Chromogranin A (CHGA) is a soluble, acidic glycoprotein stored in secretory vesicles within neuronal and neuroendocrine cells. The chromogranin/secretogranin loci have become a focus in studies of genetic mechanisms in hypertension.\(^1\)−\(^6\) Recent studies indicate that CHGA is necessary for sympathetic neurotransmission, playing critical roles in intracellular catecholamine compartmentalization and release\(^7\),\(^8\); hence, CHGA influences human sympathetic tone.\(^9\) CHGA is located on human chromosome 14q32, with 8 exons (encoding 439 amino acids) separated by 7 introns.\(^10\) The transcription of this gene is influenced by cis-acting elements in the proximal promoter.\(^1\)

Following translation, CHGA is required during the formation of catecholamine storage vesicles (chromaffin granules) at the trans-Golgi apparatus.
of chromaffin cells and postganglionic sympathetic neurons, aggregating within such secretory granules through a mechanism that is both calcium and low-pH dependent.\textsuperscript{1,5,7,8,11,12} Indeed, inhibition of the biosynthesis of CHGA results in depletions of storage vesicles in chromaffin cells.\textsuperscript{5,7,8} Within catecholaminergic cells, CHGA is co-stored and co-released with epinephrine and norepinephrine. In addition to their intracellular functions, CHGA is a pro-hormone/pro-peptide containing multiple recognition sites for specific endopeptidases, allowing enzymatic cleavage into smaller biologically active peptides.\textsuperscript{1} For example, human CHGA\textsubscript{352} to \textsubscript{372} ("catestatin") inhibits nicotinic cholinergic-stimulated catecholamine release, providing negative feedback to further sympathetic neurotransmission,\textsuperscript{13} both in cultured cells\textsuperscript{14} and in humans.\textsuperscript{9} Decreased circulating levels of catestatin occur in patients with essential hypertension, as well as their still-normotensive offspring at genetic risk of hypertension, suggesting that a deficit in this sympathetic "braking" system might be an early event in the pathogenesis of human hypertension.\textsuperscript{9}

However, to date, no studies have examined whether polymorphisms at the chromogranin loci predict the development of target organ complications of hypertension, such as end-stage renal disease (ESRD).

In this study, we investigated CHGA as a potential disease-susceptibility locus for hypertensive end-stage renal disease (HT-ESRD) in blacks, a population in which HT-ESRD is particularly prevalent.\textsuperscript{15} In this association analysis, we evaluated allele and genotype frequencies of single nucleotide polymorphisms (SNPs) individually and in combination, in the form of haplotypes at CHGA; we compared such gene and haplotype frequencies in normotensive and hypertensive black controls (with normal renal function) to those in black patients with ESRD as a result of hypertension (HT-ESRD). In addition, we evaluated the impact of CHGA SNPs on a more proximate or "intermediate" biologic phenotype,\textsuperscript{16} expression of the catestatin gene product in plasma.

## RESULTS

### Study Subjects

**Original study (California)**

Demographic and anthropometric descriptions of the study populations (cases and controls) are presented in Table 1. Cases and controls differed in renal function but were similar in age and family history of hypertension. The ESRD cases had a glomerular filtration rates (GFR) of essentially zero (all were sustained by chronic hemodialysis), whereas the control group had a mean GFR of 111 ± 2.6 ml/min. Among the hypertensive ESRD cases, there were fewer men (60% \textit{versus} 94%, \textit{P} < 0.001), fewer diabetics (\textit{P} = 0.037), and lower body mass index (BMI) (25.4 ± 0.66 \textit{versus} 28.3 ± 0.53 kg/m\textsuperscript{2}, \textit{P} < 0.002), compared with the control group. Higher prevalence of hypertension and lower prevalence of diabetes result from the case selection scheme. Lower BMI in ESRD is a predictable consequence of the malnutrition found in this disease state.\textsuperscript{17}

Within the control group (\textit{n} = 150), there were 76 hypertensive and 74 normotensive individuals. Compared with the normotensives, the hypertensives had higher blood pressure (BP) (systolic BP/diastolic BP, \textit{P} < 0.0001/\textit{P} < 0.0001) were older (53 ± 1.1 \textit{versus} 43 ± 0.8 yr, \textit{P} < 0.0001) and more likely to have diabetes (28.4% \textit{versus} 2.7%, \textit{P} < 0.0001) and a positive family history of hypertension (74.7% \textit{versus} 58.1%, \textit{P} = 0.014). Hypertensive and normotensive controls did not differ significantly in gender, BMI, or serum creatinine concentration.

### Polymorphisms

Table 2 presents the polymorphic variants studied at the CHGA locus. All SNPs had minor allele frequencies of more

### Table 1. Demographic description of the California African American study populations: ESRD (end-stage renal disease) cases and controls with normal renal function

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (ESRD) (\textit{n} = 58)</th>
<th>Controls (\textit{n} = 150)</th>
<th>\textit{P} \textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>50.2 ± 1.7</td>
<td>48.1 ± 0.8</td>
<td>0.208</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>35/23 (60%/40%)</td>
<td>141/9 (94%/6%)</td>
<td>&lt;0.0001\textsuperscript{a}</td>
</tr>
<tr>
<td>Diabetes, yes/no</td>
<td>3/55 (5%/95%)</td>
<td>24/126 (16%/84%)</td>
<td>0.037\textsuperscript{a}</td>
</tr>
<tr>
<td>Family history of hypertension, positive/negative/unknown (%)</td>
<td>31/22/5 (53%/38%/9%)</td>
<td>99/39/12 (66%/26%/8%)</td>
<td>0.206</td>
</tr>
<tr>
<td>Body mass index (kg/m\textsuperscript{2})</td>
<td>25.4 ± 0.66</td>
<td>28.3 ± 0.53</td>
<td>0.002\textsuperscript{a}</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>10.8 ± 0.47</td>
<td>0.98 ± 0.01 (all &lt;1.5 mg/dl)</td>
<td>&lt;0.0001\textsuperscript{a}</td>
</tr>
<tr>
<td>Glomerular filtration rate\textsuperscript{d} (ml/min)</td>
<td>Not calculable</td>
<td>111 ± 2.6</td>
<td>—</td>
</tr>
<tr>
<td>Hypertensive, Yes/No (%)</td>
<td>58/0 (100%/0%)</td>
<td>76/74 (51%/49%)</td>
<td>&lt;0.0001\textsuperscript{a}</td>
</tr>
<tr>
<td>Family history of ESRD, positive/negative/unknown (%)</td>
<td>7/44/7 (12%/76%/12%)</td>
<td>NA</td>
<td>ND</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Continuous variables: mean value± SE are presented. Discrete variables: ratios (%) are presented.

\textsuperscript{b}Within the controls, normotensive (NT) and hypertensive (HTN) individuals differed significantly in terms of age, prevalence of diabetes, and family history of hypertension (data not shown).

\textsuperscript{c}P value reported from ANOVA for continuous variables and \textit{X}\textsuperscript{2} for discrete variables.

\textsuperscript{d}GFR is estimated in controls using a standard algorithm.\textsuperscript{72} GFR was not calculated in ESRD because all subjects were regularly hemodialyzed.

\textsuperscript{e}Significant (\textit{P} < 0.05).
Table 2. Single nucleotide polymorphisms at the CHGA locus

<table>
<thead>
<tr>
<th>Locus</th>
<th>SNP Identity</th>
<th>Domain</th>
<th>Nucleotide Position*</th>
<th>Wild-type Allele (%)</th>
<th>Variant Allele (%)</th>
<th>HWE (black controls)</th>
<th>Amino Acid Variation</th>
<th>RefSNP No. (dbSNP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHGA</td>
<td>G-1106A</td>
<td>Promoter</td>
<td>−1106</td>
<td>G (73%)</td>
<td>A (27%)</td>
<td>0.33 0.565</td>
<td>—</td>
<td>rs9658628</td>
</tr>
<tr>
<td>CHGA</td>
<td>A-1018T</td>
<td>Promoter</td>
<td>−1018</td>
<td>A (89%)</td>
<td>T (11%)</td>
<td>2.37 0.124</td>
<td>—</td>
<td>rs9658629</td>
</tr>
<tr>
<td>CHGA</td>
<td>T-1014C</td>
<td>Promoter</td>
<td>−1014</td>
<td>T (77%)</td>
<td>C (23%)</td>
<td>5.07 0.024</td>
<td>—</td>
<td>rs9658630</td>
</tr>
<tr>
<td>CHGA</td>
<td>T-966G</td>
<td>Promoter</td>
<td>−988</td>
<td>T (77%)</td>
<td>G (23%)</td>
<td>0.62 0.430</td>
<td>—</td>
<td>rs9658631</td>
</tr>
<tr>
<td>CHGA</td>
<td>G-462A</td>
<td>Promoter</td>
<td>−462</td>
<td>G (81%)</td>
<td>A (19%)</td>
<td>0.23 0.630</td>
<td>—</td>
<td>rs9658634b</td>
</tr>
<tr>
<td>CHGA</td>
<td>T-415C</td>
<td>Promoter</td>
<td>−415</td>
<td>T (60%)</td>
<td>C (40%)</td>
<td>1.26 0.261</td>
<td>—</td>
<td>rs9658635b</td>
</tr>
<tr>
<td>CHGA</td>
<td>C-89A</td>
<td>Promoter</td>
<td>−89</td>
<td>C (87%)</td>
<td>A (13%)</td>
<td>0.16 0.690</td>
<td>—</td>
<td>rs7159323b</td>
</tr>
<tr>
<td>CHGA</td>
<td>C-57T</td>
<td>Promoter</td>
<td>−57</td>
<td>C (81%)</td>
<td>T (19%)</td>
<td>0.36 0.547</td>
<td>—</td>
<td>rs9658638</td>
</tr>
<tr>
<td>CHGA</td>
<td>T5088A</td>
<td>Intron D</td>
<td>5088</td>
<td>T (74%)</td>
<td>A (26%)</td>
<td>1.80 0.179</td>
<td>—</td>
<td>rs735726</td>
</tr>
<tr>
<td>CHGA</td>
<td>G8540C</td>
<td>Exon 6 coding</td>
<td>8540</td>
<td>G (83%)</td>
<td>C (17%)</td>
<td>2.14 0.144</td>
<td>Glu246Asp</td>
<td>rs9658655b</td>
</tr>
<tr>
<td>CHGA</td>
<td>C9610T</td>
<td>Exon 7 coding</td>
<td>9610</td>
<td>C (96%)</td>
<td>T (4%)</td>
<td>3.19 0.572</td>
<td>Arg381Trp</td>
<td>rs729940b</td>
</tr>
<tr>
<td>CHGA</td>
<td>C11825T</td>
<td>Exon 8/3'-UTR</td>
<td>11825</td>
<td>C (83%)</td>
<td>T (17%)</td>
<td>10.30 0.001</td>
<td>—</td>
<td>rs7610b</td>
</tr>
<tr>
<td>CHGA</td>
<td>G12602C</td>
<td>Downstream 457 bp</td>
<td>12602</td>
<td>G (67%)</td>
<td>C (33%)</td>
<td>4.93 0.026</td>
<td>—</td>
<td>rs875395b</td>
</tr>
<tr>
<td>CHGA</td>
<td>G17775A</td>
<td>ITPK1 (5612 bp downstream)</td>
<td>177757</td>
<td>G (89%)</td>
<td>A (11%)</td>
<td>1.19 0.276</td>
<td>—</td>
<td>rs11446</td>
</tr>
</tbody>
</table>

*Position with respect to CAP (transcription initiation, exon 1 start) site in bp (CHGA source clone NM_001275.2 or NT_026437.10).

SNPs also genotyped in the replication (North Carolina) population. Allele frequencies are values in black controls (non-ESRD subjects).

than 4%, and were in Hardy Weinberg Equilibrium (HWE) at $P > 0.05$ after adjusting for multiple comparisons. The genotype frequency distribution of all SNPs was evaluated, with particular attention to SNPs with unadjusted HWE $0.05 > P > 0.01$. One SNP in particular, C11825T, had a HWE value lower than the Bonferroni cut point ($P = 0.004$). This SNP was genotyped locally via pyro-sequencing and genotype calls were visually inspected to ensure accuracy. When the same assay was used to genotype this SNP in a white population ($n = 417$), the SNP was found to be in HWE ($\chi^2 = 1.676$, $P = 0.195$). We conclude that this SNP’s HWE departure did not result from assay artifact.

Disease–Genotype Associations: Linkage Disequilibrium (LD), Haplotypes, and “Sliding Windows”

Visual inspection (Figure 1) of the Graphical Overview of Linkage Disequilibrium (GOLD) plots of the LD structures at the CHGA (14 SNP) locus in blacks revealed that the locus displays regions of long-range LD, with blocks of particularly high LD ($D^\prime > 0.8$) at the $3'$ (promoter) and $3'$ (terminal exon) ends of each gene.

A “sliding window” analysis of the CHGA locus evaluated not only individual SNPs but also haplotypes spanning 2 to 5 adjacent SNPs. This analysis suggested peak associations of ESRD status with two regions (Figure 1). Because of the relative infrequency of individuals homozygous for any given haplotype (i.e., bearing 2 copies of that haplotype), we approached haplotype effects by grouping diploid haplotype pair (diplotype) configurations into 2 categories: presence versus absence of any particular haplotype (Table 4).

SNP Haplotypes

We analyzed 2- and 3-SNP haplotypes spanning locus regions identified by the sliding window analyses (Figure 1). On initial sliding window analysis (Figure 1), two regions of CHGA were significantly associated with HT-ESRD: $5'$ and $3'$. Chromosomes bearing CHGA promoter haplotype ATC (over G-462A→T-415C→C-89A) were more common in cases than controls ($\chi^2 = 10.36$, $P = 0.044$), indicating that ATC was associated with disease status. The ATC haplotype was also found in only 20 of 142 (0.14) individuals without renal failure, but in one third (18 of 53, 0.34) of subjects with hypertensive ESRD, thus constituting a risk factor for developing ESRD.

The magnitude of the association was attenuated but remained significant after adjusting for age, BMI, and gender.
Figure 1. CHGA locus structure, linkage disequilibrium, and sliding window analysis in California ESRD and control populations. Sliding window plots of SNP haplotype combinations in ESRD patients (n = 58) versus controls with normal renal function (n = 150) at CHGA. (Top panel) Sliding window analysis across the CHGA locus. Significance of association graded by $-\log_{10} (P\ value)$. The $P$ value is derived from likelihood ratio statistic of comparison of individual SNP and haplotype combination frequencies in ESRD patients and controls across the locus, based on 10,000 permutations. The x-axis indicates the position (in bp) of the center of each SNP window in relation to the gene cap (transcription initiation) site. Significance (horizontal black line): at $P < 0.05$, $-\log_{10} (P\ value) > 1.301$. (Middle panel) Eight exon (solid bar)/7 intron structure of the CHGA gene on human chromosome 14q32. (Bottom panel) GOLD (Graphical Overview of Linkage Disequilibrium) plot of point-by-point linkage disequilibrium (LD) among 14 SNPs with minor allele frequencies more than 5%, spanning the CHGA locus, and proceeding approximately 1 kb upstream (5’; promoter region) and approximately 5 kb downstream (3’; adjacent locus: ITPK1). The white diagonal is the line of identity ($Y = X$). LD plot constructed using both cases (ESRD) and controls (n = 208). $D’$, LD parameter, pseudocolor-scaled from 0→1.
haplotype were more common in cases (21 of 53, 0.40), than control (22 of 149, 0.15; \( \chi^2 = 27.95, P = 0.001 \)), indicating that TC was associated with ESRD (Figure 3; Table 4; OR = 2.69; 95% CI, 1.29 to 5.73; \( P = 0.001 \)). After adjustment for BMI and gender, the significance and magnitude of this association persisted (OR = 2.73; 95% CI, 1.16 to 6.34; \( P = 0.0196 \)).

### Gene \times Gene (Epistasis) and Gene \times Covariate Interactions

In light of these novel associations between two different CHGA region haplotypes and ESRD status, we considered the possibility of an interaction (i.e., nonadditive effects) between 5’ and 3’ CHGA haplotypes in predicting risk of hypertensive ESRD in the California subjects. The haplotype \times haplotype interaction term did not reach statistical significance (\( P = 0.21 \)).

Finally, we tested for the possibility of gene \times covariate interactions, between the associated haplotypes and age, diabetes status, family history of hypertension, or sex. All such gene \times covariate interactions were nonsignificant (\( P > 0.15 \)). Although there were no significant interactions detected, small sample size may preclude our ability to detect subtle interactions.

### Genetic Admixture

The possibility that cases and controls differed in degree of genetic admixture was approached by comparison of genotype frequencies at widely dispersed markers (23 genes on 11 different chromosomes) other than the loci under primary investigation because admixture is a genome-wide phenomenon18.
In the California subjects, the Pritchard method, testing the summed \( \chi^2 \) for genotype frequency differences between cases and controls, did not reveal differential admixture (\( \chi^2 = 45.08, 46 \text{ df}, P = 0.511 \)). Likewise, a “genetic distance” measure (Slatkin’s linearized \( F_{ST} \)) calculated using Arlequin failed to reveal differences between cases and controls (\( F_{ST} = -0.00099, P = 0.721 \)). Therefore, the observed CHGA allele and haplotype frequency differences cannot be attributed to differential admixture between the case and control groups.

Endogenous CHGA Peptide Catestatin Expression in ESRD

We measured the circulating concentration of the CHGA gene product catestatin in a sample of California black ESRD patients (\( n = 14 \)) and control subjects with documented normal renal function (\( n = 27 \)). Unexpectedly, the catestatin concentration was reduced in blacks with ESRD versus controls (Figure 4). In contrast, elevated plasma peptide levels are usually reported in subjects with ESRD, consistent with reduced plasma peptide elimination as GFR declines.

CHGA 3′-UTR Variant: Effect on Gene Expression

To probe the effect of the C11825T (C +87T) variant on CHGA gene expression, PC12 cells were transfected with plasmids in which a firefly luciferase reporter was succeeded by the CHGA 3′-UTR region bearing either the major allele (C11825, C +87) or minor allele (11825T, +87T) in the 3′-UTR; 48 h after transfection, the firefly/Renilla luciferase activity ratio revealed markedly reduced gene expression (by approximately 42%) in the variant (+87T) as compared with the wild-type (C +87) plasmid (\( P < 0.001 \)) (Figure 5).

Population Genetics: Haplotype Frequencies in Additional Human Ethnic Groups

Haplotype frequencies of disease-associated regions were estimated in 2 additional ethnic groups: white (European ancestry, \( n = 131 \)) and Hispanic (Mexican American ancestry, \( n = 40 \)). In the CHGA promoter (G-462A → T-415C → C-89A), haplotype frequencies differed substantially among the ethnic groups. The risk haplotype ATC was notably absent in both the white and Hispanic population control groups (\( \chi^2 = 24.1, P < 0.0001 \)). In the CHGA downstream-region C11825T (3′-UTR, C +87T) → G12602C, haplotype frequencies did not differ among the ethnic groups (results not shown).

Replication Case/Control Sample: North Carolina Haplotype ORs

Seven SNPs, encompassing the 5 California study ESRD-associated SNPs and 2 additional nonsynonymous SNPs (G8540C/exon 6, and C9610T/exon 7), were genotyped in a replication sample from North Carolina (noted in Table 2). In the promoter region, chromosomes bearing CHGA pro-

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Figure 2. CHGA promoter haplotype frequency distributions between ESRD cases and controls in California. The disease-associated 3-SNP haplotype was located in the promoter region of the CHGA gene: G-462A → T-415C → C-89A. The reference state was defined as the absence of the risk haplotype (ATC), “ATC absent.”

Figure 3. CHGA downstream (3′) haplotype frequency distributions between ESRD cases and controls in California. The disease-associated 3-SNP haplotype was located toward the 3′ end of the CHGA gene: C11825T (3′-UTR, C +87T) → G12602C. The reference state was defined as the absence of the risk (TC) haplotype.
moter haplotype TA (over T-415C C-89A) were more common in controls than cases; the TA haplotype was found in 66 of 286 (0.23) of individuals without renal failure but less frequently (45 of 277, 0.16) in subjects with hypertensive ESRD (Table 5; OR = 0.65; 95% CI, 0.42, 0.99; P = 0.0425). After adjusting for age, BMI, and gender, the association remained significant (OR = 0.62; 95% CI, 0.40, 0.98; P = 0.0394).

Similarly, the downstream region also reproduced the significant association with risk of HT-ESRD. Downstream haplotype TC, at CHGA C11825T (3'-UTR, C 87T) G12602C, was relatively rare but more common in cases (8 of 270, 0.030), than controls (1 of 277, 0.004) (Table 5, OR = 8.43; 95% CI, 1.05, 67.84, P = 0.0452). After adjustment for age, BMI, and gender, the significance and magnitude of this association persisted (OR = 11.77; 95% CI, 1.43, 96.77; P = 0.0218). Both study groups showed significant associations in both the promoter and the downstream/3' regions of the CHGA locus.

**DISCUSSION**

**Heredity and ESRD**

The past 25 yr have seen a substantial rise in the number of people living with ESRD within the United States, from a population of 42,324 in 1978 to 406,081 in 2001. Increasing incidence of ESRD has been especially evident among blacks, who, despite making up only 12% of the general population of the United States, represent 29% of patients on chronic dialysis. Epidemiologic studies have repeatedly demonstrated an excessive risk of ESRD among blacks compared with whites, even when controlling for such factors as socioeconomic status, access to health care, and prevalence of causative disease, such as diabetes and hypertension.

Essential hypertension is a common syndrome, likely polygenic, which occurs with far higher prevalence in blacks compared with the general U.S. population. ESRD is also especially common in blacks, and hypertension is a particularly prominent antecedent of the nephrosclerosis diagnosis, which seems to aggregate in black families. Recent studies of the epidemiology of black ESRD, and the search for sources of genetic risk, coincide with studies of genetic (essential) hypertension. In light of these observations, there is growing interest in the search for sources of genetic risk of ESRD in blacks. Familial aggregation of ESRD from a number of causes has repeatedly been observed. Although some cases of ESRD result from single gene mutations (approximately 8% of cases), most of the available evi-
gence from pedigree studies suggests ESRD more typically behaves as a complex trait, potentially influenced by multiple genes and environmental factors and lacking a simple correlation between genotype and phenotype.41

There is emerging interest in the role of susceptibility genes in complex human disease traits, such as ESRD, which displays familial aggregation yet whose genetic underpinnings are still obscure.32,34 Genetic variants so far associated with nondiabetic ESRD include polymorphisms at renal kallikrein (KLK1),38 Wilms' tumor proto-oncogene (WT1),42 podocin (NPHS2),43 IL-1A,44 and TRPC6.45 Because blacks may display exaggerated autonomic responses to environmental stressors46–49 and the sympathetic nervous system plays a role in creating or accelerating renal injury and thereby determining the rate of progression of renal disease,50–52 we explored whether polymorphisms at loci encoding components of the sympathetic neuroeffector junction53 influenced risk of ESRD in a population enriched for hypertensive ESRD: blacks.26,29–35Our search began with the major Disease Associations: Potential Mechanisms

The ESRD-associated CHGA regions (Figure 1) included variants with the potential for quantitative consequences on protein function. CHGA promoter variants at G-462A→T-415C→C-89A lie in a domain with transcriptional activity, and polymorphic variation in this region clearly alters CHGA promoter strength in vitro.4 Indeed, in previous studies, we have found that plasma CHGA overall immunoreactivity (both the intact molecule and immunoreactive fragments) is substantially elevated in ESRD20,53; thus, the decline in catestatin in blacks with ESRD seems to be quite selective. Such catestatin deficiency in blacks with ESRD suggests yet another mechanism for increased sympathetic tone; indeed, we have observed that endogenous catestatin deficiency is associated with increased catecholamine secretion and adverse BP responses to environmental stress, and is a risk factor for future development of hypertension.9

How might catestatin production be diminished? Here we focused on the CHGA 3′-UTR polymorphism, since the marker-on-trait association in this region was especially prominent on SNP-EM analysis (Figure 1). CHGA 3′-UTR common variant C +87T was associated with ESRD in both the California and North Carolina samples (Tables 3 through 5; Figure 3), with the T allele conferring risk of ESRD. When we tested wild-type (C +87T) versus variant (+87T) 3′-UTRs in luciferase reporter plasmids (Figure 5), the +87T variant displayed approximately 42% decline in expression. We propose that the effect of this variant on CHGA gene expression might explain the diminution in catestatin expression (Figure 4), eventuating in elevated risk of ESRD in +87T carriers (Table 3).

Table 5. North Carolina association of haplotype regions (5′ and 3′) identified via sliding window analysis between ESRD replication cases and controls

<table>
<thead>
<tr>
<th>Locus Segment/Haplotype Configuration</th>
<th>Control (n)</th>
<th>ESRD (n)</th>
<th>Univariate Odds Ratio of ESRD (CI)</th>
<th>Univariate Diplotype (P)</th>
<th>Adjusted Odds Ratio of ESRD* (CI)</th>
<th>Adjusted Diplotype (P)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHGA promoter haplotype: T-415C→C-89A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of TA</td>
<td>66</td>
<td>45</td>
<td>0.65 (0.42, 0.99)</td>
<td>0.0425</td>
<td>0.62 (0.40, 0.98)</td>
<td>0.0394</td>
</tr>
<tr>
<td>Absence</td>
<td>220</td>
<td>232</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>CHGA Exon/3′-UTR and downstream haplotype: C11825T→G12602C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of TC</td>
<td>1</td>
<td>8</td>
<td>8.43 (1.05, 67.84)</td>
<td>0.0452</td>
<td>11.77 (1.43, 96.77)</td>
<td>0.0218</td>
</tr>
<tr>
<td>Absence</td>
<td>276</td>
<td>262</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

*Association analysis are adjusted for age, BMI and gender.
The findings of this study can be viewed in the context of a growing literature implicating sympathetic nervous system (SNS) hyperactivity as a mechanism of renal injury and disease progression.50,56 Sympathetic hyperactivity has been well described in patients with chronic renal failure57 and may represent both a consequence of the disease57 and a factor in its origin and progression.56 Blacks are the population at highest risk of developing hypertensive ESRD,26,29–34 and this same population may display exaggerated autonomic responses to environmental stressors.46–49 Johnson et al.58 suggested a model in which increased sympatoadrenal tone may be starting point for a chain of events leading to renal parenchymal damage. The authors also suggested that consequent tubular injury and preglomerular arteriolopathy may result in increased sodium reabsorption and salt-sensitive hypertension, a syndrome common in blacks.

Blankestijn et al.59 demonstrated elevated sympathetic nervous tone in patients with renal failure through measurement of muscle sympathetic (peroneal) nerve activity and further showed that SNS hyperactivity is diminished by angiotensin-converting enzyme (ACE) inhibition in these patients.59,60 Elevated SNS tone is itself a mediator of renal failure progression both in animal models52,61,62 and in renal failure patients.63 Indeed, even sub-antihypertensive doses of the sympathetic outflow inhibitor monoxide diminish progressive glomerulosclerosis in animals after subtotal nephrectomy.52 While ACE inhibition may be superior to calcium channel blockade in preventing progression of nephrosclerosis in black hypertensives,64,65 some authors suggest that diminution of sympathetic tone represents a mechanism for the observed renoprotective effects of ACE inhibitors and angiotensin receptor blockers.56 Although our CHGA associations with ESRD were not accompanied by physiologic measures of autonomic tone in the same patients (such as muscle sympathetic nerve activity, baroreflex, Valsalva, or cold pressor responses), the responsible genetic variants are associated with observed biochemical markers of SNS activity: plasma CHGA peptide levels (Figure 4). As such, our findings are consistent with a model of altered sympathetic tone as a contributing factor to the pathogenesis and/or progression of hypertensive nephrosclerosis in blacks, enabled by augmented catecholamine storage vesicle biogenesis.

The contribution of hereditary sympathoadrenal changes to hypertensive ESRD suggests the possibility that previously underexplored therapeutic strategies might be especially effective for slowing progressive hypertensive nephrosclerosis. While the recent African American Study of Kidney Disease and Hypertension trial highlighted the benefits of ACE inhibition for progressive hypertensive nephrosclerosis,63 we propose that sympatho-inhibition with agents such as clonidine or ganglionic blockers might be especially effective in minimizing the sympathetic impetus toward progressive renal injury. Indeed, the observed deficiency of the endogenous nicotinic cholinergic antagonist56 catestatin (CHGA352 to 372) in blacks with ESRD reinforces the notion that sympathetic inhibition might be especially beneficial in this setting. Finally, our results raise that possibility that CHGA genetic profiling of patients with progressive nephrosclerosis might yield practical pharmacogenetic predictors of patients most likely to benefit from sympatholytic therapy.66

Population Genetics: Clues to Disease Origins

The haplotype frequencies in the ESRD-associated promoter (5’) region differed significantly by ethnicity; in the CHGA promoter region. Of particular note was the CHGA promoter risk haplotype ATC, which was relatively common in black controls (20 of 142 individuals, 14%; Table 4) but was not observed in substantial numbers of either white (n = 131 individuals) or Hispanic (n = 40 individuals) population control groups. These haplotype frequency differences in the black population (excess risk, diminished protection) seem to correspond to differences in frequency of hypertensive nephrosclerosis among the ethnic groups.32

However, the chromosomal region showing the strongest association with ESRD (Table 5; Figures 3 and 6), the CHGA downstream segment (C11825T[C + 87T]→G12602C), did not differ in frequency among control populations, whether black, white, or Hispanic. Thus, polymorphism in this region might influence risk of ESRD in multiple ethnic populations; future studies of hypertensive nephrosclerosis in additional population groups would be required to document such risk.

Strengths and Limitations of This Study Diagnosis

In this study, we examined differences in haplotype frequencies between blacks with normal kidney function and those with ESRD attributed to hypertension. The diagnosis of ESRD from hypertensive nephrosclerosis was generally a clinical one, rather than one based on renal biopsy. Some authors have questioned the accuracy of hypertensive nephrosclerosis as a clinical diagnosis in patients with chronic renal failure and ESRD.67,68 However, the accuracy of clinical diagnosis for hypertensive renal disease was supported recently by results of the African American Study of Kidney Disease and Hyperten-
Admixture
Another potential pitfall in genetic studies of blacks is the possibility of differential admixture between cases and controls,\textsuperscript{66,70} which may lead to artifact conclusions based on comparisons of genetically dissimilar populations. In this study, we estimated admixture for non-ESRD and ESRD patients groups by comparing genotype frequencies at 23 loci outside the genes of interest. Using 2 different statistical methods of analysis (one based Slatkin’s linearized $F_{ST}$ in Arlequin,\textsuperscript{19} the other based on the sum of $\chi^2$ method described by Pritchard and Rosenberg\textsuperscript{28}; Table S1), we found no evidence of differential admixture that could explain observed differences in CHGA genotype frequencies between the ESRD patients and the control group.

Sample Size and Homogeneity
Our findings suggest sympathetic nervous system (SNS) hyperactivity as a mechanism of renal injury and disease progression, in a homogeneous sample of modest size. The limited size of the sample may reduce the power to determine genetic or environmental interactions with disease-associated haplotypes. The potential for gene X environment or other gene X gene (epistatic) interactions, or the influence of other potential confounders may warrant further study. Moreover, an association study, such as ours warrants additional studies of genetic variants in larger cohorts, and perhaps monitoring the effects of such variants on progression of nephrosclerosis in hypertensive populations. Finally, the clinical diagnosis of hypertensive nephrosclerosis is not confined exclusively to patients of African ancestry; thus similar genetic association studies of this disease would be of interest in other ethnicities.

Heterogeneity of Controls: BP
Sensitivity analyses were performed, separately comparing the hypertensive ($n = 76$) and normotensive ($n = 74$) control (normal renal function) subgroups to the ESRD cases in the California sample. Sliding window analyses for each of the 2 control subgroups resulted in essentially identical plots, and peak associated regions to those originally obtained for the entire control group versus ESRD (results not shown). By contrast, sliding window analyses comparing control subgroups did not identify significant differences between normotensives and hypertensives at $CHGA$ (all $P > 0.5$). Genotype and haplotype analysis were also performed comparing control subgroups (hypertensive and normotensive) separately to ESRD cases. The reported OR associations in Tables 3 through 5 were essentially unchanged in the subgroup analyses. Thus, the allelic and haplotype associations we report seem to be specifically with the trait of hypertension ESRD, rather than hypertension itself. Finally, removal of subjects with diabetes from the case ($n = 3$) and control ($n = 24$) groups did not alter the results observed in Tables 3 through 5 for all loci.

Statistical Confidence and Replication
With all genetic studies, a common concern arises, that of reproducibility and robustness of conclusions.\textsuperscript{71–73} We began by undertaking a 2-stage haplotype analysis. The first stage evaluated chromosomal haplotype frequencies \textit{via} a sliding window analysis with permutation tests, to determine the lengths of associated regions and preclude false positive conclusions arising from multiple comparisons. We then focused on haplotype–phenotype associations at the level of the individual human subject. Finally, we replicated the marker-on-trait associations in an independent (North Carolina) ESRD case/control sample.

Biologic Plausibility
To establish a biologic rationale for our findings, we next probed the likely role of the $CHGA$ locus in sympathochromaffin function, both \textit{in vivo} (Figure 4) and \textit{in vitro} (Figure 5). Our results on catestatin and the effect of the C \textsuperscript{+87T} 3′-UTR variant on gene expression suggest a coherent biologic picture (Figure 6): 3′-UTR variation alters $CHGA$ and hence catestatin expression, eventuating in altered sympathoadrenal activity eventuating in enhanced risk for hypertensive ESRD. Nonetheless, in the associated regions of $CHGA$, the peak haplotypes associations span substantial exon/intron regions, perhaps because of extensive local patterns of linkage disequilibrium (Figure 1); thus, the precise causative variants in $CHGA$ are not yet definitively established.

Conclusion and Perspectives
In conclusion, common polymorphisms at $CHGA$ are associated with the diagnosis of hypertensive ESRD in blacks. This is the initial study to examine the role of the chromogranins as disease-susceptibility loci in ESRD. The associations suggest that a specific subpopulation of patients with hypertension may be especially susceptible to nephrosclerosis because the associations occur with hypertensive ESRD, rather than for hypertension itself. These polymorphisms may therefore represent potential clinical markers for hypertensives at risk for ESRD secondary to nephrosclerosis, particularly among blacks.

These $CHGA$ variants may prove to be useful clinical markers to identify hypertensives at particular risk for developing ESRD, and perhaps to derive specific benefit from treatment strategies targeting the sympathoadrenal system; such pharmacogenetic profiling may increase the likelihood of rational targeting of particular therapies to groups most likely to benefit.\textsuperscript{66} For example, $\alpha_2$-adrenergic agonists achieve their anti-hypertensive effects by inhibition of efferent sympathetic outflow,\textsuperscript{74} and such inhibition is particularly effective at slowing progressive glomerulosclerosis in rodent models.\textsuperscript{84} Indeed, heredity and genetic polymorphism are already known to play substantial roles in the response to $\alpha_2$-adrenergic ligands in
humans.75,76 Whether CHGA polymorphisms can effectively guide therapy in this setting remains to be evaluated.

CONCISE METHODS

Initial Case/Control Population: California
The study protocol was approved by the institutional review board of the University of California at San Diego. Fifty-eight black patients with hypertensive ESRD ("cases") were recruited from outpatient ESRD treatment (hemodialysis) units located at 3 medical centers in southern California: the Martin Luther King Jr/Charles R. Drew Medical Center in Los Angeles, CA, the Veterans Administration San Diego Healthcare System, and the University of California at San Diego Medical Center. Inclusion criteria for ESRD subjects were: age ≥18 yr, self-identification as black, and a clinical diagnosis of ESRD secondary to hypertensive nephrosclerosis (HT-ESRD).64 Exclusion criteria for ESRD subjects include clinical diagnosis of ESRD secondary to other (nonhypertensive) causes, such as diabetic nephropathy, glomerulonephritis, polycystic kidney disease, cocaine, or other intravenous drug abuse.15 Thus, none of these cases had a renal biopsy. All dialysis patients were interviewed regarding their personal and familial medical histories on a single dialysis treatment day, and each patient’s age, gender, height, weight, vital signs, and medication list were recorded. Past medical history and medication lists were confirmed by chart review.

A comparison (control) group consisting of 150 self-identified black subjects with normal renal function was enrolled through our clinical hypertension research unit. Both normotensive and hypertensive controls were enrolled. To be designated a normotensive control, subjects had no history of hypertension, and had a measured BP <140/<90 mmHg during evaluation. If hypertensive, controls reported a history of essential hypertension and displayed a systolic BP >140 mmHg or diastolic BP >90 mmHg, or be on antihypertensive therapy (or both). All control subjects reported no history of renal disease, confirmed by a serum creatinine level <1.5 mg/dl. In controls, glomerular filtration was estimated by a standard algorithm, taking into account serum creatinine, age, sex, and ethnicity.72 To promote homogeneity in the samples (case and control), ethnicity was determined by self-identification of the subject, as well as reported ethnicity of both parents and all 4 grandparents; subjects of mixed ethnicity, were excluded.78

Genotyping
A sample of EDTA-anticoagulated whole blood was obtained from all participants and stored at ~70 °C before DNA extraction and SNP genotyping. A total of 14 candidate SNPs were genotyped at CHGA and are presented in Table 2. The SNPs that showed a significant association in the initial study population were subsequently genotyped in the replication population. SNPs were either discovered through our own resequencing efforts,73 or selected from the published literature79 or public dbSNP databases (http://www.ncbi.nlm.nih.gov/SNP). SNP genotypes were established by bp extension and mass spectrometry on the extension product 2-stage base-extension assays.75,80 During stage 1, PCR primers flanking the polymorphism were used to amplify the target region from 5 to 15 ng of genomic DNA. In stage 2, an oligonucleotide primer flanking the variant was annealed to the amplified template and extended across the variant base. In the Sequenom system, the mass of the extension product (wild-type versus variant) was scored by MALDI-TOF mass spectrometry (low mass allele versus high mass allele); in the pyrosequencing system,81 the incorporation of wild-type versus variant alleles was monitored by luminescence. Candidate CHGA SNPs selected for study had minor allele frequencies of approximately ≥5% and passed genotyping QC. HWE was assessed after adjustment for multiple comparisons (for 14 SNPs, P < 0.004 required).

Statistical Analyses
Allele and genotype associations
Analyses were performed using the Statistical Analysis System statistical package, version 9.1 (SAS Institute; Cary, NC), Statistical Package for the Social Sciences (SPSS, version 11.0; Chicago, IL), SNPEM82 program (http://polymorphism.scripps.edu/snpem), and Arlequin19 version, 2.001 population genetics software (http://www.ubine.ch/arlequin). Differences in the distribution of CHGA genotypes and other covariates between ESRD cases and controls were assessed with a Wald χ² statistic (2 df test) implemented in the PROC LOGISTIC routine in SAS. A P value of <0.05 was considered significant. Frequencies of individual SNP alleles and haplotypes were also compared between HT-ESRD cases and controls using the EM83-based program SNPEM.82 ORs were used to report the direction and strength of significant associations, and Wald 95% confidence intervals were calculated for homozygous variant (minor allele) and heterozygous genotypes, with the homozygous wild-type (major allele) genotype set as the “reference” for each SNP. In instances where the NCBI variant allele was the most common allele in our sample, the reference category was set at as the high frequency variant category. The impact of potential confounders was considered, including age, sex, and type 2 diabetes. Additionally, potential interactions among SNP genotypes (or haplotypes) and covariates on ESRD were evaluated by introduction of an interaction term into the logistic model, using cutoff values of P < 0.10.

Haplotype associations
Analyses of haplotypes were performed through 2 separate but complementary methods. Comparison of haplotype frequencies between ESRD cases and controls was performed using the SNPEM program.82 SNPEM estimates haplotype frequencies for each group using the EM algorithm, taking into account the probability of all possible haplotype pairs, and calculates a likelihood statistic to compare haplotype frequencies between 2 groups (cases versus controls) and a permutation test to determine significance in the face of multiple comparisons (set at 10,000 permutations). SNPEM was used to perform a “sliding window” analysis to identify associated haplotype lengths (from 1 to 5 SNPs) within the locus.84 Potential shortcomings of this method include limitation of analysis to dichotomous traits; focusing on the chromosome, rather than the individual, as the unit of analysis; no measure of direction or magnitude of a genetic effect (other than P value); lack of adjustment for potential confounding covariates; and lack of testing for interactions (gene × gene, or gene × environment).
The second approach used haplotype assignment to individuals. Haplotype frequencies were determined in additional ethnic groups from our research activities. Additional genetic samples were collected from 131 white (European ancestry) and 40 Hispanic (Mexican American) individuals. Ethnicity was established by self-identification of the individual, as well as both parents and all 4 grandparents of that individual. All individuals in the white sample had normal renal function (serum creatinine ≤1.5 mg/dl). A \( \chi^2 \) was calculated for comparison of haplotype frequencies between nondisease populations.

Biochemical (CHGA) Phenotyping
To assess the potential impact of CHGA polymorphisms on an “intermediate” phenotype \( ^{16} \) of chromogranin gene expression in vivo, plasma levels of the CHGA-derived peptide fragment cestatin (CHGA \( ^{361} \text{to} ^{372} \); epitope: CHGA \( ^{361} \text{to} ^{372} ; A \text{YGR} \text{GPGQL} \text{372} \) were measured in EDTA-anticoagulated plasma in a subset of cases and controls genotyped at the SNPs of interest. Previous studies by our group and others have indicated that renal failure is associated with retention of chromogranin proteins within plasma, likely the result of altered metabolism and/or excretion. \( ^{20,55,88} \) Control subjects had documented unimpaired renal function (serum creatinine <1.5 mg/dl) to avoid the possible effects of renal failure on plasma peptide levels. Peptides were measured by radioimmunoassay of EDTA-anticoagulated plasma as described previously by Stridsberg \( ^{89} \) and by our group. \( ^{90,91} \) [\( ^{125} \)I]-radiolabeling of the peptide was enabled by the endogenous Tyr \( ^{363} \) residue. Polyclonal rabbit antisera were developed to the synthetic CHGA region CHGA \( ^{361} \text{to} ^{372} \). The intra-assay coefficient of variation was 3.5%, and the interassay coefficient was 4.7%. The utilization of CHGA region-specific radioimmunoassays has been described in detail previously. \( ^{92,93} \) Associations of plasma peptide levels with SNP genotypes and haplotypes were tested as described in the statistical analysis section below.

Function of a CHGA 3‘-UTR Variant
The 407 bp CHGA 3‘-UTR was subcloned into the XbaI site of the pGL3-Promoter vector (Promega, Madison, WI), just downstream (3‘) of the luciferase open reading frame, with transcription under the control of the SV40 early promoter. Single nucleotide variant C +87T was recreated by site-directed mutagenesis (QuikChange, Stratagene). Inserts were sequence-verified (for both orientation and the correct point mutation) before use. PC12 rat pheochromocytoma cells were transfected with 1 µg of each construct, as well as 10 ng of the Renilla luciferase expression plasmid pRL-TK (Promega), as an internal transfection efficiency control in each well, by the cationic liposome method (Superfect, Qiagen, Valencia, CA). Firefly and Renilla luciferase activities in the cell lysates were measured 48 h after transfection, and the results were expressed as the ratio of firefly/Renilla luciferase activity (“Stop &Glow,” Promega). Each experiment was repeated a minimum of 3 times.

Replication Case/Control Population: North Carolina Subjects
An additional study sample of cases and controls was ascertained for replication of findings and is described in detail elsewhere. \( ^{43} \) In brief, the replication sample consisted of 301 hypertension-associated ESRD cases and 305 controls, ascertained from North Carolina using a protocol approved by the Wake Forest University School of Medicine Institutional Review Board. ESRD cases were ascertained from dialysis facilities after a clinical diagnosis of hypertension-associated ESRD and absence of diabetes, based on diagnosis by treating physi-
cian and review of medical charts. Renal biopsy was performed on 10 of the cases, revealing 5 subjects with FSGS lesions and 5 with arterio-
lar nephrosclerosis. Of the remaining cases, 10 subjects also had evi-
dence of coexisting systemic disease that may predispose to glomeru-
lonephritis (systemic lupus erythematosus or HIV), but in each case the primary clinical diagnosis was hypertensive nephropathy. Healthy controls were sampled from the general black population. Cases and controls differed in age (P < 0.001), cases slightly older at 54.6 ± 0.75 yr and 50.2 ± 0.58 for controls. Sex did not differ significantly be-
tween groups (P = 0.862); 56% of cases and 55.3% of controls were male. BMI was lower (P < 0.001) in cases (26.5 ± 0.42 kg/m²) than controls (29.4 ± 0.39 kg/m²), as expected for ESRD. Controls re-
ported normal kidney function and denied a family history of renal disease. These study subjects were born in the southeastern United States and resided in North Carolina.

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DISCLOSURES

None.

REFERENCES

1. Taupenot L, Harper KL, O’Connor DT: The chromogranin-secretogra-
2. Takypuddin MA, Parme RJ, Kailasam MT, Cervenka JH, Kennedy B, Ziegler MG, Lin MC, Li J, Grim CE, Wright FA, O’Connor DT: Chro-
morganin A in human hypertension. Influence of heredity. Hyperten-
sion 26: 213–220, 1995
3. O’Connor DT, Takypuddin MA, Printz MP, Dinh TQ, Barbosa JA, Rozansky DJ, Mahata SK, Wu H, Kennedy BP, Ziegler MG, Wright FA, Schlager G, Parme RJ: Catecholamine storage vesicle protein expres-
DT, Hamilton BA: Both rare and common polymorphisms contribute functional variation at CHGA, a regulator of catecholamine physiol-
9. O’Connor DT, Kailasam MT, Kennedy BP, Ziegler MG, Yanaihara N, Parmer RJ: Early decline in the catecholamine release-inhibitory pep-
11. Videen JS, Mezger MS, Chang YM, O’Connor DT: Calcium and cate-
3073, 1992
16. O’Connor DT, Insel PA, Ziegler MG, Hook YV, Smith DW, Hamilton
18. Pritchard JK, Rosenberg NA: Use of unlinked genetic markers to
25. Whittle JC, Whelton PK, Seidler AJ, Klag MJ: Does racial variation in risk factors explain black-white differences in the incidence of hypo-
27. Tarver-Carr ME, Powe NR, Eberhardt MS, LaVeist TA, Kington RS, Coresh J, Brancati FL: Excess risk of chronic kidney disease among


See related editorial, “Naturally Too Sympathetic to a Bad Diet?,” on pages 420–422.