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Transforming growth factor-beta 1 gene polymorphisms and cardiovascular disease in hemodialysis patients

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Transforming growth factor-beta 1 gene polymorphisms and cardiovascular disease in hemodialysis patients.

Background. Atherosclerotic vascular disease is a leading cause of morbidity and mortality in patients with end-stage renal disease (ESRD) on maintenance hemodialysis (HD). Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that inhibits the atheromatous process. We studied coding region polymorphisms of the TGF-β1 gene (+869 T → C at codon 10 and +915 G → C at codon 25) as genetic susceptibility factors for prevalent vascular disease and cardiac outcomes in a cohort of HD patients enrolled in the HEMO Study.

Methods. Genotyping was carried out using polymerase chain reaction-sequence specific primer (PCR-SSP) methods with a cytokine genotyping tray. Prevalent vascular disease was coded from the Index of Disease Severity (IDS) scores for ischemic heart disease (IHD), peripheral vascular disease (PVD), cerebrovascular disease (CVD), and congestive heart failure (CHF), 0 indicating absence, and 1 to 3 increasing grades of severity. The presence of any vascular disease (VD) (i.e., any degree of IHD/PVD/CVD), and the number of coexistent vascular system diseases per patient were derived. Cardiac outcomes, one of the secondary outcomes of the HEMO Study, were expressed as a composite of the first hospitalization for, or death from, cardiac causes.

Results. The cohort consisted of 183 patients at enrollment, 56% male, 44% African American (AA), and 40% diabetic. The mean age was 62.4 ± 12.2 years, and median dialysis vintage 2.02 years. The most frequent genotype at codon 10 was T/C (67%), and at codon 25 was G/G (72%). IHD was present in 52% of patients; 65% had at least one vascular system involvement, and 31% had 2 or more. On both univariate and multivariate analysis, the G/C genotype at codon 25 was significantly associated with the presence and extent of vascular disease at enrollment. The median time to cardiac outcome, defined as a composite of the first hospitalization for, or death from, cardiac causes, was 411 days in patients with the G/C genotype compared with 851 days in those with the G/G genotype ($P = 0.03$). Patients with the G/C genotype had a 1.6-fold increased hazard for cardiac outcomes after adjustment for baseline covariates ($P = 0.04$).

Conclusion. The G/C substitution at codon 25 was associated with an increased risk for prevalent vascular disease, new onset cardiac morbidity, and cardiac mortality in HD patients, and may be a genetic susceptibility factor for the development of atherosclerosis. Further studies are required to evaluate the role of TGF-β1 as a candidate gene.

Key words: TGF-β1, hemodialysis, gene polymorphism, atherosclerosis, vascular disease.

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Atherosclerotic vascular disease represents a significant cause of morbidity and mortality in patients with end-stage renal disease (ESRD). It is approximately 10 to 30 times more prevalent in patients treated by dialysis compared with patients in the general population, after stratification for gender, race, and the presence of diabetes mellitus [1]. Among incident hemodialysis (HD) patients, approximately 40% have a history of ischemic heart disease (IHD) and congestive heart failure (CHF) [1, 2]. The high incidence of vascular complications in these patients also involves peripheral vascular disease and stroke, which are thought to be related to accelerated atherogenesis [3, 4].

No combination of traditional risk factors has provided a convincing explanation for this high prevalence of atherosclerotic vascular disease. Atherosclerosis is a slowly progressive, inflammatory, proliferative process in which various cellular elements such as macrophages, smooth muscle, and endothelial cells are involved. In recent years, experimental evidence has suggested that TGF-β1 plays a key regulatory role in atherogenesis by inhibiting both migration and proliferation of vascular smooth muscle cells and macrophages, and by protecting endothelial function [5, 6]. These in vitro observations have been confirmed by several clinical studies indicating a cardioprotective effect for high serum levels of TGF-β1, both in the general population [7] and in HD patients [8]. It has also been shown that a high proportion of the circulating concentration of TGF-β1 is under genetic control [9]. Thus, polymorphisms involving the TGF-β1 gene may provide a genetic susceptibility factor for atherosclerotic

vascular disease. This study was undertaken to study the association between single nucleotide polymorphisms at the TGF-β1 locus (+869 T → C at codon 10 and +915 G → C at codon 25) and the prevalence of atherosclerotic vascular disease and cardiac outcomes on follow-up, including new onset cardiac hospitalization events and cardiac death events, in a chronic hemodialysis population.

METHODS

Subjects

The study cohort consisted of 217 patients with ESRD on maintenance HD recruited to the baseline phase of the Hemodialysis (HEMO) Study from two Boston centers. The HEMO Study was sponsored by the U.S. National Institute of Diabetes, Digestive, and Kidney Diseases, and the full-scale trial began in 1995. Details regarding the design of the HEMO Study have been published elsewhere [10]. In brief, this was a multicenter, prospective, randomized clinical trial designed to evaluate the effect of dialyzer urea and β_2-microglobulin clearances on the morbidity and mortality of patients. Patients were eligible for inclusion into the HEMO Study if they were between the ages of 18 and 80 years, had been receiving chronic hemodialysis three times per week, and had residual renal urea clearance of less than 1.5 mL/min/35 L of urea distribution volume. Those patients in acute or chronic care hospitals with active malignancy, decompensated cardiac, hepatic, or pulmonary disease, serum albumin <3.0 g/dL, interdialytic urea clearance >1.5 mL/min, pregnancy, or a scheduled or recently (<6 months) failed transplant, were excluded.

Blood samples for genotyping were available for 187 patients. Of these, 102 patients were Caucasian and 81 were African American. Because there were only four patients representing other races, analysis of characteristics at enrollment was confined to these 183 patients. Randomization to either a standard or high dose of dialysis, and to either a low or high flux dialyzer was carried out in 176 patients in whom cardiac outcomes on follow-up were studied. This ancillary study was approved by the Human Investigation Review Committee, and all participants provided written, informed consent.

Data procurement

Vascular disease definition at baseline. Demographic, medical, and socioeconomic information was obtained once patients were enrolled into the baseline phase of the study. During the fifth week of the study, while the patients were still in the baseline phase, their comorbid medical conditions were assessed and recorded on a standardized form. The form was completed at each clinical center by specially trained study coordinators. Comorbidity measures used in this study were obtained from the Index of Disease Severity (IDS) scores used in computing the Index of Coexistent Disease (ICED), a coding system that classifies the presence and severity of 19 different diseases and 11 physical impairments [11]. The information available for completion of the form included chart progress notes, list of current medications, the most recent laboratory data, chest x-ray report, electrocardiogram, and hospital discharge summary. This assessment was supplemented by interviews of the patients, family members, primary nephrologists, and dialysis staff, as necessary, to provide an assessment of the presence and severity of disease. The present analysis focused upon those scores related to vascular disease—namely, congestive heart failure (CHF), ischemic heart disease (IHD), peripheral vascular disease (PVD), and cerebrovascular disease (CVD) scores. A score of '0' indicated that the condition was absent; '1' indicated presence of the condition with little or no morbidity, '2' was symptomatic, active disease requiring active treatment, but controlled by treatment, and '3' signified moderate to severe manifestations despite treatment. Two composite variables were derived: (1) aVD, defined by the presence of any grade of IHD, PVD, or CVD; and (2) nVD, defined by the total number of coexistent vascular diseases, and representing the extent of atherosclerotic vascular disease in the individual patient. This variable was expressed as a numerical score of 0 to 3.

Cardiac outcomes during follow-up. Cardiac outcomes were one of the secondary outcomes defined in the HEMO Study. Time to a composite of the first hospitalization for cardiac causes or death from cardiac causes was used as the end point for analysis. Cardiac death referred to deaths caused by IHD, CHF, arrhythmias, or other heart disease. Thus, this composite outcome represented new onset cardiac hospitalization events, as well as cardiac death events.

Laboratory data

Blood samples were obtained predialysis as part of the routine clinical protocol within one month of enrollment for measurement of routine laboratory parameters (including serum total cholesterol and albumin). In addition, a 30 mL heparinized blood sample was also collected as part of the ancillary study. Isolated peripheral blood mononuclear cells (PBMC) aliquots were utilized for DNA extraction and genotyping for the present study.

PBMC isolation and DNA extraction. PBMC were harvested from whole blood as previously described using Ficoll-Hypaque density gradient separation technique [12]. Cells (2.5×10^6 PBMC/mL) were resuspended in RPMI-1640 cell culture medium supplemented with L-glutamine, $NaHCO_3$, HEPES, penicillin, and streptomycin and stored at −80°C until DNA extraction.

Genomic DNA was extracted using a spin column method (QIAamp DNA Mini Kit, Qiagen, Valencia, CA, USA). In brief, 2.5×10^6 PBMC were treated with 20 μL of proteinase K (Qiagen), followed by the addition of 200 μL of sodium dodecyl sulfate to lyse the cells. The homogeneous solution was incubated at 56°C for 10 minutes, and 200 μL of 100% ethanol was added to precipitate DNA. The mixture was then applied to the QIAamp spin column, and after two washes with 500 μL of wash buffer, genomic DNA was eluted by the addition of 200 μL of elution buffer. Final DNA concentrations were 50 to 200 ng/mL, determined by mini-gel electrophoresis.

TGF-β1 genotyping. The PCR-SSP approach was used to analyze single nucleotide polymorphisms in the coding regions of TGF-β1. The studied polymorphisms were +869, $Leu^{10} \rightarrow$ Pro (T→C) at codon 10, and +915, $Arg^{25} \rightarrow$ Pro (G→C) at codon 25. The genotypes included T/T, T/C, and C/C at codon 10 and G/G, G/C, and C/C at codon 25, respectively. PCR was performed on the purified DNA using a Cytokine Genotyping Tray (One Lambda, Inc., Canoga Park, CA, USA), which provided sequence-specific oligonucleotide primers. In brief, 20 μL of genomic DNA solution was added to D-mix, which contains the dNTPs and reaction buffer, for the cytokine genotyping. Taq polymerase (1.1 μL; Gibco BRL, Grandland, NY, USA) was then added to the D-mix, vortexed for 15 seconds, and 10 μL of the D-mix mixture transferred to a 96-well microtiter genotyping tray with dried primers in each reaction well. A Perkin-Elmer 9600 thermocycler (Perkin-Elmer-Cetus, Norwalk, CT, USA) was used to amplify the promoter regions by PCR. Samples were subjected to 10 cycles at 96°C for 10 seconds, and 63°C for 60 seconds, followed by 20 cycles at 96°C for 10 seconds, annealing temperature of 59°C for 50 seconds, and 72°C for 30 seconds. After the PCR process, the amplified DNA fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide and exposure to ultraviolet light in an UV transilluminator. Interpretation of PCR-SSP results is based on the presence or absence of a specific amplified DNA fragment. An internal control primer pair was included in every PCR reaction to exclude nonspecific amplification affected by several factors, such as pipetting errors, poor DNA quality, and presence of inhibitors. A positive reaction for a specific cytokine allele or allele group is visualized on the gel as an amplified DNA fragment between the internal control product band and the unincorporated primer band.

Statistical analysis

Analysis was performed using SAS software, version 8.2 (Cary, NC, USA). Data were expressed as mean and standard deviation for continuous variables, and proportions for categorical data.

Vascular disease at enrollment was represented by the following individual outcome variables: CHF, each category of vascular disease (IHD, PVD, CVD), the presence of any type of vascular disease as a composite variable (aVD), and number of coexistent vascular diseases (nVD). All except the last were coded dichotomously (0 = absent, 1 = present), and logistic regression models were used for univariate and multivariate analyses.

The nVD was used as an ordinal outcome with 3 categories (0, 1, and ≥2). Proportional odds models were constructed to derive the risk of having a greater number of coexistent vascular diseases (i.e., a worse outcome, attributable to a particular codon 10 or codon 25 genotype, for univariate and multivariate analyses). The Mantel-Haenszel test was used to compare the proportions of patients with one or more coexistent vascular comorbidities (i.e., nVD scores 0, 1, 2, or 3) in the different genotype categories to provide the chi-square estimate for a linear trend (Epi Info 2000, version 1.1.2, Centers for Disease Control and Prevention, Atlanta, GA, USA).

The odds ratios (and 95% confidence intervals) for each outcome were computed in univariate models for the following independent variables: age, gender, race, duration on HD, serum albumin, serum total cholesterol, diabetes, smoking history (categorized as nonsmokers vs. current or past smokers), and codon 10 and codon 25 genotypes. Multivariate analyses were carried out by a process of backward elimination of variables, forcing the codon 10 and codon 25 genotypes into the models, to evaluate their association with vascular disease. Covariates included the traditional risk factors for cardiovascular disease: age, gender, race, serum total cholesterol, diabetes, and smoking history. Hypertension was not included as a risk factor because it was a near universal complication in this cohort. Results of serum cholesterol were unavailable for approximately 15% of the cohort, so the statistical technique of multiple imputations for missing values was employed in the multivariate models. However, as initial exploration showed no significant relationship between serum cholesterol and outcome, it was excluded from the final models. Serum albumin, again, was not included in the final multivariable model as a covariate, as it is closely linked to pre-existing comorbidity, and is not established as a predisposing risk factor for vascular disease.

Time to a composite of the first hospitalization for cardiac causes or death from cardiac causes was used as the end point for survival analysis. Kaplan-Meier survival curves for time to first cardiac hospitalization or death from cardiac causes, stratified by genotype, were constructed. The log-rank statistic was used to test survival time differences between genotypes. Cox proportional hazard regression was used to adjust the relationship between genotypes and outcomes for baseline covariates

Table 1. Patient and treatment characteristics

Age *years*	62 (12)
Male gender %	46
Race %	
Caucasian	56
African American	44
Diabetes mellitus %	40
Dialysis vintage *years*	3.7 (4.4)
Primary kidney disease *N* (%)	
Diabetic nephropathy	58 (32)
Hypertensive nephrosclerosis	58 (32)
Glomerular disease	25 (13)
Other	42 (23)
ICED score *N* (%)	
1	60 (34)
2	58 (32)
3	60 (34)
Serum total cholesterol *mg/dL*	174 (41)
Serum albumin *g/dL*	3.6 (0.4)
Hematocrit %	33.4 (5.1)
Equilibrated Kt/V	1.4 (0.2)
Treatment groups (randomization)	
Kt/V: Standard	90 (51)
High	86 (49)
Flux: Low	91 (52)
High	85 (48)

Data are displayed as mean (SD) or percentage.

as outlined above. Dialysis dose and flux grouping assignment from the randomization of the parent study, and the presence of baseline vascular disease derived from the IDS scores, were included in multivariate Cox models, in addition to baseline covariates. Hypertension, serum cholesterol, and serum albumin were excluded from the analysis based on the same rationale discussed above. Here again, multivariate analysis was carried out by a process of backward elimination of variables, forcing the codon 10 and codon 25 genotypes into the models. The proportional hazards assumption was met, both globally for the model, and for individual variables in the model.

Because this was a study consisting of prevalent patients, and the outcome, vascular disease, has a relationship to longevity, models were constructed both including and excluding age, and interactions between genotypes and age were explored as appropriate for each model.

RESULTS

The study population consisted of 183 patients enrolled into the baseline phase of the HEMO Study. Baseline demographic and clinical data are summarized in Table 1, and the distribution of ICED scores and vascular comorbidities in Table 2. About two thirds of the cohort had clinical evidence of IHD, PVD, or CVD, and approximately one half had a history of CHF. Hypertension was present in 96% (176/183) of the cohort. Only 3% did not receive recombinant erythropoietin therapy. Randomization to either a standard or high dose of dialysis, and to either a low or high flux dialyzer, was carried out in 176 patients of the original cohort, and therefore, outcome data were available in these subjects.

Table 2. Vascular disease scoring in the study population

	Score			
	0	1	2	3
Individual variable (IDS scores)[a]				
CHF	85 (48)	63 (35)	23 (13)	7 (4)
IHD	85 (48)	24 (13)	65 (37)	4 (2)
PVD	123 (69)	18 (10)	29 916)	8 (5)
CVD	143 (80)	10 (6)	24 (13)	1 (1)
Composite variables				
aVD[b]	63 (35)	115 (65)		
nVD[c]	63 (35)	60 (34)	42 (24)	13 (7)

Abbreviations are: CHF, congestive heart failure; IHD, ischemic heart disease; PVD, peripheral vascular disease; CVD, cerebrovascular disease; aVD, any vascular disease; nVD, total number of coexistent vascular diseases. Values represent number of patients (%). Complete data were available in 178 patients.

[a]Scoring system represents the IDS scores for vascular disease.

[b]Scoring system represents absence (score = 0) or presence (score = 1) of any vascular disease.

[c]Scoring system represents the total number of coexistent vascular diseases.

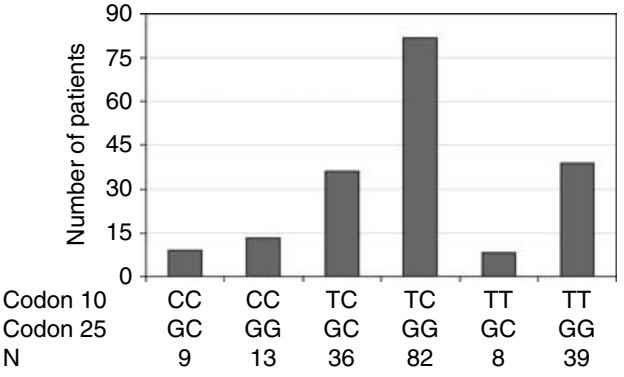

Fig. 1. Distribution of codon 10 and codon 25 genotypes in the cohort.

Genotype distribution

Figure 1 shows the distribution of the TGF-β1 coding region genotypes for the entire cohort. The T/C genotype (heterozygous state) at codon 10 (118/183, 67%) and the homozygous G/G genotype at codon 25 (140/183, 72%) were the most frequent genotypes. There were no patients who were homozygous for the G→C substitution at codon 25 (i.e., the C/C genotype). This provided three genotype categories for codon 10 and two for codon 25. The T/C-G/G combination was the most frequent (82/183, 44%) in the cohort.

Patient characteristics differed significantly by genotype and race, and hence, these demographic variables were entered into multivariable models to adjust for confounding effects. Patients with the G/C genotype at codon 25 were significantly older (67 ± 8 vs. 61 ± 13 years; $P = 0.002$), and had higher serum total cholesterol (185 ± 40 vs. 167 ± 41 mg/dL; $P = 0.02$) compared to those with the G/G genotype. The codon 25 G/C genotype was also associated with female gender (G/C vs. G/G: 65% vs. 51%; $P = 0.04$), and marginally with Caucasian race (G/C vs. G/G: 65% vs. 52%; $P = 0.09$). The distribution of diabetics and smokers did not differ by genotype. No specific

Table 3. Univariate analysis of the relationship between individual vascular comorbidities and TGF-β1 genotypes as well as other clinical variables

Predictor	IHD OR (95% CI)	CVD OR (95% CI)	PVD OR (95% CI)	CHF OR (95% CI)
G/C codon 25 (vs. G/G)	1.36 (0.71–2.62)	2.15 (1.00–4.63)	2.63 (1.33–5.18)	2.14 (1.10–4.19)
Age (per year increase)	1.05 (1.02–1.08)	1.04 (1.00–1.07)	1.05 (1.01–1.08)	1.06 (1.03–1.09)
Male gender (vs. female)	1.22 (0.68–2.21)	0.58 (0.27–1.24)	1.27 (0.67–2.41)	0.85 (0.47–1.53)
Caucasian race (vs. African American)	1.23 (0.68–2.23)	2.33 (1.04–5.21)	3.28 (1.63–6.63)	1.35 (0.75–2.44)
Diabetic (vs. nondiabetic)	2.87 (1.53–5.39)	1.01 (0.47–2.14)	1.12 (0.59–2.14)	1.93 (1.05–3.56)
Smoker (vs. nonsmoker)	1.19 (0.66–2.13)	0.70 (0.33–1.47)	1.52 (0.80–2.94)	0.90 (0.50–1.64)
Albumin (per g/dL increase)	0.62 (0.28–1.38)	0.82 (0.31–2.20)	0.77 (0.33–1.81)	0.45 (0.20–1.00)
Total cholesterol (per mg/dL increase)	1.00 (1.00–1.01)	1.00 (0.99–1.01)	1.00 (1.00–1.01)	1.01 (1.00–1.01)

Abbreviations are: IHD, ischemic heart disease; CVD, cerebrovascular disease; PVD, peripheral vascular disease; CHF, congestive heart failure. 95% CI, 95% confidence interval for the odds ratio (OR). Codon 10 genotypes (T/C vs. T/T, or C/C vs. T/T) did not show a significant association with above outcomes.

Table 4. Factors associated with the presence of any vascular disease (aVD)

	Univariate analysis		Multivariate analysis	
Variable[a]	*P* value	OR (95% CI)	*P* value	OR (95% CI)
G/C Codon 25 (vs. G/G)	<0.01	3.05 (1.40–6.62)	0.045	2.51 (1.08–5.79)
Age (per year older)	<0.01	1.06 (1.03–1.09)	0.01	1.04 (1.01–1.07)
Diabetic (vs. nondiabetic)	<0.01	3.04 (1.53–6.03)	<0.01	3.08 (1.48–6.44)
Caucasian race (vs. African American)	0.03	2.02 (1.08–3.76)	0.04	2.04 (1.02–4.07)
Male gender (vs. female)	0.60	0.85 (0.46–1.57)		
Smoker (vs. nonsmoker)	0.46	1.22 (0.71–2.08)		
Total cholesterol (per mg/dL increase)[b]	0.11	1.01 (1.00–1.02)		
Serum albumin (per g/L increase)[b]	0.04	0.39 (0.16–0.95)		

The final multivariable model was obtained by backward elimination of variables. 95% CI, 95% confidence interval for the odds ratio (OR).
[a]Codon 10 genotypes (T/C vs. T/T, or C/C vs. T/T) did not show a significant association to vascular disease.
[b]Variables not included in selection.

associations with demographic or clinical variables were noted for codon 10 genotypes. Caucasian subjects were older (64.2 ± 12.9 vs. 60.1 ± 11.0, $P = 0.03$), and a lower proportion was female (41% vs. 70%, $P < 0.01$). The proportion of diabetics did not differ by race.

Association between TGF-β1 genotypes and vascular disease at enrollment

The G/C genotype at codon 25 was associated with higher odds for the presence of CHF, PVD, and CVD, although not for IHD. Older age was significantly associated with each individual outcome. IHD and CHF were more prevalent in those with diabetes, and PVD and CVD more prevalent in Caucasians. Codon 10 substitutions did not show a significant relationship to any of the individual vascular outcomes.

The G/C genotype at codon 25 was associated with a three-fold increase in the odds for aVD on univariate analysis. The final model after backward elimination of variables is shown in Table 4. The G/C genotype at codon 25 provided a 2.3-fold greater risk (95% CI 1.02–5.3; $P = 0.045$) for the presence of vascular disease. Older age, diabetes, and Caucasian race were the other significant variables in the model. There was no association between aVD and gender or smoking history.

Patients with the G/C genotype were significantly older as compared to those with the G/G genotype. Because both older age and the presence of the G/C genotype were associated with higher odds for aVD, the effect of the G/C genotype in different categories of age was explored. In patients younger than 60 years, 92% (11/12) of those with the G/C genotype had aVD, compared to 78% (31/40) in patients aged 60 years or older. Respective proportions for the G/G genotype were 40% in patients younger than 60 years, and 70% in patients aged 60 years or older. The odds of vascular disease in relation to the G/C genotype were significantly higher in younger patients ($P = 0.02$ for homogeneity of the odds ratios).

The G/C genotype was also associated with a higher nVD score ($P = 0.004$, Mantel-Haenszel chi-square for linear trend) (Fig. 2). The proportional-odds model constructed to identify factors associated with a higher number of coexistent vascular diseases as an ordinal outcome (nVD score) showed that the GC genotype was associated with a 2.4-fold increase in odds for a higher nVD score (95% CI 1.32–4.44; $P = 0.004$). The final multivariable model showed older age, diabetes, and Caucasian race to be the other significant variables associated with a higher nVD score; their inclusion in the model diminished the strength of association between the G/C genotype and the nVD score (Table 5). Figure 3 shows the log-odds of a higher nVD score from the codon 25 genotypes as a function of increasing age, race, and the age-codon 25 interaction, which was significant ($P = 0.04$) in this model.

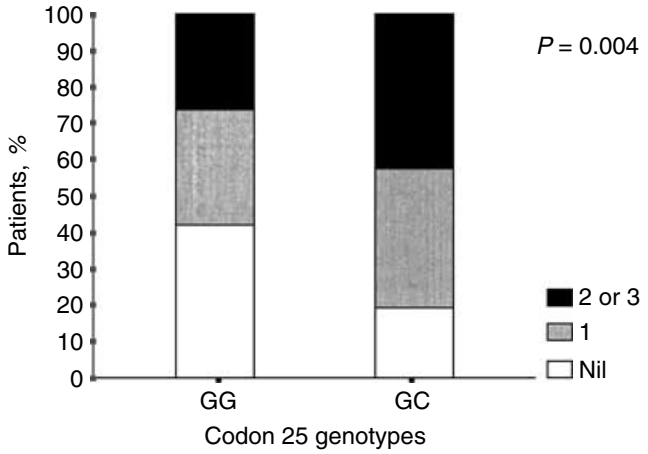

Fig. 2. Association between codon 25 genotypes and number of concomitant vascular comorbidity (ischemic heart disease, peripheral vascular disease, and cerebrovascular disease) (Mantel-Haenszel chi-square test for linear trend).

There was no demonstrable relationship between codon 10 substitutions and either of the composite outcomes representing prevalence and extent of vascular disease at baseline, in this cohort.

Association between TGF-β1 genotypes and cardiac outcomes during follow-up

The median duration of follow-up was 825 days (1 to 2376 days). During the follow-up period there were 80 patients [45% with a first cardiac hospitalization event, and 39 (22%) with a cardiac death event; a total of 91 patients (52%) had a composite event of either first cardiac hospitalization or cardiac death. The median follow-up to the composite event was 450 days (1 to 2376 days).

Kaplan-Meier analysis demonstrated (Fig. 4) that patients with the G/C genotype had a higher risk for the first cardiac hospitalization or cardiac death compared to patients with the G/G genotype (log-rank, $P = 0.02$). Median time to event was 851 days in patients with the G/G genotype, and 411 days in those with the G/C genotype. In univariate analysis, the G/C genotype was associated with a HR of 1.6 for the outcome compared to the G/G genotype (95% CI 1.06–2.51; $P = 0.03$). Multivariate models were constructed using backward elimination of variables, with and without the inclusion of age, as age appeared to confound the effect of genotype on the outcome (Table 6). Significant predictors of the outcome besides codon 25 genotype and older age were, Caucasian race, a history of smoking, and assignment to the low-flux dialyzer limb of the study. Dialysis dose assignment, gender, diabetes, and the presence of vascular disease at enrollment did not enter the models.

The role of codon 25 genotypes was also explored in diabetics versus nondiabetics, given that diabetes was a strong risk factor for vascular disease at enrollment, but not for new onset cardiac events or death. There were 35 (50.7%) events in diabetics and 56 (52.3%) events in nondiabetics ($P = 0.8$). In the subgroup of diabetics, the G/C genotype at codon 25 had no relationship to cardiac outcomes, but assignment to the high-flux dialyzer limb of the study had a HR of 0.48 (95% CI 0.23–0.98; $P = 0.04$), and Caucasian race a HR of 2.10 (95% CI 1.05–4.18; $P = 0.04$). On the other hand, in nondiabetic patients, the G/C genotype at codon 25 was a significant risk factor for cardiac outcomes on follow-up (HR = 1.96, 95% CI 1.13–3.42; $P = 0.02$) in addition to the presence of pre-existing vascular disease at enrollment (HR = 2.66, 95% CI 1.45–4.88; $P < 0.01$).

There was no demonstrable relationship between codon 10 genotypes and time to the composite event of first cardiac hospitalization or cardiac death in this cohort.

DISCUSSION

The present study assessed the relationship between single nucleotide polymorphisms at the TGF-β1 locus and atherosclerotic vascular disease in a cohort of maintenance hemodialysis patients. Prevalence of vascular comorbidity was established at the point of enrollment into the HEMO Study using a previously validated scoring system [11]. Composite outcome variables derived from individual IDS scores for IHD, PVD, and CVD provided additional sensitive markers for the presence, and extent of, pre-existing atherosclerotic vascular disease. Secondary outcomes defined in the HEMO Study included a composite of first cardiac hospitalization events and cardiac death events, allowing an estimate of new onset cardiac events, as well as death resulting from IHD, CHF, and other heart disease. These were the study end points on follow-up used in the analysis. There was a significant association between the G/C genotype at codon 25 and the presence and extent of pre-existing vascular disease; the associations with PVD and CVD individually were also significant. In addition, patients with the G/C genotype showed a higher risk of developing new onset cardiac hospitalization events or cardiac death on follow-up.

TGF-β1 is a member of a family of dimeric polypeptide growth factors that influences cellular proliferation and differentiation and the production of extracellular matrix. Experimental evidence shows that TGF-β1 inhibits migration and proliferation of smooth muscle cells, endothelial cells, and macrophages, and may play a protective role against atherosclerosis [13–16]. Experimental inhibition of TGF-β1 signaling by either antibody to TGF-β1 [17], or TGF-β1 receptor fusion protein that acts as a competitive inhibitor of TGF-β1 binding [18], transformed atherosclerotic plaques in ApoE null mice to an inflammatory and unstable phenotype. Low serum levels of TGF-β1 have been reported in some studies of human atherosclerotic disease, both in patients without renal

Table 5. Factors associated with a higher number of concomitant vascular diseases (nVD)

Variable[a]	Univariate analysis		Multivariate analysis	
	P value	OR (95% CI)	*P* value	OR (95% CI)
G/C codon 25 (vs. G/G)	0.004	2.42 (1.32–4.44)	0.07	1.78 (0.95–3.36)
Age (per year older)	0.003	2.79 (1.42–5.47)	<0.01	1.04 (1.02–1.07)
Caucasian race (vs. African American)	0.003	2.35 (1.35–4.11)	<0.01	2.30 (1.28–4.13)
Diabetes mellitus (vs. nondiabetic)	0.01	2.03 (1.16–3.54)	0.02	2.00 (1.11–3.58)
Male gender (vs. female)	0.79	1.08 (0.63–1.85)		
Smoker (vs. nonsmoker)	0.34	1.30 (0.76–2.22)		
Total Cholesterol (per mg/dL increase)[b]	0.34	1.00 (0.99–1.01)		
Serum albumin (per g/L increase)[b]	0.12	0.56 (0.27–1.15)		

The final multivariable model was obtained by backward elimination of variables. 95% CI, 95% confidence interval for the odds ratio (OR).
[a]Codon 10 genotypes (T/C vs. T/T, or C/C vs. T/T) did not show a significant association with a higher number of concomitant vascular system diseases.
[b]Variables not included in selection.

insufficiency [7] and in ESRD patients [8], but not in others [19–21].

An association between allelic polymorphisms in the coding region of the TGF-β1 gene and the atheromatous process may be related to the effect of the genotype on TGF-β1 production, genotypes associated with lower TGF-β1 production, providing a higher elevated risk. However, because allelic substitutions may potentially influence any step between transcription to activation of TGF-β1 [22], the relationship between TGF-β1 genotypes and putative function may be more complex. Grainger et al [9] recently showed that circulating levels of TGF-β1 were under genetic control. In a study of heart transplant recipients, Aziz et al [23] showed that the codon 25 G/G genotype was associated with plasma levels about one and half times higher than the G/C genotype. Awad et al [24] showed in lung transplant patients that the G/G genotype at codon 25 was associated with higher production of TGF-β1 by stimulated peripheral blood mononuclear cells (PBMC) than the G/C genotype. An association between the G/C genotype at codon 25 and coronary artery disease was seen in the Etude Cas-Temoin de l'Infarctus du Myocarde (ECTIM) Study, which enrolled a non-ESRD population [22], but other studies [25] have not demonstrated this association.

We believe that our results are especially significant, given that we were able to demonstrate an association in a prevalent hemodialysis population consisting of older subjects. Although the G/C genotype was associated with an elevated risk for vascular disease, both pre-existing and new, it is noteworthy that patients with the G/C genotype were significantly older. Thus, models that adjusted for age attenuated the effects of the codon 25 genotype on vascular disease outcomes. The high case fatality rate of vascular disease implies that we were already encountering a survival bias in this cohort, and it is conceivable that many patients with the G/C genotype and vascular disease succumbed before reaching ESRD. This is supported by our observations that in subjects <60 years of age, over 90% of those with the G/C genotype had pre-existing vascular disease, and that the risk for disease accorded by the G/C genotype differed with age, being higher in younger patients. Furthermore, the association of the risk factor (codon 25 GC genotype) with incident events (cardiac hospitalization or cardiac death) on prospective follow-up clarifies the presence of this association. It is also significant that there was a total

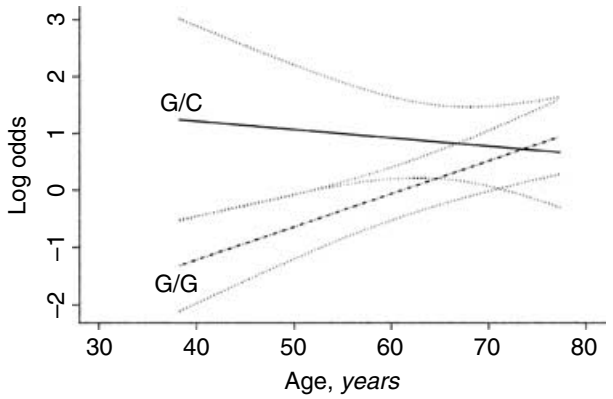

Fig. 3. Plot of the log-odds for a higher number of concomitant vascular diseases (higher nVD score) vsersus age for the G/G and G/C genotypes at codon 25. Proportional odds model adjusted for age (linear), race, codon 25, and including an interaction term for age and race. There is a significant interaction between age and codon 25 ($P = 0.04$).

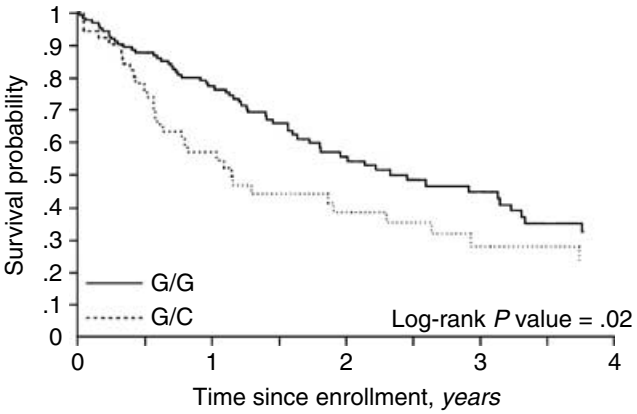

Fig. 4. Kaplan-Meier survival plot of time to composite outcome event of first cardiac hospitalization and cardiac death, by codon 25 genotypes (log-rank $P = 0.03$).

Table 6. Cox proportional hazards model of factors associated with the composite event of first cardiac hospitalization and cardiac death

	Univariate analysis		Multivariate analysis			
			Model excluding age		Model including age	
	P value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)
Codon 25 (G/C vs. G/G)	0.03	1.63 (1.06–2.51)	0.04	1.56 (1.01–2.41)	0.12	1.42 (0.91–2.20)
Age (linear)	0.01	1.03 (1.01–1.05)			0.02	1.03 (1.00–1.05)
Caucasian race	0.001	2.15 (1.38–3.34)	<0.01	2.10 (1.34–3.29)	<0.01	1.98 (1.26–3.13)
Smoking	0.10	1.43 (0.94–2.17)	0.01	1.73 (1.12–2.66)	<0.01	1.78 (1.16–2.75)
Flux group (high vs. low)	0.02	0.61 (0.40–0.94)	0.05	0.65 (0.42–0.99)	0.03	0.62 (0.40–0.95)
Kt/V group (high vs. standard)	0.47	1.16 (0.77–1.75)				
Female gender	0.19	0.76 (0.50–1.15)				
DM	0.25	0.78 (0.51–1.19)				
Vascular disease at enrollment	0.04	1.63 (1.03–2.58)				
Total Cholesterol (per mg/dL increase)[a]	0.82	1.00 (0.99–1.01)				
Serum albumin (per g/L increase)[a]	0.89	1.05 (0.59–1.84)				

The final multivariable model was obtained by backward elimination of variables.
[a]Variables not included in selection.

absence of the C/C genotype in this cohort. Although the C/C genotype is infrequent—0.7% in the ECTIM Study in a population without renal insufficiency [22], and 3% in the study by Stefoni et al [8] in a Caucasian ESRD cohort—it may be speculated that a heightened risk for vascular disease from one or more copies of the C-allelle at this locus compromises survival to ESRD and dialysis.

Although diabetes was strongly associated with pre-existing vascular disease in this cohort, it did not predict cardiac events or death on follow-up. However, subgroup analysis by the presence of diabetes showed that the G/C genotype tended to associate with cardiac outcomes in nondiabetics, but not in diabetics. It can be argued that in the absence of risk factors such as older age and diabetes, specific genetic polymorphisms such as the G/C genotype at codon 25 become important modulators of the risk of vascular disease.

We were also able to demonstrate in this cohort that assignment to high-flux dialyzers significantly decreased the likelihood of adverse cardiac outcomes on follow-up. Indeed, observations from secondary analyses of the HEMO Study indicated a reduction in all cause mortality in patients dialyzing >3.7 years [26, 27], as well as a reduction in specific cardiac related events [28].

We found no significant association between codon 10 genotypes and vascular disease outcomes, although Yokota et al [20] noted a lower risk of IHD with the C/C genotype compared to either the T/T or T/C genotypes. Suthanthiran et al [29] found that the T/C and C/C genotypes at codon 10 were more common in African American population groups who also hyperexpressed TGF-β1. This is especially significant, as the present study found Caucasian race to be a consistent risk factor for vascular disease outcomes. We were, however, unable to demonstrate any variation in codon 10 or codon 25 genotype frequency by race.

We appreciate that this study is a secondary analysis on a preselected cohort of patients enrolled in the HEMO Study, and this may limit the generalizability of our inferences. However, such a cohort provided us the advantage of a relatively homogenous study population. Further, as ESRD patients manifest accelerated atherosclerosis [1], the previous probability for vascular disease as an outcome was likely to be higher than in study populations without renal insufficiency, where similar associations have been addressed [20, 22, 25, 30]. Serum levels of TGF-β1 were unavailable, and this limited our ability to describe the phenotype more fully.

CONCLUSION

The relationship between TGF-β1 genotypes and atherosclerotic vascular disease is complex, especially in the context of CKD, and needs further study. There are competing effects of this cytokine on fibrogenesis, vascular smooth muscle cell, and macrophage function, as well as extracellular matrix production, in different target tissues. In addition, interactions with demographic factors, type and severity of kidney disease, clinical variables, and physiologic pathways such as the renin-angiotensin system would have a bearing on patient outcomes. Our study has demonstrated that coding region polymorphisms in the TGF-β1 gene are associated with both pre-existing atherosclerotic vascular disease, and new onset cardiac hospitalization events and cardiac death. Thus, TGF-β1 may be a candidate gene for atherosclerosis, a major cause of morbidity and mortality in ESRD. The role of these genetic factors need to be better defined because this would allow for superior risk profiling of the individual patient, and therefore, lead to more informed and targeted decisions regarding therapy.

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