Association Analysis of the *Cubilin* (*CUBN*) and *Megalin* (*LRP2*) Genes with ESRD in African Americans

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Abstract

Background and objectives Genetic variation in the *cubilin* (*CUBN*) gene is associated with albuminuria and CKD. Common and rare coding variants in *CUBN* and the gene encoding its transport partner *megalin* (*LRP2*) were assessed for association with ESRD in blacks.

Design, setting, participants, & measurements Sixty-six *CUBN* and *LRP2* single–nucleotide polymorphisms (SNPs) were selected and analyzed in this multistage study. Exome sequencing data from 529 blacks with type 2 diabetes (T2D) –associated ESRD and 535 controls lacking T2D or nephropathy (the Type 2 Diabetes Genes [T2D-GENES] Consortium) were first evaluated, focusing on coding variants in *CUBN* and *LRP2*; 15 potentially associated SNPs identified from the T2D-GENES Consortium as well as 51 other selected SNPs were then assessed in an independent T2D-ESRD sample set of blacks (the Affymetrix Axiom Biobank Genotyping Array [AXIOM]; 2041 patients with T2D-ESRD, 627 patients with T2D without nephropathy, and 1140 nondiabetic, non–nephropathy controls). A meta-analysis combining the T2D-GENES Consortium and the AXIOM data was performed for 18 overlapping SNPs. Additionally, all 66 SNPs were genotyped in the Wake Forest School of Medicine samples of blacks with nondiabetic ESRD (885 patients with nondiabetic ESRD and 721 controls). Association testing with ESRD was performed in models including age, sex, African ancestry proportion, and *apolipoprotein L1* gene renal-risk variants.

Results *CUBN* SNP rs1801239 (I2984V), previously associated with albuminuria, was significantly associated with T2D-ESRD in blacks (the T2D-GENES Consortium and the AXIOM meta-analysis, P=0.03; odds ratio, 1.31; 95% confidence interval, 1.03 to 1.67; minor allele frequency =0.028). A novel *LRP2* missense variant, rs17848169 (N2632D), was also significantly protective from T2D-ESRD (the T2D-GENES Consortium and the AXIOM, $P<0.002$; odds ratio, 0.47; 95% confidence interval, 0.29 to 0.75; meta–analysis minor allele frequency =0.007). Neither SNP was associated with T2D when contrasting patients with T2D with controls lacking diabetes. *CUBN* and *LRP2* SNPs were not associated with nondiabetic etiologies of ESRD.

Conclusions Evidence for genetic association exists between a cubilin and a rare megalin variant with diabetes-associated ESRD in populations with recent African ancestry.

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Introduction

Increasing evidence supports that inherited factors make major contributions to ESRD susceptibility (1,2). This is particularly true in blacks who have high rates of ESRD with marked familial aggregation of nephropathy (3). The incidence rate of ESRD in blacks is 3.3-fold higher than that in whites (4). *Apolipoprotein L1* (*APOL1*) gene renal-risk alleles associate with approximately 70% of nondiabetic ESRD in blacks (5–7); however, they do not explain the excess risk for type 2 diabetes (T2D) –associated ESRD (8). Additional genetic loci likely contribute to this risk (9–11).

The *cubilin* (*CUBN*) gene was identified as a novel locus for albuminuria from a genome–wide association study–based meta–analysis (12). The missense single–nucleotide polymorphism (SNP) rs1801239 (I2984V) in *CUBN* was associated with elevated urine albumin-to-creatinine ratio in individuals of European and recent African ancestry. Another intronic *CUBN* variant, rs10795433, in moderate linkage disequilibrium with rs1801239 (r^2=0.54), was associated with the urine albumin-to-creatinine ratio in patients with diabetes (13). Cubilin forms a functional receptor complex with megalin (encoded by the *megalin* [*LRP2*] gene) in the proximal tubule to reabsorb filtered urinary albumin (14,15). Megalin is important in facilitating the internalization of the cubilin-albumin complex (16).

Because albuminuria is an important risk factor for progression of kidney disease, we hypothesized that variation in *CUBN* and *LRP2* could contribute to nephropathy susceptibility. A recent analysis in European kidney transplant donors and recipients showed that *CUBN* SNP rs7918972 was significantly

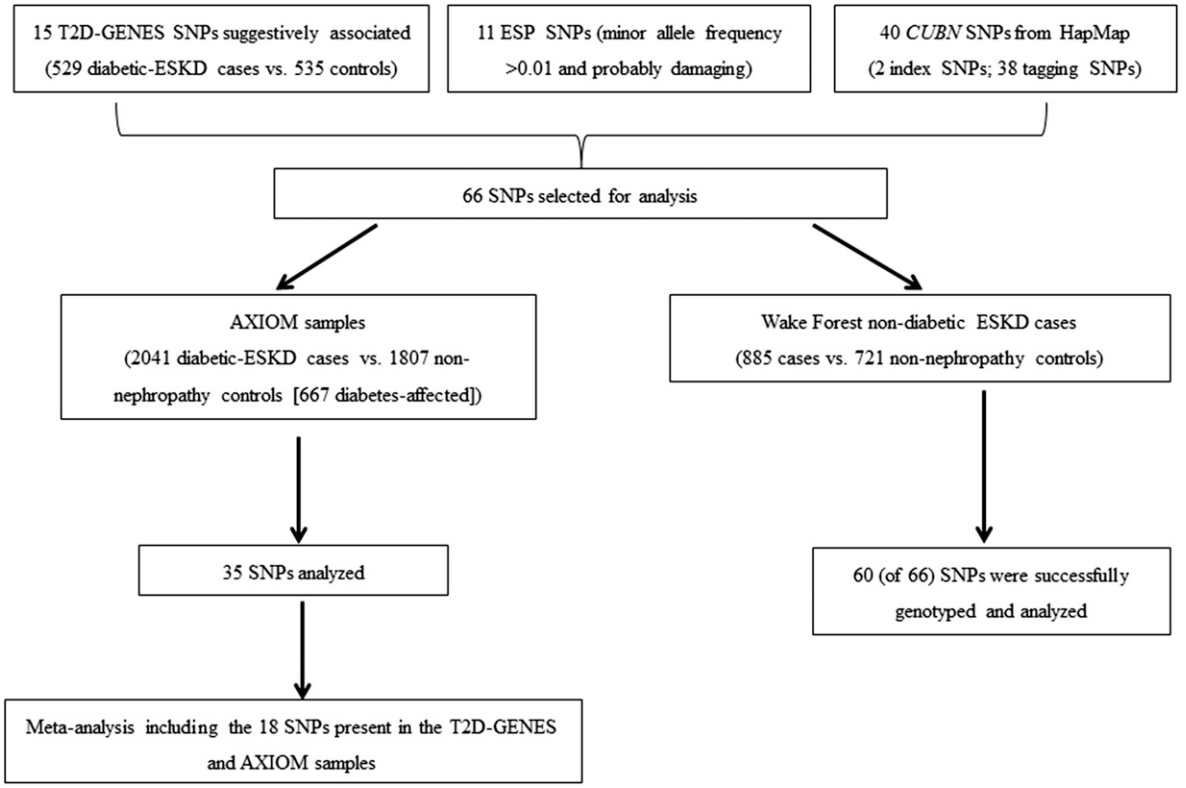

Figure 1. | *Cubilin* (*CUBN*) gene and *megalin* gene single–nucleotide polymorphism (SNP) selection and genetic association analysis workflow. AXIOM, Affymetrix Axiom Biobank Genotyping Array; ESKD, ESRD; ESP, Exome Sequencing Project; T2D-GENES, Type 2 Diabetes Genes Consortium.

associated with risk for ESRD (17). However, the role of *CUBN* genetic variation in ESRD susceptibility among blacks remains unknown. We analyzed next generation exome sequencing (NGES) data to survey the *CUBN* and *LRP2* genes to determine whether variation in these genes affected risk for ESRD in blacks beyond reducing renal proximal tubule reabsorption of albumin.

Materials and Methods

Study Participants

This study was approved by the Institutional Review Board at the Wake Forest School of Medicine; all participants provided written informed consent. Detailed recruitment and sample collection procedures have been reported (11). T2D was diagnosed in those whose illness developed after 25 years of age and who lacked diabetic ketoacidosis or receipt of insulin alone since diagnosis. ESRD was attributed to T2D with ≥5 years diabetes duration before the start of RRT in the absence of other causes of nephropathy. Patients with nondiabetic ESRD had nephropathy caused by chronic glomerulosclerosis, FSGS, or HIV-associated nephropathy, attributed to hypertension, or because of unknown causes. Those with ESRD caused by urologic or surgical causes, polycystic kidney disease, IgA nephropathy, or membranous or membranoproliferative GN were excluded. Blacks with T2D lacking nephropathy were receiving insulin and/or oral hypoglycemic agents, had a hemoglobin $A1_C$ ≥6.5% or a fasting plasma glucose >126 mg/dl, and had a serum creatinine concentration ≤1.5 mg/dl (men) or ≤1.3 mg/dl (women). Unrelated blacks without diabetes or kidney disease (eGFR≥60 ml/min per 1.73 m^2 and urine albumin-to-creatinine ratio <30 mg/g) were recruited as controls (described as non-T2D, non-nephropathy, or healthy controls). Genomic DNA was extracted with the PureGene System (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. Ethnicity was self-reported and confirmed using African ancestry proportions calculated with 70 ancestry informative markers (18,19).

SNP Selection, Genotyping, and Quality Control

In total, 66 SNPs in *CUBN* (n=50) and *LRP2* (n=16) were selected to evaluate potential ESRD associations in blacks. Figure 1 displays the study design, and Supplemental Table 1 displays the SNPs and their sources of selection. Fifteen SNPs were selected from samples provided by the Wake Forest School of Medicine to the Type 2 Diabetes Genes [T2D-GENES] Consortium Exome Sequencing Project (https://t2d-genessph.umich.edu/), which included 529 blacks with T2D-ESRD and 535 nondiabetic, non–nephropathy controls. The T2D-GENES Consortium genotyping and quality control methods have been reported (10). Among the total of 407 *CUBN* SNPs and 581 *LRP2* SNPs identified in the T2D-GENES Consortium, we selected four *CUBN* and 11 *LRP2* SNPs for analysis on the basis of association results with T2D-ESRD with P values

≤0.10. Eleven additional coding variants were selected from the Exome Sequencing Project data based on minor allele frequencies (MAFs) in blacks >0.01 and probably damaging effects using Polyphen2 prediction; six were *CUBN* SNPs, and five were in *LRP2*. From the literature, two *CUBN* SNPs previously associated with kidney disease phenotypes, rs1801239 and rs7918972 (hereafter referred to as index SNPs), were included (12,17). In addition, 38 haplotype-tagging SNPs with MAF>5% across the HapMap region of linkage disequilibrium (chromosome 10: 16897609—17012313) and inclusive of the index variants were selected. Because the index SNPs were primarily identified in European populations, the borders of their linkage disequilibrium blocks in Utah residents with Northern and Western European ancestry were determined in Haploview, and tagging SNPs were selected between these regions in Yoruba in Ibadan, Nigeria to tag differential linkage disequilibrium block structures in populations with recent African ancestry.

Association analyses were performed in 2041 independent blacks with T2D-ESRD and 1807 independent non–nephropathy controls (667 controls with T2D lacking nephropathy and 1140 controls without T2D) who had been genotyped on the Affymetrix Axiom Biobank Genotyping Array (AXIOM) samples (Affymetrix, Santa Clara, CA; none of these individuals overlapped with those in the T2D-GENES Consortium). Detailed SNP information, genotyping methods, and the AXIOM quality control data are reported in Supplemental Material. Of the initial 66 SNPs selected in the discovery analysis, 35 were present in the AXIOM samples: 27 in *CUBN* and eight in *LRP2*. Of these, 10 *CUBN* SNPs and eight *LRP2* SNPs were present in both the T2D-GENES Consortium and the AXIOM data; these 18 SNPs were included in a meta-analysis from both datasets (Figure 1).

To assess associations in nondiabetic ESRD, 885 blacks with nondiabetic ESRD and 721 nondiabetic, non–nephropathy controls were genotyped for the 66 *CUBN* and *LRP2* SNPs. Genotyping was performed using the Sequenom MassArray System (Sequenom, San Diego, CA). PCR primers were designed using MassARRAY Assay Design 3.1 (Sequenom), and genotypes were analyzed using MassARRAY Typer (Sequenom). Of all 66 SNPs, 60 were successfully genotyped, had call rates >95%, and met quality control standards on the basis of 100% concordance with blind duplicates and Hardy–Weinberg Equilibrium *P* value =0.001. Two *APOL1* G1 nephropathy risk SNPs (rs73885319 and rs60910145) and an insertion/deletion for the *APOL1* G2 risk allele (rs71785313) were genotyped in all samples on the same platform.

Statistical Analyses

For data from the AXIOM custom array, a linear mixed model–based method was used to correct for population structure and cryptic relatedness (20). This resulted in an inflation factor <1.002 computed from 315,610 high–quality autosomal SNPs with an MAF>0.05. Because all samples in the T2D-GENES Consortium and the nondiabetic ESRD datasets from the Wake Forest School of Medicine were from unrelated individuals, logistic regression was performed using PLINK for the NGES and directly genotyped data.

Single SNP association tests in all sets were computed using an additive genetic model. The fully adjusted model for association with ESRD included participant age, sex, African ancestry proportion, and recessive *APOL1* G1/G2 risk alleles. The adjusted model for association with T2D *per se* in the AXIOM samples (T2D only versus non–T2D, non–nephropathy controls) included participant age, sex, and African ancestry proportion (not *APOL1*). A corrected *P* value (*P*corr) was calculated for SNP associations by adjusting for the number of SNPs tested in each study. *P*corr values <0.05 were considered statistically significant.

Meta-Analyses

Summary statistics from 18 overlapping SNPs in the T2D-GENES Consortium and the AXIOM samples (10 *CUBN* and eight *LRP2*) were combined using the fixed effects meta–analysis method implemented in METAL (21). Of these, the *CUBN* index SNP rs1801239 was included, but the second *CUBN* index SNP rs7918972 was not present in the T2D-GENES Consortium and could not be meta-analyzed.

Results

Demographic data from patients and controls in the T2D-GENES Consortium, the AXIOM data, and the Wake Forest School of Medicine nondiabetic ESRD samples are summarized in Table 1. Participant characteristics were generally similar among patients with T2D-ESRD; however, age at onset of T2D was younger in patients with T2D-ESRD than in individuals with T2D lacking nephropathy. Patients with T2D-ESRD had older ages at enrollment compared with individuals with T2D lacking nephropathy and nondiabetic, non–nephropathy controls. Mean ages at enrollment and body mass index were lower in patients with nondiabetic ESRD than in those in the T2D-ESRD group, whereas the duration of ESRD was longer.

The initial evaluation of 15 SNPs in the T2D-GENES Consortium in 529 blacks with T2D-ESRD versus 535 nondiabetic, non–nephropathy controls identified six variants (three in *CUBN* and three in *LRP2*) nominally associated with T2D-ESRD ($P<0.05$ in additive models adjusted for age, sex, African ancestry proportion, and *APOL1*). Among these, common synonymous *CUBN* variant rs1873469 showed the strongest association: MAF=26% in patients with T2D-ESRD and MAF=33% in controls ($P=0.003$; odds ratio [OR], 0.72; 95% confidence interval [95% CI], 0.58 to 0.90). In addition, two low–frequency protective missense *LRP2* variants were identified: rs17848169 (N2632D; $P=0.01$; OR, 0.19; 95% CI, 0.05 to 0.68) and rs34291900 (G669D; $P=0.02$; OR, 0.17; 95% CI, 0.04 to 0.71); MAFs were 0.39% and 0.29% in patients and 1.2% and 1.1% in controls, respectively. The other nine SNPs showed trends toward association ($P<0.10$) (Supplemental Table 2).

Thirty-five of the 66 SNPs selected for analysis could be surveyed in the AXIOM replication sample consisting of 2041 patients with T2D-ESRD, 667 individuals with T2D lacking nephropathy, and 1140 nondiabetic, non–nephropathy controls (Supplemental Table 1). In fully adjusted models,

Table 1. Demographic and clinical characteristics of study samples

Sample Source	T2D-GENES Consortium Samples		AXIOM Samples			Non–T2D-ESRD Samples	
	Patients with T2D-ESRD	Healthy Controls	Patients with T2D-ESRD	T2D Only (No Kidney Disease)	Healthy Controls	Patients with Non–T2D-ESRD	Healthy Controls
No.	529	535	2041	667	1140	885	721
Women, %	61.2	57.3	57.1	64.5	51.7	44.1	48.8
Age at recruitment, yr	61.6±10.5	49.0±11.9	61.4±10.8	55.7±11.6	46.5±12.0	55.3±14.4	45.9±12.2
Age at T2D, yr	47.3±9.9	NA	38.6±12.7	46.2±12.3	NA	NA	NA
Duration of T2D before ESRD, yr	12 (6, 19)	NA	19 (13, 26)	NA	NA	NA	NA
Duration of ESRD, yr	3.77±3.8	NA	3.64±3.51	NA	NA	6.26±6.15	NA
Blood glucose, mg/dl	NA	88.8±13.1	NA	NA	96.5±20.9	NA	89.3±13.5
Serum creatinine, mg/dl	NA	0.99±0.25	NA	0.94±0.20	0.97±0.20	NA	0.97±0.19
BMI at recruitment, kg/m^2	29.7±7.0	30.0±7.0	30.8±7.1	33.1±7.8	29.7±7.4	27.1±7.0	29.2±7.3
African ancestry, %	80.13±11.44	78.02±11.25	83.87±11.11	82.01±12.58	82.34±10.90	84.35±11.79	82.29±10.75

Categorical data are expressed as percentages. Continuous data are presented as means±SDs. Durations of type 2 diabetes before ESRD are presented as medians (25th, 75th percentiles). T2D-GENES Consortium, Type 2 Diabetes Genes Consortium; AXIOM, Affymetrix Axiom Biobank Genotyping Array; T2D, type 2 diabetes; NA, not applicable; BMI, body mass index.

LRP2 SNP rs17848169 (P=0.02; OR, 0.54; 95% CI, 0.32 to 0.90) and *CUBN* index SNP rs1801239 (P=0.02; OR, 1.37; 95% CI, 1.06 to 1.78) were associated with T2D-ESRD (versus non-nephropathy controls). The *LRP2* SNP rs34291900 detected in the T2D-GENES Consortium data showed a weak trend toward association (P=0.18; OR, 0.67; 95% CI, 0.38 to 1.20) (Table 2). Other SNPs tested in the AXIOM samples, including the second *CUBN* index SNP rs7918972, were not associated with T2D-ESRD (Supplemental Table 1).

A meta-analysis considering 10 *CUBN* and eight *LRP2* SNPs genotyped in the T2D-GENES Consortium and the AXIOM samples was performed for association with T2D-ESRD (Table 2). *LRP2* SNP rs17848169 was significantly associated with T2D-ESRD (versus non-nephropathy controls) after correction for multiple testing with Pcorr=0.04 (P value = 0.002×18=0.04), and *LRP2* SNP rs34291900 was nominally associated (P=0.03); both variants showed the same directions of effect in each sample set. *CUBN* index SNP rs1801239 also replicated association in the meta-analysis with P=0.03 and the same direction of effect in each set. The meta-analysis was repeated by removing patients and controls felt likely to be at risk for nondiabetic ESRD on the basis of possession of two *APOL1* renal risk variants. Despite a smaller sample, results generally remained consistent (Supplemental Table 3).

Associations between *CUBN* and *LRP2* variants with nondiabetic ESRD were next assessed. The 66 selected SNPs were genotyped in 885 blacks with nondiabetic ESRD and 721 nondiabetic, non–nephropathy controls at the Wake Forest School of Medicine (independent from the T2D-GENES Consortium); 60 SNPs were successfully genotyped and met quality control standards for analysis. Among these, two SNPs in *LRP2* and four SNPs in *CUBN* were nominally associated with nondiabetic ESRD (versus non-nephropathy controls) in the fully adjusted model with P values of 0.02–0.05 under the additive model (Table 3). None remained significantly associated after correction for multiple comparisons (Pcorr>0.05). One *CUBN* index SNP rs7918972 trended toward significant association with non-T2D ESRD (P=0.06; OR, 1.25; 95% CI, 0.99 to 1.58), whereas the second *CUBN* index SNP rs1801239 was not associated (P=0.90; OR, 1.03; 95% CI, 0.61 to 1.74) (Supplemental Table 1).

To determine whether SNPs associated with T2D-ESRD reflected association with T2D *per se* or kidney disease, trait discrimination analyses were performed in the AXIOM samples. None of the associated variants were associated with T2D *per se* comparing patients with T2D lacking nephropathy with nondiabetic, non–nephropathy controls (for example, P=0.40 and P=0.78 for *LRP2* SNP rs17848169 and *CUBN* index SNP rs1801239, respectively) (Table 4). Furthermore, *CUBN* SNP rs1801239 was associated with nephropathy in patients with T2D-ESRD compared with those with T2D lacking nephropathy (P=0.04). These findings support risk or protective allele associations with nephropathy and do not support risk or protective allele associations with diabetes.

To assess the potential for synthetic association (association with one or more rare causal variants in long-range linkage disequilibrium (LD), where D' is approximately one, but r^2 is modest), we evaluated LD between rs1801239 and

Table 2. Association analysis between *cubilin* gene and *megalin* gene variants with type 2 diabetes and ESRD (additive, fully adjusted model)

Gene	SNP	Minor Allele	T2D-GENES Consortium Samples (Patients with T2D-ESRD Versus Healthy Controls)					AXIOM Samples (Patients with T2D-ESRD Versus T2D Only and Healthy Controls)					Meta-Analysis			
			N Patients/ Controls	MAF Patients/ Controls	*P* Value	OR	95% CI	*N* Patients/ Controls	MAF Patients/ Controls	*P* Value	OR	95% CI	*P* Value	OR	95% CI	Direction
LRP2	rs17848169	C	512/502	0.004/0.012	0.01	0.19	0.05 to 0.68	1997/1579	0.005/0.01	0.02	0.54	0.32 to 0.90	0.002	0.47	0.29 to 0.75	− −
LRP2	rs34291900	T	511/501	0.003/0.011	0.02	0.17	0.04 to 0.71	1997/1584	0.005/0.006	0.18	0.67	0.38 to 1.20	0.03	0.56	0.33 to 0.95	− −
LRP2	rs4667591	G	511/502	0.24/0.23	0.74	1.04	0.81 to 1.34	1991/1582	0.20/0.22	0.17	0.93	0.83 to 1.03	0.26	0.94	0.85 to 1.04	+ −
LRP2	rs144081819	T	512/502	0.036/0.033	0.63	1.15	0.65 to 2.01	1997/1584	0.036/0.038	0.95	0.99	0.79 to 1.24	0.91	1.01	0.82 to 1.25	+ −
LRP2	rs143367996	A	512/502	0.022/0.032	0.30	1.41	0.74 to 2.70	1995/1584	0.023/0.023	0.39	1.13	0.85 to 1.50	0.23	1.17	0.91 to 1.52	+ +
LRP2	rs61995913	C	512/502	0.046/0.047	0.59	0.87	0.53 to 1.43	1995/1582	0.043/0.046	0.37	0.91	0.74 to 1.12	0.30	0.90	0.75 to 1.09	− −
LRP2	rs116456291	T	512/502	0.036/0.030	0.90	1.04	0.58 to 1.88	1993/1580	0.037/0.037	0.81	1.03	0.82 to 1.29	0.79	1.03	0.83 to 1.27	+ +
LRP2	rs144864408	T	512/502	0.001/0.002	0.09	0.09	0.01 to 1.47	1997/1584	0.001/0.001	0.74	0.73	0.12 to 4.48	0.23	0.39	0.09 to 1.80	− −
CUBN	rs1873469	A	512/502	0.26/0.33	0.003	0.72	0.58 to 0.90	1994/1581	0.28/0.29	0.66	0.98	0.89 to 1.07	0.12	0.93	0.86 to 1.02	− −
CUBN	rs144360241	C	512/502	0.002/0.001	0.02	16.1	1.51 to 172.2	1998/1584	0.001/0.002	0.88	0.92	0.28 to 2.97	0.31	1.61	0.56 to 4.64	+ −
CUBN	rs148100631	C	512/502	0.001/0.005	0.04	0.07	0.01 to 0.88	1997/1583	0.001/0.001	0.63	1.48	0.29 to 7.48	0.47	0.60	0.16 to 2.34	− +
CUBN	rs1801239	C	512/502	0.029/0.029	0.95	1.02	0.55 to 1.91	1993/1582	0.031/0.024	0.02	1.37	1.06 to 1.78	0.03	1.31	1.03 to 1.67	+ +
CUBN	rs74431427	A	512/502	0.023/0.018	0.64	1.18	0.59 to 2.33	1989/1581	0.020/0.019	0.88	1.02	0.76 to 1.38	0.75	1.05	0.80 to 1.38	+ +
CUBN	rs111265129	C	512/502	0.038/0.041	0.95	0.98	0.58 to 1.66	1996/1579	0.043/0.041	0.46	1.08	0.88 to 1.34	0.51	1.07	0.88 to 1.30	− +
CUBN	rs780807	A	511/502	0.31/0.29	0.52	1.08	0.86 to 1.35	1988/1582	0.30/0.30	0.70	1.02	0.93 to 1.12	0.55	1.03	0.94 to 1.12	+ +
CUBN	rs2271460	C	512/502	0.016/0.015	0.41	1.43	0.61 to 3.34	1993/1578	0.01/0.007	0.37	1.24	0.78 to 1.96	0.24	1.28	0.85 to 1.92	+ +
CUBN	rs2271462	T	512/502	0.14/0.13	0.63	1.08	0.80 to 1.44	1991/1580	0.14/0.14	0.90	1.01	0.89 to 1.14	0.76	1.02	0.91 to 1.14	+ +
CUBN	rs12259370	T	512/502	0.071/0.061	0.42	1.19	0.78 to 1.83	1955/1544	0.068/0.067	0.72	1.03	0.87 to 1.22	0.53	1.05	0.90 to 1.23	+ +

Sample sizes may be smaller than those displayed in Table 1 because of missing *apolipoprotein L1* genotypes. Direction reflects effect in T2D-GENES and AXIOM, respectfully. SNP, single-nucleotide polymorphism; T2D-GENES Consortium, Type 2 Diabetes Genes Consortium; T2D, type 2 diabetes; AXIOM, Affymetrix Axiom Biobank Genotyping Array; MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval; LRP2, *megalin*; *CUBN*, *cubilin*.

lower–frequency *CUBN* variants (rs74431427, rs148100631, rs2271460, and rs144360241) in both the 1000 Genomes Project and our own data (22–24). No evidence of LD (D' or r^2) was observed, in part because of the low frequency of rs1801239 (MAF=0.028) and the small number of rare variants. A conditional analysis was performed with rs1801239 incorporated in the statistical model, and no major differences in significance were detected. In addition, rs1801239 was tested for association with T2D-ESRD by adjusting for each of the four rarer SNPs, and it remained significant, suggesting that the association was not caused by these rarer SNPs (data not shown). Therefore, there was no evidence supporting synthetic association for rs1801239.

Discussion

This study investigated genetic association between *CUBN* and *LRP2* gene variants with diabetic and nondiabetic etiologies of ESRD in blacks from the T2D-GENES Consortium, independent AXIOM array–based samples, and additional patients with nondiabetic etiologies of ESRD and controls from the Wake Forest School of Medicine. Because NGES is a powerful technology that allows one to comprehensively identify and test genetic variations in coding sequences of genes for disease association, we used NGES data (the T2D-GENES Consortium) to survey *CUBN*/*LRP2* genes as a first step and identified 15 SNPs suggestively associated with T2D-ESRD ($P<0.10$). Considering that most of the SNPs from the T2D-GENES Consortium were rare variants, 11 additional coding variants from the Exome Sequencing Project were selected as a supplement on the basis of their allele enrichment (MAF>0.01) and *in silico* prediction. To explore the role of common variants in disease susceptibility, 38 tagging SNPs (MAF>0.05) across the HapMap region of linkage disequilibrium with the two index variants were also selected. Thus, this study provided a locus–wide association analysis instead of simple replication for the identified variants.

CUBN index SNP rs1801239 replicated risk for association with T2D-ESRD; this variant was previously associated with albuminuria. A novel *LRP2* missense variant rs17848169 (N2632D) was also found to be protective from T2D-ESRD. In contrast, no *CUBN* or *LRP2* SNPs were significantly associated with nondiabetic ESRD in blacks.

Trait discrimination analyses supported that the associated *CUBN* and *LRP2* variants play roles in nephropathy susceptibility in blacks and not diabetes *per se*. These results suggest an important role of the cubilin-megalin complex in development of progressive diabetic kidney disease in populations with recent African ancestry beyond albuminuria caused by reduced proximal tubule reabsorption. Recent evidence supports the importance of endocytotic reabsorption of filtered albumin in health, because glomerular filtration of albumin seems to be greater than initially appreciated (25–27). Albumin reabsorption occurs in proximal tubule cells, where the cubilin-megalin receptor complex is expressed on the apical brush border and plays a critical role in receptor-mediated endocytosis (16,28,29). Although albuminuria often leads to nephropathy progression, roles of the *CUBN* and *LRP2* genes in T2D-ESRD in blacks were not previously studied.

Several *CUBN* and cubilin–associated amnion–less gene variants cause Imerslund Grasbeck syndrome, a rare autosomal recessive disease characterized by megaloblastic anemia, recurrent infections, failure to thrive, and proteinuria (30,31). However, the common *CUBN* variants rs1801239 and rs7918972 were only recently found to associate with albuminuria and nephropathy. In initial studies, rs1801239 was associated with albuminuria and not associated with eGFR or ESRD (12). In this report, rs1801239 was associated with T2D-ESRD with the same direction of effect as for albuminuria, indicating that the C allele carries risk for T2D-ESRD in blacks. We did not replicate association with the rs7918972 index SNP in *CUBN* in these black patients; this SNP was previously associated with ESRD in a European sample (17). Additional studies with large sample sizes and in different ethnic groups are necessary to clarify the correlations between genetic variation in *CUBN* and *LRP2* and diabetic ESRD.

An *LRP2* missense variant, rs17848169 (N2632D), which is protective for T2D-ESRD in blacks, was identified for the first time. Although present at low frequency, concordance for MAFs in patients and controls was present in independent T2D-GENES Consortium (<0.004 patients and 0.012 controls) and AXIOM array (<0.005 patients and <0.01 controls) samples. The same trend was observed in the nondiabetic ESRD samples (<0.008 patients and 0.011

Table 3. Strongest genetic associations in patients with nondiabetic ESRD (additive, fully adjusted model)

Gene	SNP	Minor Allele	*N* Patients/ Controls	MAF Patients/ Controls	*P* Value	OR	95% CI
LRP2	rs11898106	G	868/713	0.30/0.26	0.04	1.21	1.01 to 1.46
LRP2	rs78750385	G	885/720	0.11/0.09	0.03	1.34	1.03 to 0.76
CUBN	rs3808925	C	873/715	0.17/0.15	0.02	1.30	1.05 to 1.62
CUBN	rs2796838	T	883/718	0.40/0.43	0.05	0.85	0.72 to 1.00
CUBN	rs11254267	A	882/717	0.092/0.07	0.02	1.43	1.06 to 1.93
CUBN	rs7921129	T	876/714	0.32/0.35	0.05	0.84	0.71 to 1.00

SNP, single-nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval; LRP2, *megalin*; *CUBN*, *cubilin*.

Table 4. Trait discrimination analysis of type 2 diabetes– and ESRD–associated single–nucleotide polymorphisms in the Affymetrix Axiom Biobank Genotyping Array samples

Gene	SNP	Minor Allele	Subgroups	*N* Patients/ Controls	MAF Patients/ Controls	*P* Value	OR	95% CI
LRP2	rs17848169	C	Patients with T2D-ESRD versus healthy controls	1997/976	0.005/0.010	0.02	0.54	0.32 to 0.90
LRP2	rs17848169	C	Patients with T2D-ESRD versus T2D only controls	1997/603	0.005/0.008	0.16	0.58	0.27 to 1.25
LRP2	rs17848169	C	Patients with T2D only versus healthy controls[a]	662/1140	0.008/0.011	0.40	0.75	0.39 to 1.45
CUBN	rs1801239	C	Patients with T2D-ESRD versus healthy controls	1993/975	0.031/0.025	0.24	1.18	0.90 to 1.55
CUBN	rs1801239	C	Patients with T2D-ESRD versus T2D only controls	1993/607	0.031/0.022	0.04	1.57	1.03 to 2.40
CUBN	rs1801239	C	Patients with T2D only versus healthy controls[a]	666/1138	0.023/0.024	0.78	0.94	0.63 to 1.42

SNP, single-nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval; LRP2, *megalin*; T2D, type 2 diabetes; *CUBN*, *cubilin*.
[a]Adjusted for age, sex, and African ancestry proportion; other comparisons included *apolipoprotein L1* adjustment. Sample numbers varied because of missing *apolipoprotein L1* genotypes.

controls; P=0.14). Therefore, the association with T2D-ESRD seems credible.

Cubilin is a 460-kD multipurpose receptor that can bind to a range of ligands, including intrinsic factor/vitamin B12, transferrin, hemoglobin, HDL cholesterol, apolipoprotein A1, megalin, and albumin (32). As a peripheral membrane protein, cubilin contains a 110–residue N–terminal domain, an eight EGF–like repeat domain, and 27 CUB domains (33). Because cubilin lacks transmembrane and cytoplasmic domains, internalization of albumin is thought to be mediated *via* its interaction with megalin, a 600-kD transmembrane protein in the LDL receptor family (34).

LRP2 variant rs17848169 (N2632D) is located in the extracellular LDL receptor repeat segments of megalin, where ligand binding sites exist (35). Although megalin can bind albumin (36), animal studies reveal that the major role of megalin in albumin reabsorption is to drive internalization of cubilin-albumin complexes (16). *CUBN* index SNP rs1801239 (I2984V) is located in the 22nd CUB domain of cubilin, one of the three fragments that bind to megalin (37). Therefore, I2984V and N2632D may interfere with the interaction between cubilin and megalin to alter albumin reabsorption. Functional studies will be required to clarify potential mechanisms.

A recent report revealed that the *CUBN* rs1801239 risk variant appeared on a derived low–frequency European haplotype consisting of 19 SNPs and that the frequency of each SNP differed significantly in Africans (and was absent in West Africans). This European haplotype may represent a region of extended linkage disequilibrium, conceivably reflecting the effect of positive selective pressure under nutritional influences during evolution (38). On the basis of results in admixed blacks, we observed that variation at rs1801239 was slightly higher than that in the 1000 Genomes Project or the HapMap Yoruba data (0.024–0.031 in our AXIOM dataset versus 0.018 in public datasets). Among the 19 *CUBN* variants assessed by Tzur *et al.* (38), only rs1801239 and rs62619939 were available in this study on the basis of the differential selection strategy.

Therefore, additional association studies using variants in continental African cohorts lacking this European origin haplotype will be important to clarify the causative variant.

It is noteworthy that none of the *CUBN* or *LRP2* SNPs were associated with nondiabetic etiologies of ESRD in this sample of blacks. Although the nondiabetic ESRD sample was smaller than the T2D-ESRD cohorts with reduced statistical power (significance level of 8.3×10^{-4} on the basis of the 60 successfully genotyped SNPs in nondiabetic ESRD samples), the expected power to detect SNPs with a frequency of 0.05 and an OR of 1.5 was 0.31, and we feel that it is likely that mechanisms beyond impaired albumin reabsorption in proximal tubule cells contribute to nephropathy in blacks with nondiabetic kidney disease. Studies have shown that the *APOL1* G1 and G2 renal risk alleles markedly increase risk for FSGS, focal global glomerulosclerosis, HIV-associated nephropathy, and lupus nephritis (6,7). The younger age of controls relative to patients in this report warrants comment, because this could bias results toward the null hypothesis. In analyses of T2D-ESRD, the mean age of controls was older than the age at onset of T2D in patients. Therefore, the controls are less likely to develop T2D and/or subsequent T2D-ESRD. In analyses of nondiabetic ESRD, the mean age of controls was nearly 4 years younger than the age at onset of ESRD in patients ([age at recruitment] − [ESRD duration]); therefore, they are also far less likely to develop ESRD within this short timeframe given that they were nondiabetic, were non-nephropathic, and had a normal serum creatinine concentration (0.97 mg/dl).

In conclusion, genetic association was explored between the *CUBN* and *LRP2* genes for susceptibility to advanced nephropathy in blacks. *CUBN* variant rs1801239, previously associated with albuminuria in predominantly European populations, was associated with T2D-ESRD in individuals with recent African ancestry. A novel *LRP2* missense variant rs17848169 (N2632D) was also found to be associated with lower risk for T2D-ESRD in this population. Variants in *CUBN* and *LRP2* were not associated with T2D or nondiabetic etiologies of ESRD in blacks.

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Disclosures

None.

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