Functional Polymorphisms in the Vascular Endothelial Growth Factor Gene Are Associated with Development of End-Stage Renal Disease in Males

Kent Doi,*† Eisei Noiri,* Akihide Nakao,* Toshiro Fujita,* Shuzo Kobayashi, ‡ and Katsushi Tokunaga†

Departments of *Nephrology and Endocrinology and †Human genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; and ‡Department of Nephrology and Kidney and Dialysis Center, Shonan Kamakura General Hospital, Kanagawa, Japan

This study elucidates the genetic role of vascular endothelial growth factor (VEGF) as a predisposing factor for progression of chronic kidney disease. Single-nucleotide polymorphisms were genotyped and haplotype structures were determined in the 3’ untranslated region (UTR) of VEGF gene, and the distribution of each haplotype in male patients with ESRD (n = 101) and healthy male control subjects (n = 189) was examined. The 936C/T and 1451C/T polymorphisms in the 3’ UTR were in nearly absolute linkage disequilibrium, and haplotype analysis demonstrated that they were the primary responsible single-nucleotide polymorphisms. The distribution of the 936CC-1451CC genotype was significantly more frequent among patients with ESRD than among the age-matched healthy control subjects. In addition to case-control association study, the 936CC-1451CC genotype was also associated with significantly higher plasma VEGF levels in healthy individuals, but a significant association was found only in males, not in females. We also examined the effect of the 936C-1451C haplotype on mRNA stability. Consistent with the results of plasma VEGF levels, mRNA carrying 936C-1451C haplotype showed higher stability. The 936CC-1451CC genotype in the 3’ UTR showed not only susceptibility for ESRD but also higher plasma VEGF levels and mRNA stability, indicating the contribution of VEGF to chronic kidney disease progression, especially in males.


Chronic kidney disease (CKD) is classifiable as a multifactorial disease because the accumulation and combination of genetic factors and environmental factors influence the onset and development of ESRD. Although CKD results from various pathogeneses, including glomerulonephritis, diabetes, hypertension, and urologic disorders, the most predictive factor for progression to ESRD is not the cause of glomerular injury but the degree of proteinuria and tubulointerstitial damage (1). Presumably, certain common pathways to ESRD exist, and both genetic and environmental factors seem to be responsible for these common pathways.

The kidney is one of the most highly vascularized organs and has two important microvasculatures of glomerular and peritubular capillaries. These two microvasculatures are responsible for the common pathways to ESRD because the injury of glomerular capillary causes proteinuria and the decrease of peritubular capillary results in chronic hypoxia followed by fibrosis in the tubulointerstitium. As described above, both proteinuria and decreased peritubular capillary are putatively important predictive factors for progression to ESRD (1). Vascular endothelial growth factor (VEGF) is a main regulator of blood vessel growth. It plays a critical role in promoting endothelial survival and maintaining these microvasculatures (2). However, controversy remains as to whether VEGF has vasoprotective or atherosclerogenic effects because VEGF induces chemotaxis and activation of macrophages (3), and administration of VEGF enhanced atherosclerotic plaque progression (4).

In the human kidney, VEGF expression has been demonstrated in the podocytes (5,6), tubular cells (7), and mesangial cells in kidneys of patients with glomerulonephritis (8); VEGF receptors have been detected in the endothelium of glomeruli and peritubular capillaries (7). Furthermore, VEGF is expressed in inflammatory cells such as monocytes (9,10) and lymphocytes (11), which are responsible for fibrosis in the tubulointerstitium of kidney. Various hormones, growth factors, cytokines, and specific stimuli such as hypoxia regulate VEGF expression. Individual diversity of VEGF expression levels has also been observed (12). The VEGF gene was reported to be polymorphic, especially in the promoter region, 5’ untranslated region (UTR), and 3’ UTR (13–15). The polymorphisms in these regions were reported to be associated with VEGF levels (14,15) and diseases such as acute renal allograft rejection (16), diabetic retinopathy (17), breast cancer (18), rheumatoid arthritis (19), and sarcoidosis (20). Our study elucidates the genetic role of VEGF polymorphisms as a predisposing factor for progression of CKD.
We determined the haplotype structures in 3′ UTR of the VEGF gene and compared the distribution of each haplotype in patients with ESRD and healthy control subjects. We also examined the association of VEGF polymorphisms with plasma VEGF levels and mRNA stability to identify the functional polymorphisms that potentially influence VEGF levels.

Materials and Methods

Study Population

This study enrolled 101 male patients who had ESRD and were treated with hemodialysis at five dialysis centers in the Tokyo area. Patients who started dialysis therapy under the age of 50 (40.3 ± 11.1 yr [mean ± SD]) were investigated because they showed rapid progression of kidney disease to ESRD from the onset. The causes of ESRD were chronic glomerulonephritis (n = 70), diabetic nephropathy (n = 14), nephrosclerosis (n = 8), and miscellaneous other conditions (n = 9). Patients with polycystic kidney disease were also excluded. The control group consisted of 189 unrelated healthy male individuals. There was no statistical difference between the patients and the control subjects with respect to age (51.7 ± 10.3 versus 51.9 ± 6.0 yr). All individuals in the control group were healthy and showed no urinary abnormality, renal dysfunction, or hyperglycemia; they reported no use of medication. Genomic DNA from the healthy control subjects were obtained from a public gene bank, Health Science Research Resources Bank (Osaka, Japan). This study was approved by the Ethics Committee for Human Genome Study of The University of Tokyo. Informed consent was obtained from each individual at the time of recruitment.

Genotyping of Polymorphisms

Haplotype structures in the 3′ UTR have not been revealed precisely. Therefore, we performed direct sequencing for the entire 3′ UTR in 16 unrelated individuals and found five polymorphisms, numbered from 936C/T, 1451C/T, 1612A/G, and 1725G/A polymorphisms for haplotype analysis. A single-nucleotide polymorphism (SNP) typing system using fluorescence capillary electrophoresis–single-strand conformation polymorphism (CE-SSCP) analysis that we established recently (21) was used for the 936C/T polymorphism, were mixed into tubes at various ratios (1:11, 2:10, 3:9, 4:8, 5:7, 6:6, 7:5, 8:4, 9:3, 10:2, and 11:1). The final concentrations of mixed DNA from 136 individuals (21). This system calculated the peak heights of respective alleles using DNA analysis software (GeneScan; Applied Biosystems, Foster City, CA). Peaks that represent two alleles were previously found not to show equal height in heterozygotes (22). Therefore, 10 heterozygotes were selected randomly, and the two peaks' heights were measured to calculate the relativity factor (23). After correction using the relativity factor, the relative ratio of the DNA amount was calculated according to the relative heights of the peaks. This assay is capable of measuring all VEGF mRNA isoforms because oligonucleotide primers for the 936C/T polymorphism are located in exon 8.

Mixed DNA samples were used to validate the accuracy of estimation by quantitative CE-SSCP analysis. Genomic DNA from two homozygous individuals, 936CC and 936TT of the 936C/T polymorphism, were mixed into tubes at various ratios (1:11, 2:10, 3:9, 4:8, 5:7, 6:6, 7:5, 8:4, 9:3, 10:2, and 11:1). The final concentrations of mixed DNA were adjusted to 10 ng/μl. Using mixed DNA samples, the relative ratios of DNA carrying 936C allele were estimated in comparison with the total. Correlation between the estimated values and calculated values was determined using Pearson correlation coefficient.

Reverse Transcriptase–PCR for VEGF Isoforms

PCR amplifications were performed with cDNA samples from PBMC described above to examine the contributions of VEGF isoforms in mRNA stability assay. Oligonucleotide primers were designed to amplify VEGF mRNA to identify unique VEGF isoforms as described previously. The sense primer was located in exon 3, and the antisense primer was located in exon 8 (24). Products were analyzed using agarose gel electrophoresis with ethidium bromide staining.

Statistical Analyses

The χ² test was used to compare frequencies of the SNP alleles in the study groups. Conformity of the genotype proportion to Hardy-Weinberg equilibrium was examined in the patient group and the control group. The frequencies of haplotypes were estimated using an expectation-maximization algorithm based on a maximum-likelihood method (25). Permutation-based hypothesis testing (permutation test) procedures were performed to examine associations of estimated haplotype frequencies with ESRD (26,27). The χ² statistics were derived from a series of simple 2 × 2 tables based on respective haplotypes’ frequencies versus all others combined between case and control groups. The null distribution of the χ² test statistics then was approximated via a randomization test in which the case/control status indicators were permuted randomly among the individuals in the sample; then the χ² statistics were recomputed. The P value of the permutation test was determined empirically using the ratio of the data sets above the null χ² statistics for 10,000 permutations. These calculations were per-
formed using a commercial program (SNPAlalyze Ver3.2 Pro; Dynacom Co. Ltd., Chiba, Japan).

The Mann-Whitney U test was used to compare plasma VEGF levels, and the paired t test was used to compare the ratio of 936C/936T in the mRNA stability assay (StatView-J5.0; SAS Institute Inc., Cary, NC). P < 0.05 was considered significant.

**Results**

**Polymorphisms in VEGF Gene Were Associated with Development of ESRD, Especially in Males**

The frequencies of genotypes in the patients with ESRD and the healthy control subjects did not differ significantly from those expected under Hardy-Weinberg equilibrium. Table 1 shows that four common haplotypes were presumed in the promoter-3' UTR. In the 3' UTR, four polymorphisms were genotyped: 936C/T and 1451C/T polymorphisms were in nearly absolute linkage disequilibrium. The 936C allele almost exclusively co-occurred with the 1451C allele, and the 936T allele was found with the 1451T allele. A permutation test demonstrated that the frequency of one haplotype (936T-1451T-1612A-1725G) in 3' UTR significantly decreased in patients with ESRD compared with healthy control subjects (Table 1). This haplotype was determined distinctly with these two polymorphisms. Moreover, mutant carrier frequencies showed a significant association with patients with ESRD only in these two polymorphisms (936C/T: P = 0.013, odds ratio [OR] 2.02; 95% confidence interval [CI] 1.15 to 3.53; 1451C/T: P = 0.0045, OR 2.24, 95% CI 1.27 to 3.94). The other polymorphisms showed no significant association in terms of the frequencies of genotypes, alleles, or mutant carriers. These results indicated that the 936C/T and 1451C/T polymorphisms were the primary responsible SNP.

Next we calculated the sample size required for case-control association study as described previously (28). Considering that the frequency of the disease-susceptible allele (936C-1451C), which showed a recessive mode of inheritance, was approximately 80% and morbidity of ESRD in Japan was 0.2%, we recalculated the penetrances for genotypes 936CC-1451CC, 936CT-1451CT, and 936TT-1451TT as 0.25, 0.1, and 0.1%, respectively. With these parameters, the sample size required for the power of 1 – β above 0.8 was calculated as 104 under the assumption that the number of patients and control subjects was equal. This indicated that the number of our samples provided virtually enough detection power in this study. Finally, the distribution of the 936CC-1451CC genotype was shown to be significantly higher in patients with ESRD compared with the age-matched control subjects by logistic analysis (P = 0.0030; OR 2.37; 95% CI 1.34 to 4.18; Table 2).

**Association of 936C/T and 1451C/T Polymorphisms with Plasma VEGF Levels**

Plasma VEGF levels were measured in 32 healthy males and 31 healthy premenopausal females. Those levels were significantly higher in the 936CC-1451CC individuals than the other genotypes in male samples (936CC-1451CC [n = 16]: median 44.5 pg/ml, range 16.3 to 154.2 pg/ml; 936CT-1451CT [n = 11]/936TT-1451TT [n = 5]: median 32.5 pg/ml; range 9.0 to 54.6 pg/ml; P = 0.026; Figure 1). However, no significant difference was found in female samples (936CC-1451CC [n = 17]: median 36.5 pg/ml; range 10.4 to 74.6 pg/ml; 936CT-1451CT [n = 12]/936TT-1451TT [n = 2]: median 49.0 pg/ml; range 20.8 to 80.8 pg/ml; P = 0.20; Figure 1). Furthermore, no significant association was shown for other polymorphisms with plasma VEGF levels (data not shown).

**Association of 936C/T and 1451C/T Polymorphisms with VEGF mRNA Stability**

Mixed DNA samples were used to validate the accuracy of estimation by quantitative CE-SSCP analysis. Figure 2 shows the calculated ratio by peak heights. The mixing ratio of 936C allele to the total was very highly correlated (r = 0.99). Using quantitative CE-SSCP analysis, the influence of 936C/T and 1451C/T polymorphisms on mRNA stability was examined on PMBC that were isolated from 10 individuals who were heterogonous for 936C/T and 1451C/T polymorphisms. After the inhibition of transcription by actinomycin D, the relative ratio of cDNA carrying the 936C allele increased (Figure 3). This result indicated that the transcript with 936C-1451C haplotype was more stable than that with the 936C-1451T haplotype. We also performed reverse transcription–PCR to examine isoforms that were transcribed by isolated PBMC. Transcripts of the unbound isoforms of VEGF121 and VEGF165 were dominantly found even after placenta growth factor and actinomycin D treatment (Figure 4). VEGF189 and VEGF206 isoforms were not detected clearly.

**Discussion**

We performed a case-control association study including a total number of 290 ESRD male patients and healthy male

---

**Table 1. Estimated haplotype frequencies in 3’ UTR of VEGF gene**

<table>
<thead>
<tr>
<th>936C/T</th>
<th>1451C/T</th>
<th>1612A/G</th>
<th>1725G/A</th>
<th>ESRD (%; n = 101)</th>
<th>Control (%; n = 189)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>75.7</td>
<td>70.1</td>
</tr>
<tr>
<td>T</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>12.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.0</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>6.4</td>
<td>6.9</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>4.5</td>
<td>2.7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>99.0</td>
<td>98.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>UTR, untranslated region; VEGF, vascular endothelial growth factor.

<sup>b</sup>p = 0.039 versus control based on 10,000 permutations.
control subjects and found significant associations of the 936C/T and 1451C/T polymorphism in 3’ UTR of the VEGF gene. These polymorphisms were in nearly absolute linkage disequilibrium, and the 936C-1451C haplotype was inferred as a risk haplotype for ESRD (Table 1). The distribution of the 936CC-1451CC genotype was demonstrated to be significantly higher in patients with ESRD compared with age-matched healthy control subject (Table 2). Therefore, we concluded that the 936CC-1451CC genotype was associated with ESRD. To our knowledge, this is the first such report, but three reports have shown significant associations of the 936C/T polymorphism with certain diseases such as breast cancer (18), rheumatoid arthritis (19), and sarcoidosis (20).

In addition to the association with ESRD in case-control association study, the 936CC-1451CC genotype showed significantly higher plasma VEGF levels and mRNA stability. These results indicated that the 936CC-1451CC individuals who show higher VEGF levels than the others might be susceptible to ESRD. VEGF seems to play an important role in maintaining normal kidney vasculature as a strong survival factor for endothelium. Ostendorf et al. (29) showed the blockade of capillary repair in a Thy-1 glomerulonephritis model by administration of an aptamer that inhibits VEGF. Kang et al. (30,31) showed that VEGF preserved endothelium and attenuated renal dysfunction and histologic damage in experimental rat models of CKD. However, not only mice that lack one VEGF allele but also mice that overexpress VEGF in renal podocyte developed glomerular disease (32). Moreover, VEGF has a chemotactic effect for monocytes and macrophages and could amplify the inflammatory reactions. Celletti et al. (4) demonstrated that administration of VEGF to hyperlipidemic mice promoted progression of atherosclerosis with increased monocytes in the aortic plaque. Analysis of patients with type 1 diabetes revealed that plasma VEGF levels were higher in patients with nephrop-
Development of CKD to ESRD seems to be influenced by many genetic and environmental factors. The contribution of the 936CC-1451CC genotype might be significant but moderate, as shown in other multifactorial diseases such as hypertension and diabetes (34,35). In other words, the 936CC-1451CC genotype would not engender an all-or-nothing event. This study demonstrated the functional significance of the 936CC-1451CC genotype using plasma of healthy individuals and PBMC of the 936C/T-1451C/T heterozygous individuals to exclude influences of other factors that regulate VEGF production. We examined plasma of healthy individuals because plasma and serum VEGF levels would differ in patients with hypertension (36) and peripheral arterial occlusive disease (37). The influence by VEGF polymorphisms might be inapparent using patient samples. We also examined mRNA stability using PBMC of heterozygotes and found a significant difference in the relative mRNA amount. Examining heterozygous samples is useful for detecting subtle but significant differences because heterozygotes express virtually equal amounts of mRNA. Furthermore, possible modifications by experimental procedures are excluded.

We found a significant association of the polymorphisms in 3' UTR with mRNA stability (Figure 3). Generally, the 3' UTR is known to contain important sequences that contribute directly and significantly to the longevity of mRNA transcripts (38), and many factors that bind to functional sequences within the 3' UTR of VEGF have been reported (39–43). Although the 936C/T and 1451C/T polymorphisms are not included directly in the binding sequences of the known factors, the binding sites of hypoxia-inducible RNA-protein (39) and heterogeneous nuclear ribonucleoprotein L (41) are located close to the polymorphic sites. Thus, it is possible that unknown regulatory proteins bind to these polymorphic sites.

Alternative splicing in VEGF gene generates four major isoforms: VEGF121, VEGF165, VEGF189, and VEGF206. The expression of unbound isoforms VEGF165 and VEGF121 have been demonstrated in podocytes, isolated glomeruli, and tubular cells (6,24). We examined these isoforms of PBMC that were used for mRNA stability assay and showed the VEGF165 and VEGF121 expression in PBMC (Figure 4). Those results indicated that alternative splicing in the VEGF gene in PBMC was similar to that in renal intrinsic cells. Moreover, all VEGF mRNA transcripts of respective isoforms and cells including PBMC and the intrinsic renal cells such as podocytes, tubular cells, and mesangial cells contain the 936C/T and 1451C/T polymorphisms in 3' UTR because they have the same genome DNA sequence. Therefore, the 936CC-1451CC genotype potentially influences VEGF production in all types of renal cells and contributes to progression to ESRD. For instance, the 242C/T polymorphism in the CYBA gene encoding the p22 phox component is assumed to play an important role in NAD(P)H oxidase activity. Several reports have demonstrated the functional significance of this genetic polymorphism by using neutrophils that were isolated from healthy individuals (44), saphenous vein and internal mammary artery from patients who underwent coronary artery bypass surgery (45), and vasodilator function of epicardial arteries (46). These reports indicated that genetic polymorphisms can make an impact in the different types of cells, although regulation of cell biology, including degeneration of mRNA, might differ according to cell type and circumstances around cells. We demonstrated the contribution of the 936CC-1451CC genotype for mRNA stability by using isolated PBMC. Further investigations are required to demonstrate whether the polymorphisms in VEGF gene can differently affect renal cells under physiologic and pathologic conditions. Recently, an inhibitory isoform of VEGF has been reported (47). This isoform inhibited proliferation and migration of endothelial cells. It was found in immortalized human podocytes (48). Antibodies that were used for plasma VEGF levels by enzyme immunoassay and oligonucleotide primers in this study were incapable of distinguishing this inhibitory isoform.

The effect of VEGF genetic polymorphisms might be undetectable in females because VEGF transcription is regulated by estrogens, and this regulation was mediated by transcriptional activation of the estrogen receptor (49). Our preliminary data showed no significant association between ESRD female patients and young female healthy individuals (data not shown). Therefore, we investigated the association of VEGF genetic polymorphisms only with male individuals. Further investigations are indispensable for confirmation of the association of the 936CC-1451CC genotype with ESRD in females. In this study, significant associations of VEGF plasma levels with the 936C/T and 1451C/T polymorphism were found only in males, not in females (Figure 1). With VEGF plasma levels, two previous reports also demonstrated significant associations of the 936C/T polymorphism in healthy males (14) and healthy postmenopausal females (18). We found reproducible results in males. In contrast, we found no significant association in premenopausal females, who differed from postmenopausal females in terms of their estrogen levels. Moreover, males are known to be vulnerable to progression of renal disease in experimental animal models (50–52) and in a meta-analysis of human clinical studies (53). Recently, Antus et al. (54) demonstrated protective effects of estrogen on rat remnant kidney, and Kang et al. (55) introduced the possibility that estrogen protects female rats in a remnant kidney model by regulating
VEGF expression. Our results indicate that VEGF regulation both by genetic polymorphisms and by sex hormones such as estrogens plays an important role in progression to ESRD.

Potential limitation of this study is related to a reported high 10-yr survival rate that exceeds 75% among the younger incident dialysis patients in Japan (56,57). Because the average duration of dialysis therapy of our patients with ESRD was approximately 10 yr, our case-control study design cannot rule out potential confounding as a result of ascertainment bias in the case in which the 936C-1451CC genotype is associated with a survival advantage in male patients with ESRD. Prospective cohort studies are needed to confirm a direct association of the 936CC-1451CC genotype with ESRD development.

Conclusion

Our case-control association study demonstrated a significant association of 936C/T and 1451C/T polymorphisms located in the 3’ UTR of the VEGF gene with ESRD in males. The 936C-1451C haplotype showed not only susceptibility to ESRD but also higher plasma VEGF levels and mRNA stability, advocating that these functional polymorphisms contribute to the development to ESRD by regulating VEGF production.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C “Medical Genome Science” (K.T.); grant #14370315 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (E.N.); and grants from the Cell Science Research Foundation (E.N.) and Takeda Medical Research Foundation (E.N.).

We are grateful to Dr. Jun Ohashi (University of Tokyo) for suggestions about statistical analysis and to Drs. Yasushi Yukiyama, Hitoshi Miyake (Fujitsu Kawasaki Hospital), Tadahiro Nishi (Nishi Clinic), Takashi Ozawa (Kodaira Kitaguchi Clinic), Hisakazu Degawa (Sinoiwa Clinic Funabori), and Hiroshi Nonaka (Nonaka Clinic) for providing samples. We also thank Ms. Mami Haba, Ms. Rui Maeda, Takashi Ozawa (Kodaira Kitaguchi Clinic), Hisakazu Degawa (Sinoiwa Clinic Funabori), and Hiroshi Nonaka (Nonaka Clinic) for providing samples. We also thank Ms. Mami Haba, Ms. Rui Maeda, and Ms. Yoshimi Ishibashi (University of Tokyo) for technical assistance.

References


34. Luft FC: Geneticism of essential hypertension. *Hypertension* 43: 1155–1159, 2004


Access to UpToDate on-line is available for additional clinical information at http://www.jasn.org/