Association of the Distal Region of the Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 Gene With Type 2 Diabetes in an African-American Population Enriched for Nephropathy

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OBJECTIVE—Variants in the ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) gene have shown positive associations with diabetes and related phenotypes, including insulin resistance, metabolic syndrome, and type 1 diabetic nephropathy. Additionally, evidence for linkage for type 2 diabetes in African Americans was observed at 6q24-27, with the proximal edge of the peak encompassing the ENPP1 gene. Our objective was to comprehensively evaluate variants in ENPP1 for association with type 2 diabetic end-stage renal disease (ESRD).

RESEARCH DESIGN AND METHODS—Forty-nine single nucleotide polymorphisms (SNPs) located in the coding and flanking regions of ENPP1 were genotyped in 577 African-American individuals with type 2 diabetic ESRD and 596 African-American control subjects. Haplotypic association and genotypic association for the dominant, additive, and recessive models were tested by calculating a χ^2 statistic and corresponding P value.

RESULTS—Nine SNPs showed nominal evidence for association ($P < 0.05$) with type 2 diabetic ESRD in one or more genotypic model. The most significant associations were observed with rs7754586 ($P = 0.003$ dominant model, $P = 0.0005$ additive, and $P = 0.007$ recessive), located in the 3′ untranslated region, and an intron 24 SNP (rs1974201: $P = 0.004$ dominant, $P = 0.0005$ additive, and $P = 0.005$ recessive). However, the extensively studied K121Q variant (rs1044498) did not reveal evidence for association with type 2 diabetic ESRD in this African-American population.

CONCLUSIONS—This study was the first to comprehensively evaluate variants of the ENPP1 gene for association in an African-American population with type 2 diabetes and ESRD and suggests that variants in the distal region of the ENPP1 gene may contribute to diabetes or diabetic nephropathy susceptibility in African Americans. ***Diabetes*** **57:1057–1062, 2008**

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AIM, admixture informative marker; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; ESRD, end-stage renal disease; HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; MAP, minor allele frequency; SNP, single nucleotide polymorphism.

The ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) gene, also referred to as plasma cell membrane glycoprotein (PC-1), spans over 83 kb and is located on chromosome 6q22-23. ENPP1 was first described as a mediator of insulin resistance by Goldfine et al. (1), and the K121Q variant was subsequently determined to be associated with insulin resistance in humans using euglycemic clamp studies (2). Variants in ENPP1, primarily K121Q, have shown positive associations with obesity and BMI (3–5), metabolic syndrome (6), and type 2 diabetes (3,7,8). Negative association results for these variants in large meta-analyses of Caucasian populations (9–11) question the reproducibility of these positive findings, although a recent meta-analysis of >40,000 Caucasian individuals from 30 studies detected a modest association with 121Q under a recessive model (12).

Three previous association studies of the ENPP1 gene in populations of African descent, two in African Americans (5,13) and one in Afro-Caribbeans from the Dominican Republic (14), have focused exclusively on associations with the K121Q variant. These studies found a much higher frequency of the 121Q allele (74–78% in African Americans and 54.2% Dominicans) than that observed in other populations, and two of the three studies found an association with type 2 diabetes (13,14) but the third did not (5). These equivocal results suggest that further examination of variants in the ENPP1 gene in additional populations with African ancestry is warranted.

A genome-wide scan for type 2 diabetes in African Americans provided evidence of linkage at chromosome 6q24-27 (15). Although ENPP1 lies proximal to the logarithm of odds (LOD)-1 interval, it is located under the linkage peak. ENPP1 remains a logical type 2 diabetes candidate gene due to its inhibitory actions on insulin receptor function (16) and positive associations with diabetes and related phenotypes. Additionally, ENPP1 is an attractive candidate gene for end-stage renal disease (ESRD) due to its expression in kidney tubules and previous associations with type 1 diabetic nephropathy (17,18).

We have analyzed 49 single nucleotide polymorphisms

TABLE 1
Characteristics of African-American subjects

Trait	Type 2 diabetic ESRD case subjects (n = 577)	Control subjects (n = 596)
% Female (n)	61% (351)	51% (305)
Age at exam (years)	62.2 ± 10.3 (541)	49.3 ± 9.8 (448)
Age at type 2 diabetes diagnosis (years)	41.8 ± 11.6 (544)	NA
Age at ESRD diagnosis (years)	59.0 ± 10.5 (560)	NA

Data are means ± SD (number of subjects with data available), unless otherwise indicated. All case subjects were diagnosed with type 2 diabetes and ESRD. NA, not applicable.

(SNPs) spanning >91 kb across the ENPP1 gene in an African-American case-control population consisting of 577 African-American individuals with type 2 diabetic ESRD and 596 African Americans without a known diagnosis of type 2 diabetes. To date, ENPP1 genetic studies in African Americans have been limited to the K121Q variant or a three-SNP haplotype containing K121Q. This study represents the most comprehensive evaluation of the ENPP1 gene in an African-American population with type 2 diabetic ESRD.

RESEARCH DESIGN AND METHODS

Subjects. This study was conducted under Institutional Review Board approval from Wake Forest University School of Medicine and adhered to the tenets of the Declaration of Helsinki. Identification, clinical characteristics, and recruitment of African-American and European-American patients and control subjects have been described previously (19). Briefly, 577 unrelated African-American patients with type 2 diabetes born in North Carolina, South Carolina, Georgia, Tennessee, or Virginia were recruited from dialysis facilities. Case subjects had type 2 diabetes diagnosed at least 5 years before initiating renal replacement therapy, background or greater diabetic retinopathy, and/or >3+ proteinuria on urinalysis in the absence of other causes of nephropathy. A total of 596 unrelated African-American control subjects and 39 unrelated European-American control subjects born in North Carolina, South Carolina, Georgia, Tennessee, or Virginia and undiagnosed for type 2 diabetes or renal disease were recruited. DNA extraction was performed using the PureGene system (Gentra Systems, Minneapolis, MN). DNA was also obtained from 44 Yoruba Nigerians from the National Institute of General Medical Sciences (NIGMS) Human Variation Collection (Coriell Cell Repositories, Camden, NJ).

ENPP1 SNP selection and genotyping. We used the genotypic data of the Yoruba and CEPH Europeans from the International HapMap project (20) to tag the common variants in ENPP1. Using the largest reported ENPP1 transcript plus 5 kb upstream and downstream of the gene, we uploaded the Yoruba genotypic data into Haploview 3.2 (21) and selected markers with a minor allele frequency (MAF) ≥0.05, excluding SNPs with a designability score of <1.0 on the Illumina platform. The aggressive (two- or three-SNP haplotype) tagging option of Tagger was used for SNP selection (22), resulting in 38 tagging SNPs that capture 66 SNPs with a mean $r^2 = 0.979$. Next, we uploaded the CEPH European genotypic data into Haploview 3.2 (23), forced the inclusion of the 38 Yoruba tag SNPs and exclusion of Illumina-undesignable SNPs, and identified additional CEPH European tag SNPs necessary to capture CEPH European SNPs with MAF >0.05. Thirty-five tagging SNPs captured 74 SNPs with a mean $r^2 = 0.982$ in CEPH Europeans. One previously associated SNP, K121Q (rs1044498), was also included, yielding a total of 49 SNPs at an average density of one SNP every 1.87 kb, with the largest gap being 10.2 kb and the smallest 51 bp.

Forty-nine ENPP1 SNPs were genotyped in 577 African-American individuals with type 2 diabetic ESRD and 596 African-American control subjects. Forty-two SNPs were genotyped using Illumina's Custom Genotyping Service (San Diego, CA), while seven SNPs were genotyped using iPlex methodology on a MassARRAY genotyping system (Sequenom, San Diego, CA) (24): rs1974201, rs2021966, rs7773477, rs9372999, rs9375830, rs9493120, and rs1044498. The genotyping success rates for the 49 SNPs in the African-American case and control subjects ranged from 90.4 to 100%. Concordance rates for 46 replicate pairs were 100% for all SNPs, except rs9372999, where there was one discordant genotype among 46 replicate pairs (97.8% concordance).

Genotyping for admixture analyses. Seventy biallelic admixture informative markers (AIMs) were genotyped by Illumina's Custom Genotyping Service or using a MassARRAY genotyping system (Sequenom) (24) in 577 African-American case and 596 African-American control subjects, 44 Yoruba and 39 European-American control subjects (supplementary Table 1 available online at http://dx.doi.org/10.2337/db07-0886). The genotyping success rates for the AIMs range from 94.9 to 100% in the African-American case, African-American control, Yoruba, and European-American samples. Primer sequences are available on request.

Statistical analyses. Hardy-Weinberg equilibrium (HWE) values were determined by calculating a χ^2 statistic and corresponding P value. Haplotype block structure was established using Haploview 3.2 (21), using the block definition from Gabriel et al. (25).

Unadjusted haplotypic association and genotypic association for dominant, additive, and recessive models were tested by calculating a χ^2 statistic and corresponding P value using the program SNPGWA (C. Langefeld and M. Stiegert, unpublished). Due to a lack of validity of the large sample χ^2 statistic, only the dominant model P values were considered for SNPs with 10 or fewer individuals that were homozygous for the minor allele.

Ancestral proportions were calculated using the program FRAPPE (Frequentist Estimation of individual ancestry proportion) (26) under a two-population model. Estimates of "pseudo-ancestral" allele frequencies were obtained from genotyped Yoruba and European-American samples. Individual estimates of African ancestry for African-American subjects were used as covariates for logistic regression tests of dominant, additive, and recessive models of association as implemented in the program SNPADMIX (C. Langefeld and M. Stiegert, unpublished).

RESULTS

Characteristics of the African-American case and control populations are shown in Table 1. Control subjects were significantly younger than case subjects ($P < 0.0001$), although they were significantly older than the mean age at type 2 diabetes diagnosis in case subjects ($P < 0.0001$). There was a higher proportion of females (61%) among the case than the control subjects (51%), possibly due to participation bias.

Genotyping success rates for the 49 ENPP1 SNPs were 91.4% in the African-American case and 90.4% in the African-American control subjects. Using an HWE P value threshold of 0.01, two SNPs, rs858341 ($P = 0.004$) and rs9493105 ($P = 0.001$), deviated from expected HWE proportions in the African-American control subjects, whereas no SNPs were inconsistent in the African-American case subjects. These SNPs were retained for exploratory analyses but neither showed significant evidence of association with type 2 diabetic ESRD. Eight and nine blocks of high linkage disequilibrium (LD) were identified in the African-American control subjects (Fig. 1) and case subjects (Fig. 2), respectively, using the method of Gabriel et al. (25) implemented in the program Haploview (21).

Genotype frequencies and counts are shown in supplementary Table 2, and single-SNP and two- and three-marker haplotypic association results are presented in supplementary Table 3. Nine SNPs showed nominal evi-

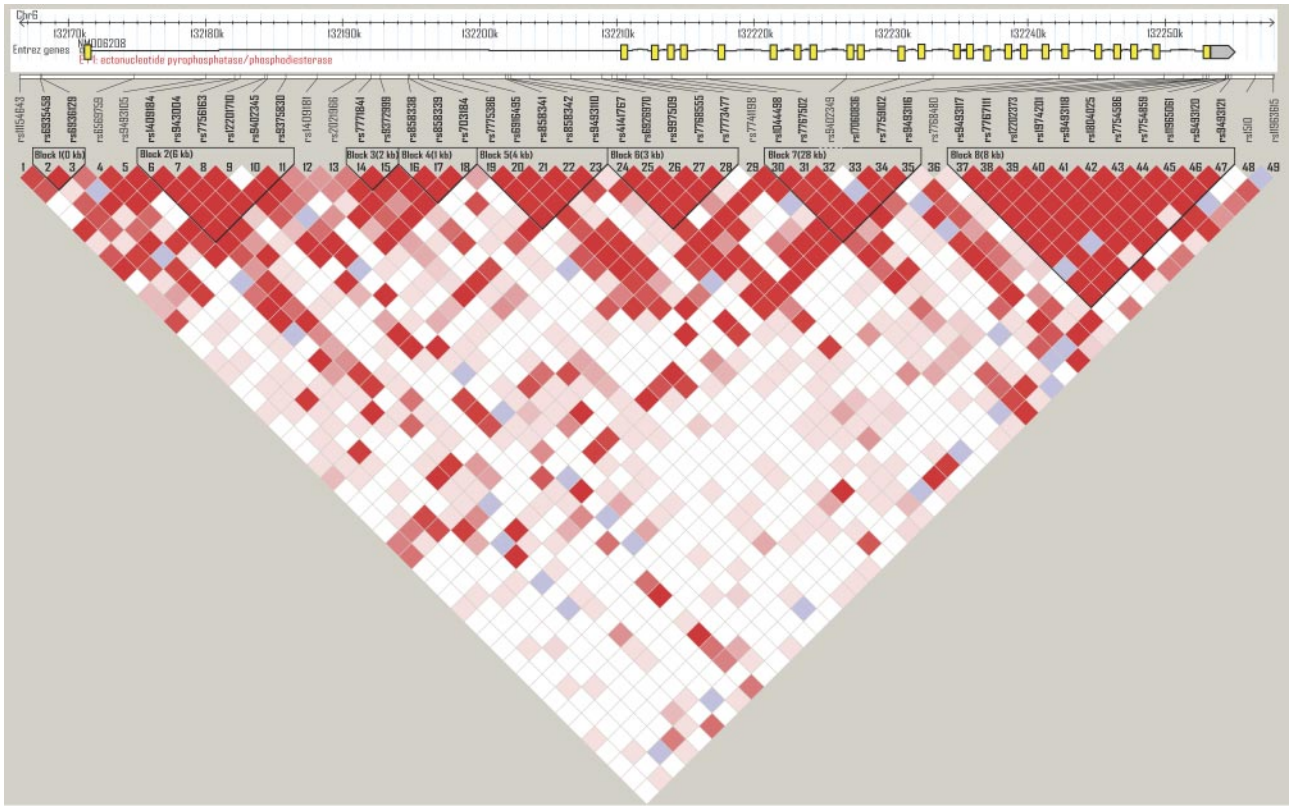

FIG. 1. LD structure of the ENPP1 gene in African-American control subjects (n = 596) with haplotype blocks based on the definition of Gabriel et al. (25) implemented in Haploview (21). D′ values are displayed in the squares. Empty squares represent a pairwise D′ = 1. Red squares represent high pairwise LD, coloring down to white squares of low pairwise LD, while blue squares indicated LOD <2.

dence for association ($P < 0.05$) with type 2 diabetic ESRD in one or more genotypic model (Table 2). The most significant associations were observed for rs7754586 and rs1974201, with both SNPs being associated in all three genotypic models ($P = 0.003$, 0.0005, 0.007 and $P = 0.004$, 0.0005, 0.005 for the dominant, additive, and recessive models for rs7754586 and rs1974201, respectively). These two markers are 1,621 bp apart and are located in the 3′ untranslated region (UTR) (rs7754586) and intron 24 (rs1974201).

Haplotype analysis of two- and three-marker haplotypes showed nominal evidence of association with 6 two-marker haplotypes and 7 three-marker haplotypes (supplementary Table 3). Five of the six significant two-marker haplotypes are located in the distal region of the gene (intron 24-3′ UTR), with P values ranging from 0.044 to 0.002. All seven significant three marker haplotypes lie in the intron 24-3′ UTR region of the gene with P values ranging from 0.025 to 0.003.

Admixture proportions and individual admixture estimates for the African-American case and control subjects were calculated based on the genotyping results of 70 AIMs. The mean proportion of African ancestry was 0.817 (SD 0.133) for the African-American case subjects and 0.791 (SD 0.131) for the African-American control subjects. Seven of the nine SNPs remained significantly associated with type 2 diabetic ESRD in one or more genotypic model of association, after adjusting for admixture (Table 2).

DISCUSSION

We have comprehensively investigated genetic variation across the ENPP1 gene by genotyping 49 SNPs in 577 African-American individuals with type 2 diabetic ESRD and 596 African-American control subjects, and found nominal evidence of association ($P < 0.05$) with nine single SNPs, six 2-marker haplotypes, and seven 3-marker haplotypes (Supplementary Table 3).

The most significant genotypic associations were observed with marker rs7754586, located in the 3′ UTR, and intron 24 SNP rs1974201. These SNPs are ~1,600 bp apart and were significantly associated with type 2 diabetic ESRD in all three genotypic models of association (Table 2), conferring risk for type 2 diabetic ESRD with odds ratios between 1.34 and 1.50 (Supplementary Tables 4 and 5). After correcting for multiple tests, SNPs rs1974201 (nominal $P = 0.0005$, adjusted $P = 0.0167$) and rs7754586 (nominal $P = 0.0005$, adjusted $P = 0.0169$) maintained significance for the additive model at Experiment-wise Error Rate (EER) $P < 0.05$ (27). Numakura et al. identified mutations in exon 23 in Japanese patients with idiopathic infantile arterial calcification, leading to amino acid alterations in the ENPP1 gene (28). Individuals from Sicily, Italy that carried a cluster of three alleles in the 3′ UTR: rs1044548 A, rs11964389 C, and rs104455 T, which they labeled the “P haplotype”, had a higher risk for insulin resistance and had higher levels of plasma glucose and insulin during an oral glucose tolerance test (29). Frittitta et al. (29) also determined that individuals with type 2 diabetes from Gargano, Italy, had a higher “P haplotype” frequency (7.8% vs 1.5%) than control subjects. The 3-SNP “P haplotype was also shown to increase mRNA stability and was associated with ENPP1 overexpression (29). Using the Yoruba HapMap data, two SNPs we found to be associated with type 2 diabetic ESRD tag the three SNPs

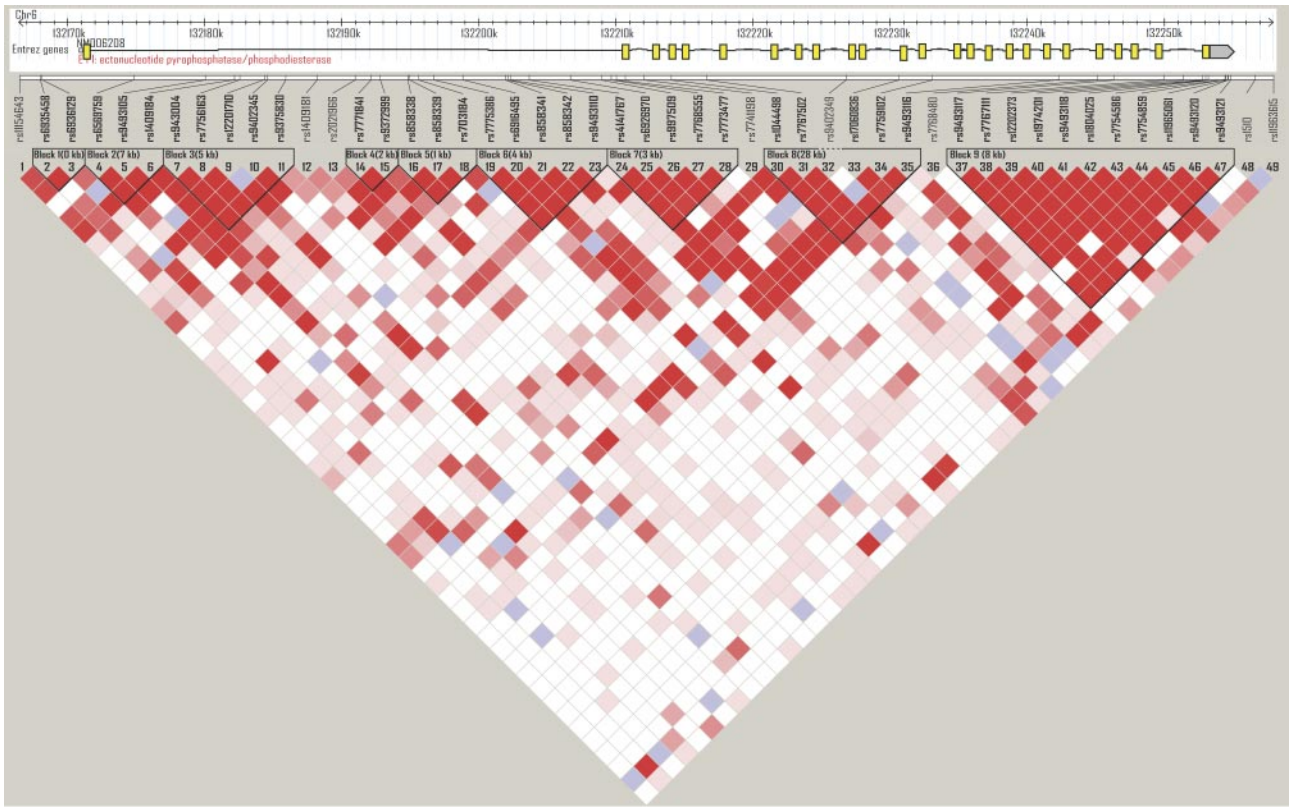

FIG. 2. LD structure of the ENPP1 gene in African-American case subjects (n = 577), with haplotype blocks based on the definition of Gabriel et al. (25) implemented in Haploview (21). D′ values are displayed in the squares. Empty squares represent a pairwise D′ = 1. Red squares represent high pairwise LD, coloring down to white squares of low pairwise LD.

contained in the "*P* haplotype": rs9493118 in intron 24 tags rs1044548 (r^2 = 0.86) and rs1044558 (r^2 = 1), while rs9493120, located in the 3′UTR, tags rs11964389 (r^2 = 0.90). Overlapping two- and three-SNP haplotypes across a 4.7-kb region encompassing parts of intron 24, exon 25, and the 3′ UTR were associated with type 2 diabetic ESRD in African Americans (supplementary Table 3). Although no functional role for the associated SNPs in this African-American study has been determined, the positive association provides encouraging support that functional variants in the distal region of the ENPP1 gene may contribute to type 2 diabetes or diabetic nephropathy susceptibility in African Americans.

The extensively studied K121Q variant (rs1044498) was analyzed in the dataset; however, it did not reveal any evidence for association with type 2 diabetic ESRD in this African-American population (supplementary Table 3). The minor allele frequency for the C allele of rs1044498 (corresponding to the Q allele) was 78.7 and 79.8% in the African-American control and case subjects, respectively. These frequencies are similar to previous African-American estimates (5,13) but are higher than estimates obtained from an Afro-Caribbean Dominican Republic population (14). Other ethnicities such as Japanese (10.5%), Chinese (4.2%), Caucasian Americans (14%), and Polish (13%) show a large divergence in frequencies for the 121Q allele (9,30), and replication has not been successful in several populations including U.K. (9,10), Japanese (30), and African Americans (5). The lack of replication with K121Q in the current study of African Americans may be due to a lack of statistical power. Power analyses using Power for Association With Error (PAWE) (31,32) and the parameters odds ratio (OR) = 1.07, 79% allele frequency (as seen in the current study of African Americans), and 7% disease prevalence suggest 10, 54.4, and 83.4% power to detect association for the additive model using 500, 5,000, and 10,000 case and control subjects. For the same sample sizes, there is 9.4, 49.7 and 78.8% power to detect association using a recessive genotypic model. These calculations suggest extremely large samples would be required to detect a significant association with ENPP1 K121Q in this population.

One limitation of this study was that our ascertainment scheme did not permit us to distinguish whether associations were with type 2 diabetes and/or nephropathy. ENPP1 is expressed in the kidney and has shown significant association with type 2 diabetes (3,7,8,12), as well as type 1 diabetic nephropathy (17,18); therefore, variants in this gene may play a role in both diabetes and nephropathy. In an effort to determine whether the observed associations were primarily due to type 2 diabetes and/or ESRD, we genotyped the nine associated SNPs and the K121Q variant in 328 individuals with type 2 diabetes in the absence of overt nephropathy ("type 2 diabetes only"), as well as 326 individuals with ESRD due to causes other than type 2 diabetes. The association results (supplementary Table 6) and odds ratios (supplementary Table 7) using these samples and the common control subjects suggest that the positive associations are largely driven by the type 2 diabetes component of our African-American type 2 diabetic ESRD population. Seven of the nine SNPs associated with the type 2 diabetic ESRD were also associated with type 2 diabetes in the absence of nephropathy in at least one model, with comparable effect sizes but reduced significance, probably due to the smaller sample size. The exception is SNP rs9493120, which was signifi-

cantly associated in all three populations with ORs ranging from 0.45 to 0.78 in the type 2 diabetes only and ESRD populations and from 0.74 to 0.83 in the type 2 diabetic ESRD analyses (supplementary Tables 4 and 7). In line with previous associations with both metabolic and nephropathy phenotypes, ENPP1 variants may plausibly have a role in both traits. Analyses of independent African-American populations and larger samples of diabetic patients without nephropathy will be needed to resolve this question. Additional analyses showed that the two SNPs most significantly associated with type 2 diabetic ESRD, rs7754586 and rs1974201, were not associated with age at type 2 diabetes diagnosis, age at ESRD onset, or duration of diabetes before onset of ESRD in type 2 diabetic ESRD cases (data not shown).

The majority of control subjects were not tested for diabetes. However, serum glucose values were obtained for 256 of the 596 African-American control subjects (mean 93.6 mg/dl). Five of these individuals had glucose levels >126 mg/dl. Four of these measures were nonfasting, and the fifth had unknown dietary status, suggesting the overall misclassification rate of control subjects is likely to be <2%. While this has not affected our ability to detect positive associations with ENPP1, it has likely reduced our power to detect more subtle influences of additional variants and has led to underestimated odds ratios.

This study represents the most comprehensive evaluation of ENPP1 variants in relation to type 2 diabetes and/or ESRD susceptibility to date. By analyzing variants throughout the entire gene, we were able to detect significant association outside of the K121Q variant. Associated SNPs, located in intron 24 and the 3′ UTR, have not been reported in previous diabetes/nephropathy association studies of ENPP1. The proximity of the two most significantly associated SNPs in high LD ($r^2 = 0.98$) and the prior identification of functional variants in exon 23 (28) support a role for variants in the distal region of ENPP1 in relation to type 2 diabetic ESRD susceptibility in African Americans. These results indicate that this region of the ENPP1 gene warrants further examination to investigate the biological relevance of variation in these regions.

TABLE 2
Unadjusted and admixture-adjusted genotypic results for SNPs associated with type 2 diabetic ESRD

Marker	Physical position*	Location	Dominant model *P* value	Dominant admixture-adjusted *P* value	Additive model *P* value	Additive admixture-adjusted *P* value	Recessive model *P* value	Recessive admixture-adjusted *P* value
rs7771841	132192798	Intron 1	0.060	0.102	**0.025**	**0.046**	0.066	0.085
rs9402349	132226801	Intron 9	**0.039**	0.143	—	—	—	—
rs17060836	132230641	Intron11	0.849	0.515	0.570	0.938	**0.020**	**0.046**
rs7759102	132233100	Intron13	**0.043**	0.083	—	—	—	—
rs7767111	132249978	Intron24	**0.045**	**0.018**	**0.031**	**0.010**	0.168	0.096
rs1974201	132252814	Intron24	**0.004**	**0.011**	**0.0005**	**0.002**	**0.005**	**0.015**
rs9493118	132253058	Intron24	**0.048**	**0.019**	**0.029**	**0.010**	0.139	0.078
rs7754586	132254435	3′ UTR	**0.003**	**0.010**	**0.0005**	**0.002**	**0.007**	**0.018**
rs9493120	132254694	3′ UTR	0.080	**0.035**	**0.047**	**0.017**	0.151	0.088

P values <0.05 are shown in bold; *NCBI Build 35 (May 2004).

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