An Exome-wide Association Study for Type 2 Diabetes–Attributed End-Stage Kidney Disease in African Americans

Meijian Guan1,2, Jacob M. Keaton1,2, Latchezar Dimitrov1, Pamela J. Hicks1, Jianzhao Xu1, Nicholette D. Palmer1,2,3, James G. Wilson4, Barry I. Freedman5, Donald W. Bowden1,2,3 and Maggie C.Y. Ng1,2,3

1Center for Genomics and Personalized Medicine Research, Wake Forest School of Medicine, Winston-Salem, North Carolina, USA; 2Center for Diabetes Research, Wake Forest School of Medicine, Winston-Salem, North Carolina, USA; 3Department of Biochemistry, Wake Forest School of Medicine, Winston-Salem, North Carolina, USA; 4Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, Mississippi, USA; and 5Department of Internal Medicine, Section on Nephrology, Wake Forest School of Medicine, Winston-Salem, North Carolina, USA

Introduction: Compared with European Americans, African Americans (AAs) are at higher risk for developing end-stage kidney disease (ESKD). Genome-wide association studies (GWAS) have identified >70 genetic variants associated with kidney function and chronic kidney disease (CKD) in patients with and without diabetes. However, these variants explain a small proportion of disease liability. This study examined the contribution of coding genetic variants for risk of type 2 diabetes (T2D)-attributed ESKD and advanced CKD in AAs.

Methods: Exome sequencing was performed in 456 AA T2D-ESKD cases, and 936 AA nondiabetic, non-nephropathy control individuals at the discovery stage. A mixed logistic regression model was used for association analysis. Nominal associations (P < 0.05) were replicated in an additional 2020 T2D-ESKD cases and 1121 nondiabetic, non-nephropathy control individuals. A meta-analysis combining 4533 discovery and replication samples was performed. Putative T2D-ESKD associations were tested in additional 1910 nondiabetic ESKD and 219 T2D-ESKD cases, as well as 912 AA nondiabetic non-nephropathy control individuals.

Results: A total of 11 suggestive T2D-ESKD associations (P < 1 x 10^-4) from 8 loci (PLEKHN1, NADK, RAD51AP2, RREB1, PEX6, GRM8, PRX, APOL1) were apparent in the meta-analysis. Exclusion of APOL1 renal-risk genotype carriers identified 3 additional suggestive loci (OTUD7B, IFITM3, DLGAP5). Rs41302867 in RREB1 displayed consistent association with T2D-ESKD and nondiabetic ESKD (odds ratio: 0.47; P = 1.2 x 10^-6 in 4605 all-cause ESKD and 2969 nondiabetic non-nephropathy control individuals).

Conclusion: Our findings suggest that coding genetic variants are implicated in predisposition to T2D-ESKD in AAs.

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Although the apolipoprotein L1 gene (APOL1) G1 and G2 alleles explain a substantial proportion (~70%) of the disparity in nondiabetic ESKD in AAs versus European Americans, they fail to account for the excess risk of T2D-ESKD in AAs.11–13

Although DKD-associated variants have been identified by genome-wide association studies (GWASs), their contributions to DKD risk are modest.6–9,14 GWASs are not efficient in testing low-frequency variants, and these may account for a portion of the missing heritability. In contrast, exome sequencing is a powerful tool to explore coding regions, particularly low-frequency variants, which are poorly imputed by GWASs. In our prior studies, several coding variants located in RREB1, NPHS1, CUBN, LRPP2, COL4A3, and CLDN8 were shown to contribute to diabetic and/or nondiabetic ESKD in AAs.15–18 Herein, we extend prior studies by performing a comprehensive association study of coding variants for T2D-ESKD risk in AAs. We examined 1392 AA individuals with or without T2D-ESKD by exome sequencing (stage 1), followed by replication (stage 2) in 3141 AA subjects, and a meta-analysis combining 4533 AAs from stage 1 and stage 2 (stage 3), and a discrimination analysis in 1003 subjects with T2D without nephropathy to exclude T2D-associated variants (stage 4). We also performed replication in an additional 3041 all-cause ESKD cases and controls (stage 5). Finally, all T2D-ESKD and nondiabetic ESKD cases as well as controls were meta-analyzed (n = 7574) to evaluate the impact of associated variants on risk of all-cause ESKD (stage 6).

MATERIALS AND METHODS

Study Participants

Study participants were recruited by Wake Forest School of Medicine (stages 1–5) and Jackson Heart Study (stage 1). The studies were approved by the institutional review board of each participating center. All participants provided written informed consent. Description of a subset of study participants (1834 T2D-ESKD, 1295 nondiabetic non-nephropathy, 850 T2D-lacking nephropathy, 1705 nondiabetic ESKD) has previously been reported.15 Participants were considered to have T2D without nephropathy when their estimated glomerular filtration rate <60 ml/min per 1.73 m² and urine albumin/creatinine ratio <30 mg/g [when urine albumin/creatinine ratio was available]. Individuals were considered to have T2D without nephropathy when their estimated glomerular filtration rate was \( \geq 60 \text{ ml/min per } 1.73 \text{ m}^2 \) and urine albumin/creatinine ratio was <30 mg/g in the presence of T2D.

Sequencing, Genotyping, and Quality Control

Discovery Cohort

A total of 456 AA T2D-ESKD cases (5 with CKD4), 936 AA nondiabetic non-nephropathy controls, and 338 AA T2D cases lacking nephropathy underwent exome sequencing as part of the T2D-GENES consortium project. Details of sequencing data generation have been described previously.19 Briefly, exome sequencing was performed using an Agilent V2 capture array platform (Agilent Technologies, Santa Clara, CA) at the Broad Institute (Cambridge, MA). Sequence data underwent multiple levels of quality control (QC) for both samples and sequence reads. A total of 357 samples with average coverage \( \leq 20x \), possible DNA contamination, showed non-AA ancestry in principal component analysis, or missing information was excluded. Aligned sequence reads were filtered based on multiple QC criteria, including number of mapped reads, fraction of properly paired reads, distribution of insertion sizes, distribution of average base quality, as well as guanine and cytosine bias. A total of 136,584 exome variants with minor allele frequency (MAF) \( \geq 0.01 \) were included in this study.

Replication and Discrimination Cohort

The replication and discrimination cohorts included 2020 AA T2D-ESKD cases (143 had CKD4 or macroalbuminuria), 1121 AA nondiabetic non-nephropathy controls, and 665 AAs with T2D who lacked nephropathy. Samples were genotyped on a custom Affymetrix Axiom Biobank Genotyping Array (Affymetrix, Santa Clara, CA). Detailed variant information, custom content design, including fine mapping of candidate regions, genotyping methods, and QC have been reported.16 In brief, this array includes approximately 264K coding variants and insertions/deletions (indels), 70K
loss-of-function variants, 2K pharmacogenomic variants, 23K expression quantitative trait loci markers, 246K multiethnic population-based genome-wide tag markers, and 115K custom content. A total of 724,530 variants were successfully called for downstream QC and analyses. No batch effect was observed. Variants with call rates <95%, departure from Hardy Weinberg Equilibrium (P < 0.0001), and monomorphic variants were removed. A total of 683,375 variants were kept for imputation. Sample QC was also performed to remove individuals with low call rate, gender discordance, DNA contamination, or non-AA ancestry. Duplicate samples were compared, and one of each duplicate pair removed. A total of 1039 samples were excluded from this study. Variants that passed QC were imputed to a combined haplotype reference panel including the 1000 Genomes phase 3 cosmopolitan reference panel (October 2014 version) and a version of the African Genome Variation Project reference panel including 640 African ancestry haplotypes kindly provided by the African Partnership for Chronic Disease Research and Wellcome Trust Sanger Institute. Prephasing was performed using SHAPEIT2 and imputation was performed using IMPUTE2. Variants with imputation info scores <0.4 were excluded from analysis.

All-Cause ESKD Replication Cohort
Stage 5 analyses included 1910 nondiabetic ESKD cases, 219 T2D-ESKD cases (7 with CKD4 or macroalbuminuria), and 912 nondiabetic non-nephropathy controls. These samples were genotyped on the Multi-Ethnic Genotyping Array (Illumina, San Diego, CA), designed to improve fine-mapping and functional variants discovery by increasing variant coverage across multiple ethnicities. In brief, the array includes variants from 2 major categories: (i) backbone content containing highly informative variants for GWAS and exome analyses in ancestrally diverse populations, and (ii) custom content used to replicate or generalize index GWAS associations, augment GWAS tagging variants in priority regions, enhance exome content in priority regions, fine-map GWAS loci, identify functional regulatory variants, explore medically important variants, and identify novel variant loci in candidate pathways. Genotyping was performed at Wake Forest School of Medicine. DNA from cases and controls was equally interleaved on 96-well plates to minimize artifactual errors during sample processing. A total of 48 samples sequenced as part of the 1000 Genomes Project at the Coriell Institute for Medical Research were included in genotyping and had a concordance rate of 98.6%. Genotype calling was performed using GenomeStudio (Illumina). A total of 1,705,970 variants were successfully called for downstream QC and analyses. No batch effect was observed. Variants with missing position or alleles, allele mismatch, call rates <95%, departure from Hardy Weinberg Equilibrium (P < 0.0001), frequency difference >0.2 compared with 1000 Genomes Project phase 3 reference panel, and monomorphic variants were removed. Multiple probe sets were compared and only one set with the highest call rate was kept. We kept 1,204,700 high-quality variants for subsequent imputation. Sample QC was performed to remove individuals with low call rates (<0.95), gender discordance, DNA contamination, or non-AA ancestry. Duplicate samples were compared, and one of each duplicate pair was removed. A total of 1519 samples were excluded from analysis. Variants and samples that passed QC were used to perform prephasing with SHAPEIT2 and imputation with IMPUTE2 using a combined haplotype reference panel from the 1000 Genomes Project phase 3 and the African Genome Variation Project, described previously. Variants with imputation info scores <0.4 were excluded from analysis.

Statistical Analysis
Discovery and Replication of Associations in T2D-ESKD (Baseline Model)
Single-variant association analyses in case-control samples (from all analysis stages) were performed using a logistic mixed model method implemented in the program GMMAT under an additive genetic model. This controlled for population structure and cryptic relatedness by incorporating a genetic relationship matrix estimated from a set of high-quality autosomal variants as a random effect. Principal component analysis was performed using EIGENSOFT (Broad Institute of Harvard and MIT, Cambridge, MA). The first eigenvector (PC1), representing African-European ancestry, along with age and sex, were used as covariates. Body mass index was not included because of changes after CKD onset.

In the stage 1 analysis, exome-wide analysis was performed in 456 T2D-ESKD cases and 936 nondiabetes non-nephropathy controls on 193,646 variants that passed QC and had MAF ≥ 0.01. Variants with nominal associations (P < 0.05) were further tested in a replication cohort with 2020 T2D-ESKD cases and 1121 nondiabetic non-nephropathy controls (stage 2 analysis). A P value of 0.05 was used as a cutoff because we had limited sample size and power in the discovery stage. Meta-analysis including the discovery and replication stages (2476 cases and 2057 controls) was performed using a fixed-effect inverse variance weighting method implemented in METAL in the stage 3 analysis. Variants with suggestive evidence of
associations \( (P < 1 \times 10^{-4}) \) were selected for discrimination analysis.

**Discrimination Analysis**

To distinguish whether the meta-analysis association results were driven by T2D alone or T2D-ESKD, 1003 AAs with T2D-lacking nephropathy and 2057 nondiabetic non-nephropathy controls from analysis stages 1 and 2 were compared in a discrimination analysis (stage 4 analysis). Variants showing nominal association with T2D \( (P < 0.05) \) were excluded.

**Replication of Associations in All-Cause ESKD**

Variants that showed suggestive association \( (P < 1 \times 10^{-4}) \) in stage 3 T2D-ESKD meta-analysis and no evidence of association with T2D in the stage 4 discrimination analysis were tested for association with all-cause ESKD in 1910 nondiabetic ESKD cases, 219 additional T2D-ESKD cases, and 912 nondiabetic non-nephropathy controls (stage 5 analysis). Variants showing nominal association with all-cause ESKD \( (P < 0.05) \) were tested in a meta-analysis of all-cause ESKD that included all of the T2D-ESKD cases, nondiabetic ESKD cases, and controls from stages 1, 2, and 5 analyses \( (n = 7574) \). This meta-analysis evaluated whether T2D-ESKD associated gene variants contributed more broadly to other causes of ESKD. Subjects with stage 1 and 2 \( (n = 4533) \) were used to calculate the power to detect variants with a range of frequency \( (MAF = 0.01 \) to 0.4) and odds ratio \( (OR: 1.2 \) to 2) for a disease with 0.1% prevalence using CaTS (http://csg.sph.umich.edu/abecasis/cats/).

**Association Analyses With Exclusion of APOL1 Risk Genotype Carriers (APOL1-Negative Model)**

APOL1 G1 and G2 risk alleles explain a substantial proportion of genetic susceptibility to nondiabetic kidney disease in AAs.\(^{12}\) To minimize effects of misclassification, the same single-variant analyses were repeated \( (APOL1\text{-negative model}) \) by excluding APOL1 renal-risk-genotype carriers and those missing APOL1 genotype data from T2D-ESKD and nondiabetic ESKD samples. Individuals were considered APOL1 renal-risk-genotype carriers if they possessed 2 G1 alleles \( (rs60910145 \text{ G allele, } rs73885319 \text{ G allele}) \), 2 G2 alleles \( (6 \text{ base pair } \text{inframe deletion}) \), or were compound heterozygotes \( (1 \text{ G1 and } 1 \text{ G2 allele}) \).\(^{12}\) This secondary analysis reduces heterogeneity in the T2D-ESKD case group, despite its smaller sample size. Specifically, 98 of 456 T2D-ESKD cases from stage 1 discovery and 386 of 2020 T2D-ESKD cases from stage 2 replication were removed. In addition, 936 of 2136 all-cause ESKD cases in the stage 5 analysis were removed, considering the impact of APOL1 in nondiabetic ESKD. This strategy may unmask effects of nondiabetic ESKD variants beyond APOL1.

**Gene-based Analysis**

Four gene-based tests implemented in RAREMETAL\(^ {28} \) were applied: Sequence Kernel Association Test,\(^ {29} \) Madsen-Browning test,\(^ {30} \) Variable Threshold test,\(^ {31} \) and Combined and Multivariate Collapsing test\(^ {32} \) to test for the joint effect of rare variants \( (MAF < 0.05) \) within a gene on the stage 1 discovery analysis with exome sequencing data. Variants were categorized into 4 groups in all methods: (i) moderate to high-impact protein structure altering variants \( (\text{transcript ablation, splicing acceptor, splicing donor, stop gained, frameshift, stop lost, start lost, transcript amplification, inframe insertion, inframe deletion, missense, } \) and protein altering); (ii) a more restricted group with only high-impact protein structure–altering variants \( (\text{transcript ablation, splicing acceptor, splicing donor, stop gained, frameshift, stop lost, start lost, transcript amplification}) \); (iii) variants predicted to be deleterious by at least 1 of the 4 prediction methods, including SIFT \( (\text{sorting intolerant from tolerate}) \),\(^ {33} \) LRT \( (\text{likelihood ratio test}) \),\(^ {34} \) MutationTaster,\(^ {35} \) and CADD \( (\text{combined annotation–depletion}) \);\(^ {36} \) and (iv) variants predicted to be deleterious by all 4 prediction methods. Age, gender, and PC1 were included as fixed-effect covariates. A P value \(< 2.5 \times 10^{-6} \) was considered exome-wide significant. Both baseline and APOL1-negative models were evaluated.

**Functional Characterization**

The publicly available expression quantitative trait loci database GTEx (http://www.gtexportal.org/home/) was used to determine potential influences of T2D-ESKD associated variants on nearby gene expression. We queried 14 top associations from either the baseline or APOL1-negative models in GTEx across multiple tissues. Additional functional annotation of genetic variants was performed with Variant Effect Predictor \( (\text{VEP}) \),\(^ {37} \) SnpEff,\(^ {38} \) and dbNSFP.\(^ {39} \)

**Multiple Comparison Correction**

We used Bonferroni correction to adjust for multiple comparisons problem for both single-variant analysis and gene-based analysis. Exome-wide significance was defined as \( P < 3.5 \times 10^{-7} \) \( (0.05/136,584) \) and \( P < 2.5 \times 10^{-6} \) \( (0.05/20,000) \) for single-variant analysis and gene-based analysis, respectively. We also calculated false discovery rate \( (\text{Q-value}) \) using the Benjamini-Hochberg algorithm for both discovery analysis and meta-analysis in single-variant analysis.

**RESULTS**

Genetic variants located in coding regions were evaluated in 3 independent AA cohorts for association with T2D-ESKD and all-cause ESKD through a multistage
study design (Figure 1). A total of 8577 individuals categorized as having T2D-ESKD, nondiabetic non-nephropathy, T2D-lacking nephropathy, and nondiabetic ESKD were included in a sequence of association analyses. Overall, 18 suggestive T2D-ESKD associations in 11 distinct regions were identified, including 2 missense variants, in either the baseline or the APOL1-negative models. Rs41302867 located in the RREB1 region revealed consistent association with T2D-ESKD and nondiabetic ESKD, which is consistent with our previous report. Additionally, gene-based associations identified 7 genes with suggestive evidence of association with T2D-ESKD.

Clinical Characteristics of Study Participants
Clinical characteristics of study participants from all analysis stages are presented in Table 1. Individuals with T2D-ESKD and T2D-lacking nephropathy were older than the nondiabetic non-nephropathy controls at recruitment. However, the average age of diagnosis of T2D in T2D-ESKD and of T2D-lacking nephropathy participants were similar or younger than healthy controls at recruitment. Overall, individuals with T2D-lacking nephropathy were more obese than individuals with T2D- or nondiabetic ESKD and healthy controls.

Stage 1 Discovery Analysis in Exome Sequencing Cohort
The stage 1 discovery analysis with exome sequencing data included 456 T2D-ESKD patients and 936 nondiabetic non-nephropathy controls (Figure 1). A total of 136,584 exome variants that passed QC and had MAF ≥ 0.01 were tested for association with T2D-ESKD using a logistic mixed model. Age, gender, and PC1 were included as fixed-effects in the model (baseline model). The association analysis yielded an inflation factor of 0.996 (Supplementary Figure S1), indicating that population structure and cryptic relatedness were sufficiently controlled. No exome-wide significant association ($P < 3.5 \times 10^{-7}, 0.05/136,584$) was observed. However, 20 variants showed suggestive significance at a $P < 1 \times 10^{-4}$. We selected 6852 variants with nominal significance.

Figure 1. Analysis workflow of single-variant association analysis for T2D-ESKD exome sequencing study (baseline model). Stage 3 combines stage 1 and stage 2 samples; stage 6 combines stage 1, stage 2, and stage 5 samples. T2D, type 2 diabetes; ESKD, end-stage kidney disease.
Table 1. Clinical characteristics of study cohorts

| Characteristics                                      | All-cause ESKD replication cohort | Replication and discrimination cohort | Discovery cohort |
|------------------------------------------------------|----------------------------------|--------------------------------------|-----------------|
| N                                                    | 1910                             | 219                                  | 456             |
| Nondiabetic non-nephropathy controls                 | 912                              | 912                                  | 936             |
| Nondiabetic T2D-lacking nephropathy controls         | 50.68                            | 41.89                                | 58.05           |
| Nondiabetic T2D-ESKD cases                          | 58.74                            | 51.74                                | 61.62           |
| Age (yr)                                             | 64.61                            | 84.75                                | 66.47           |
| Age at T2D onset (yr)                                | 46.2                             | 38.62                                | 51.74           |
| Duration of T2D (yr)                                 | 15.36                            | 38.62                                | 51.74           |
| Duration of ESKD (yr)                                | 3.11                             | 19.66                                | 46.20           |
| Fasting serum glucose (mg/dl)                        | 84.75                            | 92.10                                | 12.68           |
| Body mass index (kg/m²)                              | 29.43                            | 45.2                                | 66.47           |
| eGFR, estimated glomerular filtration rate (ml/min)  | 1.51                             | 4.62                                | 12.68           |

**Note:** Categorical data expressed as percentage; continuous data as mean ± SD.

Stage 2 Replication Analysis

An independent cohort of 2020 AA T2D-ESKD cases and 1121 AA nondiabetic non-nephropathy controls with imputed dosage data was used to replicate the selected variants from the discovery stage (Figure 1). More than 93% of the variants were imputed with imputation quality info >0.4 for association analyses.

Stage 3 Meta-analysis

A total of 6852 variants with nominal association ($P < 0.05$) were selected for meta-analysis of the discovery and replication samples. Fifteen variants with consistent directions of association with T2D-ESKD at $P < 1 \times 10^{-4}$ were identified. However, no variant reached exome-wide significance ($P < 3.5 \times 10^{-7}$).

Stage 4 Discrimination Analysis

To differentiate T2D-ESKD associations identified in meta-analysis as putative T2D-ESKD or T2D-only (non-nephropathy) associated loci, we performed a T2D discrimination analysis comparing 1003 AA T2D cases lacking nephropathy with 2057 nondiabetic non-nephropathy controls from stages 1 and 2. Four of the 15 T2D-ESKD associated variants were nominally associated ($P < 0.05$) with T2D and were removed from subsequent analyses (Supplementary Table S2).

The remaining 11 T2D-ESKD associated variants were located in 8 loci, including **PLEKH1N, NADK, RAD51AP2, RREB1, PEX6, GRM8, PRX, APOL1** ($P < 1 \times 10^{-4}$) (Table 2, Supplementary Figure S2). The appearance of 2 **APOL1** G1 alleles, rs73885319 (OR: 1.32; 95% confidence interval [CI]: 1.17–1.50; $P = 9.90 \times 10^{-6}$) and rs60910145 (OR: 1.33; 95% CI: 1.18–1.51; $P = 5.74 \times 10^{-6}$), known to be associated with nondiabetic ESKD, may be due in part to misclassification of DKD cases. $^{12}$T2D-ESKD associated variants located within **RAD51AP2** and **PRX** are highly correlated ($r^2 > 0.9$), and rs834514 at **RAD51AP2** (OR: 0.76; 95% CI: 0.67–0.87; $P = 6.56 \times 10^{-5}$) and rs268671 at **PRX** (OR: 0.75; 95% CI: 0.65–0.86; $P = 2.11 \times 10^{-7}$) were missense variants. Moreover, variants from **PLEKH1N, RAD51AP2, PEX6,** and **PRX** were associated with the expression level of nearby genes across multiple tissues, suggesting potential regulatory roles (Supplementary Table S3).

Association Analysis Excluding **APOL1** Renal-Risk Genotype Carriers

The primary analysis showed moderate association of **APOL1** G1 alleles with T2D-ESKD, suggesting misclassification of some cases. A secondary analysis excluding **APOL1** G1 and G2 risk genotype carriers...
Table 2. T2D-ESKD associated variants in meta-analysis from discovery and replication cohorts (baseline model)

| Variant | Gene | Annotation | CHR | POS | EA | Effect allele | Effect allele frequency | Evidence | OR (95% CI) | P | Q-value |
|---------|------|------------|-----|-----|----|--------------|------------------------|----------|-------------|---|---------|
| rs59928984 | - | - | - | - | - | - | - | - | - | - | - |
| rs180881514 | NADK | Intron 1 | 1685713 | T | 0.01 | 4.87 (1.96-10.05) | 0.019 | - | 4.13 (1.71-9.92) | 0.011 | 0.011 |
| rs6635127 | RAD51AP2 | Missense | 17696573 | T | 0.21 | 0.71 (0.56-0.94) | 0.0037 | - | 1.0 (0.68-1.67) | 0.21 | 0.032 |
| rs59928984 | - | - | - | - | - | - | - | - | - | - | - |
| rs180881514 | NADK | Intron 1 | 1685713 | T | 0.01 | 4.87 (1.96-10.05) | 0.019 | - | 4.13 (1.71-9.92) | 0.011 | 0.011 |
| rs6635127 | RAD51AP2 | Missense | 17696573 | T | 0.21 | 0.71 (0.56-0.94) | 0.0037 | - | 1.0 (0.68-1.67) | 0.21 | 0.032 |
| rs73885319 | - | - | - | - | - | - | - | - | - | - | - |
| rs60910145 | APOL1 | Missense | 36662034 | G | 0.23 | 1.42 (1.14-1.78) | 0.00081 | - | 1.33 (1.11-1.51) | 0.001 | 0.001 |

Table S4). In contrast, 3 additional associations were identified at OTUD7B (rs115499155; OR: 0.43; 95% CI: 0.28–0.65; \( P = 8.58 \times 10^{-5} \)), IFITM3 (rs34481144; OR: 0.67; 95% CI: 0.55–0.81; \( P = 5.25 \times 10^{-5} \)), and DLGAP5 (rs8009244; OR: 0.75; 95% CI: 0.65–0.86; \( P = 6.42 \times 10^{-5} \)). Variants in IFITM3 and DLGAP5 demonstrated association with transcript abundance of nearby genes based on GTEx (Supplementary Table S3). Excluding APOL1 risk genotype carriers from both cases and controls showed similar effect sizes despite slightly attenuated significance likely due to reduced sample size (Supplementary Table S5).

Replication Analysis in All-Cause ESKD

In the stage 5 analysis, an all-cause ESKD cohort consisting of 1910 non-diabetic ESKD cases, 219 T2D-ESKD cases, and 912 non-diabetic non-nephropathy controls was used to replicate the 14 T2D-ESKD associated signals identified from the baseline model and the APOL1-negative models. This analysis evaluated the generalizability of T2D-ESKD loci in non-diabetic etiologies of ESKD. Initially, the 14 suggestive T2D-ESKD associations were tested in 2129 all-cause ESKD cases and 912 non-diabetic non-nephropathy controls. Only 1 variant, located in RREBI (rs41302867; OR: 0.52; 95% CI: 0.31–0.88; \( P = 0.015 \)), achieved \( P < 0.05 \). Similar results were obtained after excluding 219 T2D-ESKD cases from the association analysis; only rs41302867 reached \( P < 0.05 \). Consistent results were observed after excluding APOL1 renal-risk
### Table 3. Top T2D-ESKD associations in meta-analysis after removing APOL1 renal-risk genotype carriers (APOL1-negative model)

| Variant | Gene | Annotation | CHR | POS | EA | R | OR (95% CI) | P | Info | EAF |
|---------|------|------------|-----|-----|----|---|-------------|---|------|-----|
| rs115499155 | OTUD7B | Synonymous | 1 | 149943088 | T | 0.02 | 0.44 (0.21–0.92) | 0.006 | 0.03 | 0.021 |
| rs41302867 | RREB1 | Intron 6 | 7240876 | A | 0.025 | 0.49 (0.31–0.78) | 0.0032 | 0.21 | 0.44 (0.3–0.65) | 0.0003 |
| rs74678433 | GRM8 | Intron 7 | 126173950 | G | 0.024 | 2.47 (1.29–4.73) | 0.0066 | 0.2 | 1.97 (1.26–3.07) | 0.0008 |
| rs34481144 | IFITM3 | 5'UTR | 11 | 320836 | T | 0.1 | 0.68 (0.54–0.86) | 0.0011 | 0.2 | 0.67 (0.55–0.81) |
| rs8009244  | DLGAP5 | Intron 14 | 55643752 | A | 0.23 | 0.75 (0.58–0.97) | 0.03 | 0.21 | 0.75 (0.63–0.86) |
| rs268672   | PRX    | Synonymous | 19 | 40901604 | A | 0.19 | 0.71 (0.55–0.92) | 0.0089 | 0.19 | 0.75 (0.64–0.86) |
| rs268671   | PRX    | Missense | 19 | 40901614 | A | 0.2 | 0.71 (0.55–0.92) | 0.0089 | 0.19 | 0.75 (0.64–0.86) |

**DISCUSSION**

This study presents results of an exome sequencing–based genetic association study evaluating the contribution of coding variants to risk of T2D-ESKD in AAs.
We tested the top variants for replication in additional T2D-ESKD and control cohorts, and evaluated the generalizability of T2D-ESKD associations to common forms of nondiabetic ESKD. Evidence of nominal association with T2D-ESKD was observed for 11 loci, including PLEKHN1, NADK, RAD51AP2, RREB1, PEX6, APOL1, GRM8, PRX, OTUD7B, IFITM3, and DLGAP5 in single-variant analysis of either baseline or APOL1-negative models. Two associated variants from RAD51AP2 and PRX were missense mutations. Meta-analysis of all-cause ESKD revealed strong association at RREB1, previously identified as associated with ESKD.\(^{15}\) These results suggest it plays a role in AA diabetes- and nondiabetes-associated kidney disease. Gene-based analyses identified additional suggestive T2D-ESKD loci with multiple variants having cumulative effects: TMEM5, SPATS2, ZIC4, HELZ2, ILDR2, LGALS3BP, and RSDA2. However, there were minimal overlapping associations between single-variant and gene-based tests. The only locus that revealed association with both T2D-ESKD and nondiabetic ESKD was an intronic variant at RREB1, a large complex gene encoding the ras responsive transcription factor. RREB1 has repeatedly been implicated in fasting glucose, T2D susceptibility, fat distribution, and adipocyte development in large-scale genetic studies.\(^{40-44}\) Relevant to kidney disease susceptibility, RREB1 variants had been reported to be associated with kidney function and interact with APOL1 renal-risk-alleles in nondiabetic nephropathy.\(^{15,46}\) More recently, the same variant, rs41302867, was shown to be associated with all-cause ESKD in AAs and European Americans.\(^{15}\) Despite partially overlapping participants, the present results suggested a role for RREB1 in common forms of ESKD in AAs with a substantially expanded sample. In addition, it is noteworthy that the ESKD associated variant rs41302867 had no association with T2D alone in this study ($P = 0.096$); this suggests a pleiotropic effect of RREB1 in multiple traits including T2D and kidney disease.

Among the putative T2D-ESKD associated loci, an intronic variant at GRM8 (rs74678433) showed strong and consistent associations in baseline and APOL1-negative models ($P < 5.96 \times 10^{-6}$ and $P < 6.24 \times 10^{-3}$, respectively). Metabotropic glutamate receptor 8, the protein product encoded by GRM8, is associated with weight gain in mouse models.\(^{47,48}\) Previous studies suggest that obesity is a major risk factor for kidney disease in patients with diabetes.\(^{49}\) This result may suggest potential genetic correlation between obesity and T2D-ESKD. Two missense variants showing putative association with T2D-ESKD were identified, rs834514 and rs268671 located at RAD51AP2 and PRX, respectively. RAD51 Associated Protein 2, encoded by RAD51AP2, is a recombinase that plays a critical role in both DNA repair and meiotic recombination. Rs834514 may regulate the expression of RAD51AP2, GEN1, and VSNL1 in multiple tissues, including pancreas and adipose. The missense variant (rs268671) located in the periaxin gene (PRX), is a key myelination molecule forming tight junctions between myelin loops and axons. Mutations in PRX caused late-onset Charcot-Marie-Tooth neuropathy, a common inherited neurological disorder.\(^{50,51}\) Charcot-Marie-Tooth neuropathy has been reported to be associated with kidney disease, mainly focal segmental glomerulosclerosis.\(^{52-54}\) The underlying mechanism linking these entities remains unknown.

A splice region variant (rs9986447) located in PEX6 was nominally associated with T2D-ESKD in the baseline model. Rs9986447 showed significant impact on the expression level of PEX6 in a number of tissues, including adipose and pancreas, indicating its regulatory role in this gene. This gene plays a direct role in the biosynthesis of peroxisome, a subcellular organelle...
involved in lipid metabolism. \textit{PEX6} may associate with DKD susceptibility, as dyslipidemia is thought to be a contributor.\textsuperscript{55}

Analyses excluding \textit{APOL1} renal-risk genotype carriers provided an opportunity to uncover the genetic architecture in T2D-ESKD. In the \textit{APOL1}-negative model, 4 additional variants revealing suggestive association with T2D-ESKD were identified in \textit{OTUD7B}, \textit{IFITM3}, and \textit{DLGAP5}. \textit{OTU deubiquitinase 7B}, encoded by \textit{OTUD7B}, may be involved in the inflammatory response of DKD progression via regulating the nuclear factor kappa-B signaling pathway.\textsuperscript{56} However, \textit{IFITM3} (interferon induced transmembrane protein 3) and \textit{DLGAP5} (DLG associated protein 5) do not have literature to support their role in T2D-ESKD. Further studies are required to evaluate potential biological roles.

Gene-based methods provide an opportunity to evaluate the cumulative impact of low-frequency variants (MAF < 0.05) within a region on the disease of interest. Four types of gene-based analysis methods were used to test 4 categories of variants grouped by their functional impact. The top signal was found at a transmembrane protein gene \textit{TMEM5} showing consistent association in both the baseline and \textit{APOL1}-negative models. For \textit{ILDR2} (Ig-like domain containing receptor 2), Ildr2 has been implicated in T2D susceptibility and hepatic lipid metabolism in mouse models.\textsuperscript{57,58} Potential involvement of \textit{ILDR2} in T2D-ESKD disease remains to be assessed.

This study suggests that low-frequency and rare variants located at coding regions provide crucial information on understanding the genetic architecture of complex diseases such as DKD. We identified suggestive T2D-ESKD signals in 19 loci using multiple single-variant and gene-based analyses; however, additional replication is required to confirm findings given limited power in the current study. There was lack of enrichment between the single-variant analyses and gene-based tests. This may be partially due to the deleterious variants included in gene-based tests, which tend to have low frequencies and may be excluded (or underpowered) in single-variant analyses. On the other hand, gene-based associations were performed only in exome sequencing data; thus, with limited power.

The pathophysiology and pathology of DKD is heterogeneous, with effects of glycemia, blood pressure, albuminuria, diabetes duration, serum uric acid, dyslipidemia, obesity, and smoking.\textsuperscript{49,59,60} Thus, T2D-associated DKD may share a common genetic background with other phenotypes. It also has been proposed that genetic variation in Mendelian disease genes may in part account for common disease susceptibility.\textsuperscript{61,62} Identification of \textit{GRM8}, \textit{PEX6}, \textit{ILDR2}, \textit{RREB1}, and \textit{PRX} may indicate potential genetic correlations between T2D-ESKD with obesity, dyslipidemia, T2D, and Mendelian Charcot-Marie-Tooth neuropathy.

This study has similar limitations as in other reports. First, it is difficult to exclude all individuals misclassified as DKD due to the frequent lack of kidney biopsies. However, careful removal of patients with ESKD attributed to non-diabetic etiologies, and subsequently excluding \textit{APOL1} risk genotype carriers with high risk for non-diabetic kidney disease, should minimize misclassification. In addition, although the multistage study design brings together 8577 AA individuals, study power was moderate (Supplementary Tables S7 and S8). This is especially true in the discovery exome sequencing cohort, which may result in additional variants of modest effect and low frequency that were not captured. There are few other existing collections of appropriate AA samples; this limited possible replication studies. Finally, we used imputed data to replicate exome sequencing findings in stage 2 and to evaluate their association with non-diabetic ESKD in stage 5. Although we used info < 0.4 to filter out low-quality alleles, there might be some variants with low imputation quality included. However, the highlighted variants all showed high imputation quality (0.82 to 1).

In conclusion, an exome sequencing–based, multi-phase study to identify T2D-ESKD susceptibility loci was performed in AAs, and 18 suggestive associations with T2D-ESKD were detected. \textit{RREB1} was consistently associated with diabetic and non-diabetic etiologies of ESKD in AAs. T2D-ESKD associated variants at \textit{GRM8}, \textit{PEX6}, \textit{ILDR2}, \textit{RREB1}, and \textit{PRX} may support genetic correlation between T2D-ESKD and related phenotypes. Future efforts to confirm the newly identified associations and determine their potential impact on the biological processes related to DKD requires investment in additional sample recruitment and comprehensive functional evaluation.

**DISCLOSURE**

All the authors declared no competing interests.

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**SUPPLEMENTARY MATERIAL**

**Figure S1.** QQ plot of T2D-ESKD versus controls in model 1 discovery stage (MAF ≥ 0.01).

**Figure S2.** Regional plot of top T2D-ESKD associations in baseline model.

**Figure S3.** Regional plot of top T2D-ESKD associations in APOL1-negative model.

**Table S1.** Breakdown of ESKD cases for each stage.

**Table S2.** Discrimination analysis for top associations of baseline model and APOL1-negative model.

**Table S3.** GTEx results of top associations ($P < 1 \times 10^{-4}$).

**Table S4.** Results of top associations from baseline model in APOL1-negative model.

**Table S5.** Top T2D-ESKD associations in meta-analysis after removing APOL1 renal-risk genotype carriers in both cases and controls.

**Table S6.** Gene-based analysis results for T2D-ESKD associated loci in single-variant association analysis.

**Table S7.** Power analysis of single-variant association analysis at significance level of $\alpha = 3.5 \times 10^{-7}$.

**Table S8.** Power analysis of single-variant association analysis at significance level of $\alpha = 1.0 \times 10^{-4}$.

Supplementary material is linked to the online version of the paper at [http://www.kireports.org/](http://www.kireports.org/).

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