 5.0 mo from the initiation of dialysis (95% within 8.7 mo). Their baseline demographic and clinical data were obtained from questionnaires, as well as from hospital and clinic records. Prevalent CVD was defined as medical record documentation of coronary artery disease, cerebrovascular disease, or peripheral vascular disease. The level of cardiovascular and other comorbidity was assessed by a trained nurse on the basis of medical records and clinic staff reports using the Index of Co-Existing Disease, a standardized and validated four-level scale that has been tested in multiple studies (15).

**DNA Analysis**

The resequencing data of 24 African-American and 23 European patients from the Program for Genomic Applications at the University of Washington (13) were used to choose sequence variants to genotype. On the basis of 31 retrieved variants in the IL6 gene, 11 polymorphisms were selected in this study, including three previously described polymorphisms, 174G/C, 572G/C, and 597G/A, and two novel coding variants, Pro32Ser (C>T) and Asp162Val (A>T), along with six other polymorphisms (rs2069825, rs2069827, rs2069840, rs1554606, rs2069845, and rs2069849), which differentiate the 10 common haplotypes (Figure 1). Haplotypes were constructed separately for black and white patients using the PHASE program, version 1.0.1 (16).

Because of a limited statistical power to detect effects of the multiple haplotypes and the multiple comparison issues, we sought a method to classify haplotypes. Cladistic method, in which the evolutionary history of the haplotype variation is estimated, has been proposed for phenotypic association study (17,18). Such an approach requires that the cladistic structure not be disrupted by recombination and can be estimated. In the IL6 gene, most of the variants are in linkage disequilibrium and belong to a single haplotype block (defined using the Confidence Interval Method [19] implemented in Haplovie 3.2). The cladistic approach, therefore, was chosen for grouping haplotypes. A phylogenetic tree was inferred using Molecular Evolutionary Genetic Analyses version 2.1. On the basis of bootstrap values from the phylogenetic and molecular evolutionary analyses (20,21), haplotypes were sorted into three major branches of related haplotype groups, defined as clades. These three haplotype clades can be distinguished by the two polymorphisms 174G/C (rs1800795) and 1888G/T (rs1554606).

Genotyping was performed using TaqMan (Applied Biosystems, Foster City, CA) as the primary method. A length-modified single base extension protocol (22) was used when the TaqMan method failed, as was the case for the 174G/C polymorphism. The statistic, based on 45 blindly split samples from the CHOICE cohort, ranged from 93 to 100% for the four polymorphisms.

**Figure 1.** Phylogenetic relationships of IL6 gene haplotypes on chromosome 7p21. On the basis of resequencing data of 31 single-nucleotide polymorphisms (SNP) from 23 European and 24 African-American patients (13), a phylogenetic tree of the IL6 gene that was constructed using Molecular Evolutionary Genetic Analyses reveals three major branches: Clades 1 to 3. The two polymorphisms highlighted in gray and marked with a star (174 G/C and 1888G/T) were chosen as “clade tag” SNP. The numbers to the right are the frequencies (%) for black and white CHOICE participants of the corresponding haplotypes. Dots represent the same alleles as those on the ancestral haplotype on the bottom, and dashes denote deletions. The 11 genotyped SNP are underlined in the figure. The 31 SNP on the bottom line are as follows from left to right: rs2069824, rs2069825, rs2069827, rs1800797, rs1800795 (−597G/A), rs2069830 (−174G/C), rs2069832, rs2069833, rs2069834, rs1474347, rs1524107, rs2066992, rs2069833, rs2069840, rs1554606, rs2069841, rs2069842, rs1548216, rs2069843, rs2069844, rs2069847, IL6#5602, rs2069860, rs2069849, rs2069852, rs2069855, Il6#7592, and Il6#7659. IL-6 numbers are from the Programs for Genomic Applications annotated sequence (http://pga.gs.washington.edu/data/il6/il6.ColorFasta.html).
To assess for potential population stratification, a panel of 87 ancestry-informative single-nucleotide polymorphisms were genotyped to measure admixture. The degree of individual genetic white to black admixture was estimated using Bayesian methods implemented in the STRUCTURE program ver. 2 (23).

Biochemical Measurements
IL-6 was measured in serum by an ultrasensitive ELISA method (R&D Systems, Minneapolis, MN) with a coefficient of variation of 7%. Serum albumin levels were measured using the Bromocresol Green method (coefficient of variation 1.1%).

Outcome Ascertainment
CVD events were ascertained using follow-up through the dialysis clinics and Center for Medicare and Medicaid Services data. Incident CVD events included myocardial infarction, cerebrovascular accident, coronary artery bypass graft, percutaneous coronary angioplasty, peripheral artery bypass, amputation, abdominal aortic aneurysm repair, carotid endarterectomy, and sudden coronary death. Medical records from hospitalizations were reviewed and adjudicated by two members of the study’s outcomes committee using uniformly applied criteria modified from the Cardiovascular Health Study (24). The $\kappa$ statistic for the event adjudication was 95%.

Statistical Analyses
Single-locus analyses were performed for the four polymorphisms. Dominant mode of inheritance, which was suggested in the previous studies (4), was tested here. Haplotype analyses were conducted for the two clade tagging polymorphisms −174G/C and 1888G/T. Individuals were assigned the most likely pair of haplotypes (when the probability of assignment was >90%) using the PHASE program. The distribution of IL-6 levels was highly skewed to the right, and logarithmic transformations were applied for normalization. All regression analyses were performed using STATA 7.0 statistical software (StataCorp, College Station, TX). Mean levels of inflammatory markers are presented after adjustment to avoid confounding. Adjustment was arbitrarily set to female; 60 yr; white; on hemodialysis; and no comorbidity, including diabetes, congestive heart failure, and prevalent CVD. Choosing other levels would change the absolute level but not the pattern of association. For survival analysis, follow-up time was defined as the period from initiation of dialysis to the first CVD event. Individuals were censored as a result of renal transplantation ($n = 131$), loss to follow-up ($n = 3$), or death attributed to causes other than CVD ($n = 114$). For all survival analyses, the proportional assumption of the Cox model was confirmed by inspection of log (−log[survival function]) curves and Schoenfeld residuals.

Results
Table 1 shows the characteristics of 775 individuals according to race. Black patients tended to be younger and were more likely to be female, current smokers, and on hemodialysis. Black patients less frequently presented history of CVD and congestive heart failure. Black patients also had higher body mass index (BMI) and systolic BP and lower levels of IL-6. The genotype frequencies of the 11 IL6 polymorphisms in white patients were significantly different from those in black patients, and Hardy-Weinberg expectations were met in both races. The frequency of −174C allele (0.43 in white patients; 0.09 in black patients) was similar to that in the general population. The 32Ser allele was common in black patients and absent in white patients, whereas the 162Val allele was absent in black patients and rare in white patients. Clades 1 and 2 were more frequent, and clade 3 was less frequent in black than in white patients.

IL6 Polymorphisms, Levels of Inflammatory Markers, Incident CVD, and All-Cause Mortality
Compared with G/G homozygotes, carriers of the −174C allele had higher IL-6 levels and lower albumin (markers of inflammation) overall and in white patients, although the difference was marginally significant (Figure 2). This trend was not apparent in black patients.

Compared with 32Pro allele homozygotes, the Ser allele carriers had lower IL-6 levels and higher albumin levels. The 162Val allele, present on the −174C allele background, was significantly associated with lower IL-6 levels and higher albumin levels.

Over a median of 2.5 yr of follow-up, 294 CVD events occurred. Kaplan-Meier plot (Figure 3) and Cox proportional hazards model showed that compared with individuals with the genotype −174C/G, CVD risk was higher for GC heterozygotes and CC homozygotes and the hazard ratio (HR) of CVD was 1.81 (95% confidence interval [CI] 1.39 to 2.36) for GC and 1.37 (95% CI 1.00 to 1.89) for CC. A dominant inheritance model in which CC individuals were not at higher risk than GC individuals is consistent with previous studies (4). After adjustment for demographic information, diabetes, congestive heart failure, prevalent CVD, and comorbidity score (model a), these estimates diminished somewhat (HR 1.49; [95% CI 1.15 to 1.94] and HR 1.16 [95% CI 0.81 to 1.65], respectively). Both Pro32Ser and Asp162Val polymorphisms were associated with lower risk for CVD, but these associations were not significant. The 1888G/T polymorphism was not associated with levels of inflammatory markers or CVD risk.

IL6 Haplotypes, Levels of Inflammatory Markers, Incident CVD, and All-Cause Mortality
Haplotype analyses revealed patterns that were similar to the single-locus analyses (Table 2). Given that the 162Val allele had an opposite effect from the −174C allele and is present on the background of the −174C allele, the haplotype that contained the 162Val allele ($n = 10$) was removed from clade 3 (most of the −174C carriers). Clade 3 in the absence of 162Val was associated with higher levels of IL-6 ($P = 0.03$) and lower levels of albumin ($P = 0.06$). Clade 1 and clade 2 were not related with levels of inflammatory markers. Inclusion and exclusion of the haplotype that contained the 32Ser allele from clade 1 did not change the effect of clade 1.

In a dominant association model, clade 3 was associated with higher risk for CVD in overall and white patients, and clade 2 was associated with lower risk for CVD only in white patients. When compared with carriers of two copies of clade 1 (with 79 incident CVD events), clade 3 carriers (180 CVD events) but not clade 2 carriers (42 CVD events) were significantly associated with higher CVD risk. This suggests that the clade 2 effect was most likely due to the mirror effect of clade 3. With adjustment for covariates, the relative risk for CVD was 1.44 (95% CI 1.12 to
Table 1. Patient characteristics by race (n = 775) 

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>White (n = 529 [68%])</th>
<th>Black (n = 246 [32%])</th>
<th>P Valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean age (yr [SD])</td>
<td>59 (14)</td>
<td>54 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% female</td>
<td>43</td>
<td>53</td>
<td>0.008</td>
</tr>
<tr>
<td>% former smoker</td>
<td>49</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>% current smoker</td>
<td>16</td>
<td>18</td>
<td>0.02</td>
</tr>
<tr>
<td>% baseline hemodialysis</td>
<td>76</td>
<td>88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICED comorbidity score (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>level 0 to 1</td>
<td>32</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>level 2</td>
<td>37</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>level 3</td>
<td>30</td>
<td>26</td>
<td>0.15</td>
</tr>
<tr>
<td>% prevalent CVD</td>
<td>49</td>
<td>32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% diabetes</td>
<td>53</td>
<td>56</td>
<td>0.43</td>
</tr>
<tr>
<td>% congestive heart failure</td>
<td>48</td>
<td>45</td>
<td>0.006</td>
</tr>
<tr>
<td>mean BMI (SD)</td>
<td>26.7 (6.3)</td>
<td>27.8 (7.6)</td>
<td>0.04</td>
</tr>
<tr>
<td>mean SBP (mmHg [SD])</td>
<td>149 (19)</td>
<td>153 (17)</td>
<td>0.002</td>
</tr>
<tr>
<td>Laboratory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean cholesterol (mg/dl [SD])c</td>
<td>190 (49)</td>
<td>187 (51)</td>
<td>0.39</td>
</tr>
<tr>
<td>mean albumin (g/dl [SD])</td>
<td>3.6 (0.4)</td>
<td>3.6 (0.3)</td>
<td>0.64</td>
</tr>
<tr>
<td>median IL-6 (pg/ml [IQR])</td>
<td>4.3 (2.8 to 7.5)</td>
<td>3.7 (2.4 to 5.9)</td>
<td>0.01</td>
</tr>
<tr>
<td>IL6 polymorphisms (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−174G/C (promoter), C allele</td>
<td>43</td>
<td>9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1888G/T (intron 3), T allele</td>
<td>46</td>
<td>34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pro32Ser (exon 2), Ser</td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Asp162Val (exon 5), Val</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IL-6 haplotype clade carrier</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n [%]) −174G/C 1888G/T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clade 1: G/G</td>
<td>402 (76)</td>
<td>220 (89)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>clade 2: G /T</td>
<td>30 (6)</td>
<td>105 (43)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>clade 3: C/T</td>
<td>357 (67)</td>
<td>42 (17)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*BMI, body mass index; CVD, cardiovascular disease; ICED, Index of Co-Existent Disease; IQR, interquartile range; SBP, systolic BP.

bBy χ², t test, or Mann-Whitney U test.

cTo convert values for cholesterol to mmol/L, multiply by 0.02586.

1.84; P = 0.006) for clade 3 carriers compared with clade 3 noncarriers (Table 2). This association remains significant after Bonferroni correction for three comparisons (at a significance level of 0.016). Further adjustment for systolic BP, BMI, cholesterol, HDL cholesterol, and IL-6 levels attenuated this association (HR 1.41; 95% CI 1.01 to 1.96). The pattern of increased CVD risk in individuals with clade 3 was predominantly seen in white but not in black patients. No significant interactions were detected with race, smoking, age (<60 versus ≥60 yr), gender, or diabetes for CVD or mortality. All regression models were rerun with adjustment of genetic admixture. No significant changes were observed with this adjustment.

We also conducted further analyses to identify an “at risk” haplotype in clade 3. Except for the two rare haplotypes (the first and fourth ones), the two common haplotypes in clade 3 were similarly associated with higher CVD risk (the second one: HR 1.45, P = 0.06; the third one: HR 1.67, P = 0.002). Other additional haplotype analyses did not increase the predictive value of IL6 variants for inflammatory markers and CVD risk.

Discussion

In this large, prospective study of dialysis patients, we first reported that two coding variants in the IL6 gene, pro32Ser and Asp162Val, seem to downregulate the inflammatory process by lowering IL-6 and elevating serum albumin levels. The two variants are not significantly associated with reduced CVD risk, which could be due to a limited power related to the rarity of the two variants. Information on their functional significance is lacking. Our findings suggest that these coding variants may alter IL-6’s structural stability or function at the protein level, because amino acid substitutions from aspartic acid to valine and from proline to serine can have important structural and functional consequences (25). The data confirmed that the polymorphism −174G/C pre-
dicted incident CVD and mortality in white patients. The modest dominant effect of the $-174C$ allele that was observed in the dialysis population is consistent with that in the general population reported by two large-scale prospective studies (4,7) and two case-control studies (5,6) but not with other case-control studies (8,9,26,27). The previous difficulty demonstrating a significant association highlights two common problems. One is survival bias (e.g., early death attributable to genotype), which occurs particularly in a cross-sectional or case-control study. The other one is inadequate sample size to detect true associations, which also may be the case for our black subgroup analysis given the lower frequency of the $-174C$ allele in this group. The consistency of prospective data in diverse cohorts of white patients may mitigate the concern of confounding as a result of population stratification.

Our data revealed that the $-174C$ allele in the absence of the $162Val$ variant (clade 3) predicted higher serum levels of IL-6 and lower albumin levels. Because the $-174$ polymorphism is close to a glucocorticoid receptor binding site that has a negative regulatory effect, a mutation to the C allele from the ancestral G allele might influence binding at this receptor (11,28) and lead to a decreased ability to repress transcriptional activation and result in overexpression of the IL-6 gene during an inflammatory state. This hypothesis is supported by large in vivo studies in patients who had aneurysmal disease (7) or hypertension (29) or were postoperative (10) or in newborns after birth trauma (30). Those studies and ours shared a common setting where participants were exposed to inflammatory stimuli. Mixed results have been reported in healthy individuals (8,26,31–34) and in in vitro studies (11,12) of situations in which there is little or no inflammation and glucocorticoid regulation is not critical or absent. Nevertheless, inadequate sample sizes, confounding, gene–environment interactions, allelic (e.g., the $-174C$ effect may differ in the presence or absence of the 162Val) or locus heterogeneity, or a nonfunctional variant under study also may explain this inconsistency.

Our results indicate that functional variations in the IL-6 gene may modify CVD risk by influencing serum IL-6 levels and in some cases changing the structure of the IL-6 protein. These findings support an atherogenic role of IL-6 because genotypes precede atherosclerosis and do not change over time. The acute-phase reaction, triggered by upstream cytokines such as IL-6, likely are involved in atherosclerosis through endothelial activation, adhesion molecules release (35), vascular smooth muscle cell proliferation (36), platelet aggregability, and/or coagulation (37).

Our study has several limitations. Inflammatory markers were measured on only one occasion; multiple measurements would provide a more precise estimate of the true values. Unmeasured variability of circulating IL-6 levels within individuals may partially explain the observed residual effect of IL-6 gene variants after adjustment for serum IL-6 levels. Given the
Table 2. Adjusteda difference in mean levels of inflammatory markers and adjusted HR of incident CVD associated with the clades in the IL6 gene

<table>
<thead>
<tr>
<th>Clade</th>
<th>Mean Levels (SE) Overallb</th>
<th>HR of CVD (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Comparison Group</td>
<td>Albumin (g/dl)</td>
</tr>
<tr>
<td>Clade 1 G/G</td>
<td>Two copies of G/T or C/T</td>
<td>−0.02 (0.04)</td>
</tr>
<tr>
<td>Clade 2 G/T</td>
<td>Two copies of G/G or C/T</td>
<td>−0.02 (0.04)</td>
</tr>
<tr>
<td>Clade 3 C/T</td>
<td>Two copies of G/G or G/T</td>
<td>−0.05 (0.03)</td>
</tr>
</tbody>
</table>

a Adjusted for age, gender, race, dialysis modality, cigarette smoking, comorbidity score, diabetes, prevalent CVD, and congestive heart failure. HR, hazard ratio.
bIn the absence of the 162Val variant.

References

4. Humphries SE, Luong LA, Ogg MS, Hawe E, Miller GJ:...


In this paper a relationship is established between functional variants of the IL6 gene, inflammation, and subsequent cardiovascular events in dialysis patients. The paper is related to the paper by Stenvinkel et al. in this month’s CJASN, which suggests that the link between IL6 and cardiovascular disease may involve oxidative stress indicated by elevated levels of myeloperoxidase that are reduced by statin therapy (pages 281–287).