Functional \textit{ENTPD1} Polymorphisms in African-Americans with Diabetes and End-Stage Renal Disease

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Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org
ABSTRACT

Objective: The vascular ectonucleotidase ENTPD1 protects against renal injury and modulates glucose homeostasis in mouse models. We sought to determine whether human variation in ENTPD1 influences predisposition to diabetes or diabetic nephropathy.

Research Design and Methods: We analyzed ENTPD1 single nucleotide polymorphisms (SNPs) in 363 African-Americans controls, 380 subjects with type 2 diabetes and end-stage renal disease (DM-ESRD), and 326 subjects with ESRD unrelated to diabetes. Using human cell lines, we correlated disease-associated ENTPD1 haplotypes with ENTPD1 gene expression. Finally, we studied consequences of ENTPD1 deletion in a mouse model of type 2 diabetes (db/db).

Results: A common ENTPD1 two-SNP haplotype was associated with increased risk for DM-ESRD (p=0.0027), and an uncommon 4-SNP haplotype was associated with protection against DM-ESRD (p=0.004). These haplotypes correlated with ENTPD1 gene expression levels in human cell lines in vitro. Subjects with high ENTPD1-expressing haplotypes were enriched in the DM-ESRD group. By crossing ENTPD1-null mice with db mice, we show that ENTPD1 deletion has prominent effects on metabolic syndrome traits. Specifically, deletion of ENTPD1 lowered glucose levels in control (db/-) mice with one functional leptin receptor, and dramatically lowered weights in db/db mice with no functional leptin receptors. Similar effects were seen in aged ENTPD1-null mice with normal leptin receptors.

Conclusion: ENTPD1 polymorphisms appear to influence susceptibility to type 2 diabetes and/or diabetic nephropathy in African-Americans. Studies in human cell lines and in vivo mouse data support a potential role for ENTPD1 genetic variation in susceptibility to type 2 diabetes.
Diabetes and its complications are enormous sources of mortality, morbidity, and cost in the United States. African-Americans are especially prone to diabetes and diabetic kidney disease. Genetic variation plays a major role in susceptibility to both development of diabetes and onset of diabetic renal injury. Finding genes responsible for this predisposition to disease could help predict who may be at high risk and also identify important pathways involved in disease pathogenesis.

ENTPD1, also known as CD39, is an ectonucleotidase that hydrolyzes ATP and ADP to AMP, initiating an enzymatic cascade that leads to the generation of adenosine. By regulating nucleotide levels, ENTPD1 controls the activity of both purinergic receptors (P2X and P2Y) and downstream adenosine (P1) receptors. ENTPD1 is widely expressed, particularly in blood vessels and on cells of the immune system. ENTPD1 is present in glomeruli, afferent arterioles, and larger vessels of the kidney. Mice null for ENTPD1 sustained markedly more severe renal injury than wild type mice in models of type 1 diabetes and ischemia-reperfusion injury.

Previously, a locus for susceptibility to diabetic and non-diabetic renal disease was located on human chromosome 10q24 in African-Americans. Subsequently, others have also found loci in this chromosomal region associated with renal dysfunction in Caucasians with type-2 diabetes. It appears likely that one or more genes with effects on nephropathy are located in this region. ENTPD1 is located close to the peak LOD-score marker in both of these studies.

More recently, we have reported that ENTPD1-null mice also exhibit abnormalities of glucose homeostasis. The close functional relationship between ENTPD1 and another ectonucleotidase that influences metabolic syndrome traits in humans, ENPP1, suggests that ENTPD1 could affect glucose homeostasis in humans as well.

To determine whether variation in ENTPD1 might influence the development of diabetes or diabetic renal disease, we tested the frequency of single nucleotide polymorphisms (SNPs) in ENTPD1 in three groups of African-American patients: 363 healthy controls, 326 patients with non-diabetic End-Stage Renal Disease (non-DM ESRD), and 380 patients with ESRD caused by type-2 diabetes (DM ESRD). We support these genetic association results with functional studies in vitro using human cell lines and in vivo using mouse models with non-functional leptin receptors (db/db). We show that ENTPD1 gene expression in human cell lines correlates with the disease-associated genotypes, and mice null for ENTPD1 share metabolic phenotypes with humans who have low ENTPD1-expressing genotypes.

METHODS

Human Subjects. Collection of controls and cases have been described previously. Briefly, All subjects were recruited in North Carolina, South Carolina, Georgia, Virginia, or Tennessee, and all were born in one of these 5 southeastern states. 363 unrelated African-American controls were recruited through community screening events, all without a history of type 2 diabetes or renal disease. 380 unrelated African-American patients with type 2 diabetes and end-stage renal disease were recruited (DM ESRD) from dialysis clinics. 326 unrelated African-American
patients with end-stage renal disease but without a history of diabetes (non-DM ESRD) were also recruited from dialysis clinics. A single investigator (BIF) classified all ESRD patients into DM ESRD or non-DM ESRD. Non-DM ESRD patients included those with "hypertension-associated ESRD" or primary glomerular diseases. The DM ESRD groups included patients with either 1) diabetes and histologic evidence of diabetic nephropathy or 2) with diabetes ≥ 5 years prior to the initiation of renal replacement therapy and either 2a) diabetic retinopathy or 2b) proteinuria >500 mg per 24 hours. DM ESRD patients had no other known cause of renal disease. Patients with renal cystic diseases, hereditary nephritis, or ESRD related to urologic disease were all excluded from analysis.

Genotyping. 18 SNPs tagging 80% of the known SNPs in the Yoruba (YRI) Hapmap population were selected using the program Tagger (www.broad.mit.edu/mpg/tagger). Tag SNPs all had minor allele frequency of ≥ 5%. rs3793744, the only common non-synonymous coding variant in ENTPD1, was force inserted in SNP selection. Tag SNPs spanned the ENTPD1 gene on chromosome 10 from 97.504Mb to 97.613Mb. We used a Sequenom MassArray platform(17) for genotyping.

Genetic Analysis. SNPs with call rates <96% were excluded from analysis. All SNPs were in conformation with Hardy-Weinburg Equilibrium (p<0.05) in controls. Determination of haploblock structures and associations were performed using Haploviev software at 10,000 permutations. In vitro ENTPD1 expression studies. Genotyped, EBV-transformed lymphoblasts from the YRI HapMap (www.hapmap.org) collection and Perlegen (www.perlegen.com) African-American panel were used for analysis. Cells were grown in RPMI 1640 media with 2mM L-glutamine, 15% fetal bovine serum, penicillin-streptomycin mix, and 10mM Hepes buffer. Cells were seeded at 200,000 per ml and harvested 24-48 hours later in log-phase growth.

Then, mRNA was isolated using RNeasy (Qiagen) according to the manufacturer's instructions. 0.5 ug of mRNA was reversed transcribed to cDNA using the Taqman Reverse Transcription Kit (Applied Biosystems). Probe-primer sets for ENTPD1 and the 18S ribosomal subunit (loading control) were obtained from Applied Biosystems. Real-time PCR was performed on an Applied Biosystems 7700 system. ENTPD1 mRNA expression was normalized using 18S expression.

For protein isolation, cells were washed twice with ice-cold PBS and then lysed in an NP-40 based buffer with protease inhibitors (Roche mini-tabs). 40 µg of protein were separated on a gradient gel under non-reducing conditions and transferred to PVDF paper (Millipore) using a semi-dry transfer apparatus. For ENTPD1, we used a mouse monoclonal antibody (BU-61, Ancell) and a goat anti-mouse secondary antibody (Pierce). We used anti-GAPDH antibody (Ambion) as a loading control. Band density was quantified with a Kodak ImageStation 2000M, and protein level was expressed as a ratio of ENTPD1 to GAPDH.

In silico, expanded ENTPD1 expression studies. For YRI HapMap subjects,
ENTPD1 gene expression levels were downloaded from (http://www.sanger.ac.uk/humgen/geneva r/). Methods for the generation of this data are described in Stranger et al.(18) Genotypes were obtained from www.hapmap.org. Statistics were performed using student T-test on log2 transformed data.

Enzyme Activity. 250,000 lymphocytes were harvested in log-phase growth. ATPase and ADPase activity were determined with a Malachite Green assay for phosphate production as described previously using 200μM nucleotide as the substrate during a ten minute incubation.(19) Tetramisole was used to inhibit alkaline phosphatases.(19)

Animal Studies. All mouse studies were done with IACUC approval at Beth Israel Deaconess Medical Center. Mice in all 4 db groups originated from crossing db/- heterozygote breeders on a C57/BL6 background from Jackson Labs with ENTPD1-/- breeders backcrossed >6 times on a C57/BL6 background. Diabetic mice were db/db offspring with or without ENTPD1, and db/- offspring were used as controls. In the aging study, male ENTPD1-/- mice backcrossed >6 times onto a C57/BL6 background were compared with age and strain-matched C57/BL6 wild-type controls.

Animal Phenotyping. Albuminuria was quantified using an albumin to creatinine ratio. Albumin was measured using the Albuwell-M kit from Exocell and creatinine was measured using the Creatinine Companion from Exocell. Blood glucose was measured using a One-touch Ultra 2 glucometer (Lifescan). For the db-based studies, non-fasting glucose levels were measured at 3PM. For the non-db studies (aging), glucose levels were measured after an 8-hour daytime fast.

For histopathology, mouse kidney tissue was fixed in 10% buffered formalin and embedded in paraffin, then cut into 3-micron sections and stained with PAS stain. Glomerular area was calculated using Spot imaging software. The glomerular sclerosis index has been described previously.(9)

RESULTS

Using a set of markers chosen with the program Tagger, we genotyped 18 SNPs (MAF>5%) in ENTPD1 that were predicted to cover 80% of the known SNPs in the Yoruba (YRI) Hapmap population. Seventeen of 18 SNPs passed quality standards and conformed to HWE criteria as described in Methods above. These 17 SNPs tagged 79% of YRI HapMap SNPs.

Location of SNPs in the ENTPD1 gene, minor allele frequency of each SNP, and uncorrected p-values in the populations in this study are shown in Table 1. When controls and subjects with non-DM ESRD were compared, one SNP (rs6584026) showed nominal significance (p=0.027). Comparing controls and subjects with DM ESRD revealed two SNPS (rs12763743 and rs3897983) that were nominally significant (0.041 and 0.037 respectively).

We considered the possibility that the two nominally significant SNPs for DM ESRD might have additive effects. We used the two significant SNPs discovered in the DM ESRD population to form a two-SNP haplotype for further analysis. Three common combinations accounted for all subjects tested (AG=82.9%, AA=10.8%, and TG=6.3%). The most common haplotype (AG) appeared to confer increased risk of diabetes and/or diabetic nephropathy (p=0.0027, p=0.009 after permutation; OR=1.51) compared to the other two haplotypes, which were
both nominally associated with protection from diabetes and/or diabetic nephropathy (Table 2a). We refer to the AG haplotype as the "2-SNP risk haplotype," and the combined group of AA and TG haplotypes as the "2-SNP protective haplotypes." No difference was seen with respect to these 2-SNP risk or protective haplotypes when comparing controls with non-DM ESRD subjects.

A broader, unbiased haplotype analysis using all successfully genotyped SNPs showed that 16 of the SNPs fell into a single haplotype block defining 10 haplotypes with frequency >1%. One haplotype was associated with protection against diabetes and/or diabetic nephropathy with p=0.004 (OR=0.34). This statistical significance of this protective haplotype remained unchanged when reduced to 4 SNPs (rs4918972, rs10748648, rs1342790, rs3793744; GAGA in Table 2b). After permutation analysis, the p-value remained significant at p=0.017. The GAGA haplotype is referred to as the "4-SNP protective haplotype." This protective haplotype included the uncommon variant rs3793744, a coding SNP in exon 7 causing a valine to isoleucine substitution. No association was seen for this haplotype when comparing controls to non-DM ESRD subjects.

We made several additional comparisons to help define whether our haplotypes were associated with diabetes or diabetic nephropathy. First, we compared healthy controls with all ESRD subjects (both DM ESRD and non-DM ESRD), and found much weaker associations for both the 2-SNP and 4-SNP haplotypes (supplementary table 1). Then we compared non-DM ESRD versus DM ESRD subjects: the 2-SNP (AG) risk haplotype remained robust (p=0.0038, OR=1.53) while the 4-SNP (GAGA) protective haplotype lost some statistical power (p=0.024), though the odds ratio was unchanged (OR=0.37) when non-ESRD were substituted for controls. We also pooled healthy controls and non-DM ESRD into a "non DM" group, and compared them with DM ESRD subjects. Here, the p-value of the 2-SNP (AG) risk haplotype improved to 0.0008 (OR=1.51), while the 4-SNP (GAGA) protective haplotype was similar to the control versus DM ESRD comparison (p=0.0056, OR=0.37). Taken together, these analyses tend to support a genetic association with diabetes rather than diabetic nephropathy.

To determine whether either of these disease-associated haplotypes correlated with general expression levels of ENTPD1, we took advantage of the fact that ENTPD1 is highly expressed on B-lymphocytes(6). For the 2-SNP haplotype, we examined YRI Hapmap lymphoblasts for subjects who were homozygous for the 3 different haplotype groups. While many subjects in the YRI population were homozygous for the AG risk haplotype, only 2 were homozygous for the AA protective haplotype and only one was homozygous for the TG protective haplotype. Since the AA and TG haplotypes were almost identical in terms of risk (p=0.04, nominally protective) and odds ratios (0.70 and 0.64, respectively), we pooled these into a single group and compared ENTPD1 expression from their lymphocytes with those from the risk (AG) haplotype group using real-time PCR (Fig. 1A). Lymphocytes homozygous for the AG risk haplotype had 39% higher ENTPD1 expression levels than homozygotes for the protective AA or TG haplotypes (Protective=5.62 +/- 0.36 vs. Risk=7.81
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+/- 0.36; p=0.007), with no overlap between groups (range 5.09-6.30 for Protective and 6.91-9.53 for Risk). Not enough cell lines homozygous for the 4-SNP protective haplotype (1 cell line) were available for comparison.

To provide additional support for a correlation between haplotype and expression level, we used gene expression data from Stranger et al. generated from YRI HapMap cell lines, which allowed us to incorporate haplotype heterozygotes into the analysis. For consistency, we analyze the 2- and 4-SNP haplotypes in terms of number of protective alleles. We pooled subjects with 1 or 2 protective alleles for the 2-SNP haplotype and compared them with subjects with no protective alleles (2 risk alleles). Subjects with zero protective haplotypes (n=31) expressed 16% more ENTPD1 than subjects with 1 or 2 protective haplotypes (n=29; p=0.027)(Fig. 1B). We then pooled subjects with 1 or 2 copies of the protective 4-SNP allele (n=7) and compared them to those with 0 copies (n=53). While the difference was not significant (p=0.09), those with no protective alleles expressed 16% more ENTPD1 than those with 1 or 2 protective alleles (Figure 1C). These cell lines with at least one protective 4-SNP allele had the lowest mRNA expression levels of any haplotype group, including the protective 2-SNP haplotype. To account for the fact that we were comparing subjects with protective 4-SNP alleles versus a pool that contained many more subjects with one or more protective 2-SNP allele, we then compared subjects based on their total number of protective alleles (# 2-SNP protective alleles plus # 4-SNP protective alleles) to integrate the effects of 2-SNP and 4-SNP haplotypes on ENTPD1 expression. Those with no protective alleles (n=29) expressed 18% more ENTPD1 than subjects with at least one protective 2-SNP allele or one protective 4-SNP allele (n=31)(p=0.01). Both haplotypes appear to correlate with ENTPD1 expression, and were especially predictive of mRNA expression levels when used in tandem. The small number of subjects with the protective 4-SNP allele prevented us from determining if the effects of the 2-SNP and 4-SNP haplotypes were independent.

The protective 4-SNP haplotype (GAGA) included a SNP causing an amino acid change (rs3793744: V to I at position 293). A second, uncommon haplotype (GTGA) also included the minor allele (A) at rs3793744, but was not associated with DM ESRD when compared to controls. We compared expression levels between cell lines with 1 or more copies of each of these haplotypes. Remarkably, the non-protective (GTGA) 4-SNP haplotype including the minor allele at rs3793744 expressed 45% more ENTPD1 mRNA than the protective (GAGA) 4-SNP allele with the same SNP at rs3793744 (n=3 vs n=7, p=0.0006). This finding suggests that differences in mRNA expression underly the effect of the 4-SNP haplotype rather than the amino acid substitution. We did note, though, that protein expression in the homozygotes for the protective 4-SNP haplotype were surprisingly low compared to other cell lines (Supplementary Fig. 1A-B). This finding was demonstrated in single samples from two separate collections (HapMap and Perlegen), and activity assays showed that the effect was not due to differential antibody binding at the amino acid change at position 293 (rs3793744)(Supplementary Fig. 1C-D).

To further investigate the effects of ENTPD1 on either diabetes or diabetic
nephropathy, we crossed ENTPD1-null mice with db/db mice to create a type 2 model of diabetes and type 2 diabetic nephropathy.

Assessment for renal injury at eight months of age demonstrated that in the non-diabetic mice (db/-), ENTPD1 deletion caused mildly increased albuminuria (WT=37.2 +/- 2.3 ug/mg vs. KO=54.7 +/- 6.7 ug/mg; p=0.028) and a trend toward increased glomerular sclerosis (Fig. 3A-B). In the diabetic groups, there were no differences in albuminuria, sclerosis index, or glomerular area between db/db mice with and without ENTPD1 (Fig. 3C-E).

Metabolic syndrome parameters revealed more dramatic differences. In non-diabetic mice, non-fasting (3PM) glucose was lower in ENTPD1-null mice at both 8 weeks (WT=208.1 +/- 4.8 mg/dL vs KO=176.8 +/- 7.8 mg/dL; p=0.006) and 6 months (WT=179.2 +/- 5.7 mg/dL vs KO=161.9 +/- 5.8 mg/dL; p=0.039) (Fig. 4A-B). No differences in glucose levels were seen in the db/db groups at these time points with or without ENTPD1 (Fig. 4C-D). While weights of non-diabetic mice were no different (fig. 5A-B), large differences were seen in db/db mice, with deletion of ENTPD1 protecting against obesity in the db/db mice at both 8 weeks (WT=41.4 +/- 1.0g vs KO=37.6 +/- 1.4g; p=0.026) and 6 months (WT=62.4 +/- 0.9g vs KO=52.0 +/- 1.3g; p<0.000001) (Fig. 5C-D).

We also aged WT and ENTPD1-null mice (leptin receptor normal) to see if aging would cause similar metabolic differences as we saw in the db/db model. At two years of age, ENTPD1-null mice had markedly lower fasting glucose (WT=141.6 +/- 5.1 mg/dL vs KO=112.8 +/- 3.3 mg/dL; p=0.0009) and lower weights (WT=46.4 +/- 2.7g vs KO=39.2 +/- 0.7; p=0.042) than WT controls (Fig. 6A-B). Consistent with the db experiments, we did also note increased albuminuria in aged ENTPD1-null mice despite lower weights and blood glucoses (data submitted for publication).

**DISCUSSION**

Our previous studies in ENTPD1-null mice suggested a role for this ectonucleotidase in both resistance to renal injury and in glucose homeostasis. We tested polymorphic markers in the ENTPD1 gene in humans for association with DM ESRD and non-DM ESRD in African-Americans, a group of Americans with high rates of both diseases. We found two common polymorphisms (rs12763743 and rs3897983) that are nominally associated with diabetic but not non-diabetic ESRD. When used together, these two polymorphisms showed even stronger association (p=0.0027) with subjects in the diabetic nephropathy group. These two SNPs may represent independent, additive effects or they may together tag a causal SNP better than either of the SNPs alone.

More general haplotype analysis also identified an uncommon 4-SNP haplotype that appears to protect against diabetes or diabetic nephropathy (p=0.004). The effect size of this uncommon haplotype is strong, conferring a relative risk of 0.34. Additional groupwise comparisons using pooled groups further suggested that the association was due to diabetes rather than nephropathy in the DM ESRD groups.

To support association the association of ENTPD1 with diabetes and/or diabetic nephropathy, we tested HapMap cell lines derived from African subjects for ENTPD1 expression. Cell lines homozygous for the two-SNP (AG) risk haplotype expressed 39% more ENTPD1 mRNA than those cell lines with two protective
haplotypes (AA and TG). We performed additional bio-informatic analysis that showed similar results: zero alleles of the 2-SNP protective haplotypes led to 16% greater ENTPD1 expression than 1 or 2 protective alleles. The protective 4-SNP haplotype also showed a trend toward lower ENTPD1 expression levels, though this analysis was limited by a smaller number of representative cell lines and was confounded by comparison with a group including many protective 2-SNP haplotypes. In fact, the protective 4-SNP haplotype showed the lowest ENTPD1 expression of any group. By using an index that took into account the total number of both the 2- and 4-SNP protective haplotypes, we identified a strong correlation between genotype and ENTPD1 mRNA expression level using both haplotypes derived from our genetic association study. Additional experiments measuring ENTPD1 protein levels confirmed that the protective 4-SNP haplotype was associated with very low ENTPD1 levels and may suggest an effect beyond mRNA expression, though we did not have sufficient samples available to us to prove this hypothesis definitively.

We also used genetic studies in mice to assess the role of ENTPD1 on diabetes and diabetic nephropathy. While ENTPD1-null control mice did have increased albuminuria compared to WT, perhaps the more striking findings related to metabolic syndrome traits. In particular, ENTPD1-null mice had lower glucose levels in the absence of the homozygous db/db mutation, and dramatically lower weights in the presence of the db/db mutation. Notably, previous work demonstrated that ENTPD1-null mice have higher leptin levels than WT mice,(13) potentially a compensatory response that is disabled in the absence of one or both copies of the normal leptin receptor. The exact reason for this differential effect in db/db and db/- mice on blood glucose and weight still remains to be elucidated. However, even in the presence of normal leptin signaling, using WT mice rather than db/- heterozygotes as controls, aging accentuated both phenotypes (i.e. difference in glucose homeostasis and weight gain). Our mouse findings again suggest that the effect we discovered in humans may relate to low ENTPD1 protecting against diabetes in control subjects, though increased albuminuria in the non-diabetic CD39-null mice may point toward an effect on renal disease as well.

Further support for association between ENTPD1 and diabetes and diabetic nephropathy is the finding that polymorphisms in another ectonucleotidase with a remarkably similar enzymatic mechanism, ENPP1, have also been associated with both diabetes and diabetic nephropathy. Nephropathy has been associated with ENPP1 polymorphisms, first in Caucasian populations,(20-22) and subsequently in an African-American population that included our subjects.(23) Polymorphisms in the same gene also have an important influence on metabolic syndrome traits,(14) and there appears to be an important interaction between weight and glucose homeostasis,(24; 25) just as we see with ENTPD1 in mice. While ENPP1 hydrolyzes extracellular ATP to AMP and pyrophosphate, ENTPD1 hydrolyzes extracellular ATP to AMP and 2 phosphate molecules. Low functional expression of either of these ectoenzymes could increase extracellular levels of ATP and decrease levels of the nucleoside adenosine.
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The strength of our study rests on mutually supportive data built from genetic association of human disease with genetic variants, gene expression levels that correlate with the disease-associated haplotypes, and a mouse model that shows phenotypic differences based on expression level of the gene. Ultimately, replication in larger cohorts will be needed to validate our findings. Our data, along with previous studies with ENPP1, suggest that extracellular nucleotide metabolism may be a critical determinant of diabetes and/or diabetic nephropathy.

ACKNOWLEDGEMENTS
This work was supported by NIH grants K08-DK076868 to D.J.F. and P01 HL076540 to S.C.R. We thank Barbara Stranger, Emmanouil Dermitzakis, and their colleagues for making their gene expression data publicly available. We acknowledge Coriell Institute, Perlegen, and the National Human Genome Research Institute (NHGRI) for their role in providing human cell lines to the research community.

REFERENCES
Functional ENTPD1 Polymorphisms


Fig 1. (A.) *ENTPD1* mRNA expression by real time PCR comparing YRI Hapmap cell lines homozygous for the 2-SNP risk haplotype AG (n=7) vs. the protective haplotypes AA and TG (n=3), normalized for 18S expression. *ENTPD1* mRNA levels are 39% higher in cell lines with zero protective haplotypes than in cell lines with 2 protective haplotypes. (B.) In silico *ENTPD1* expression for YRI cell lines with 0 (n=31) vs. 1 or 2 (n=29) copies of the 2-SNP protective haplotypes AA and TG. Zero copies of the protective haplotype conferred 16% higher levels of *ENTPD1* expression. (C.) *ENTPD1* expression for YRI cell lines with 0 (n=53) vs. 1 or 2 (n=7) copies of the 4-SNP protective (GAGA) haplotype. Zero copies of the protective haplotype were associated with 16% higher *ENTPD1* expression. Though non-significant, cell lines with 1 or 2 protective copies had lower expression than all other groups tested. (D.) Protective index incorporating both the 2-SNP and 4-SNP haplotypes. The total number of protective 2-SNP and 4-SNP protective alleles was summed for each cell line. Cell lines with zero protective alleles (n=29) had 18% more *ENTPD1* expression than cell lines with at least 1 protective allele (n=31). (E.) Comparison of *ENTPD1* mRNA expression of protective 4-SNP haplotype (GAGA, n=7) and uncommon 4-SNP haplotype (GTGA, n=3) that share the minor allele (A) at rs3793744 that codes for a valine to isoleucine substitution at amino acid 293. The difference indicates that rs3793744 is not itself the causal SNP.
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E.

![Bar graph showing relative ENTPD1 expression levels for GTGA and GAGA genotypes. The p-value is 0.0006.](image)

Minor allele (A) at rs3793744
Fig 2. (A.) Albumin:creatinine ratio for db/- non-diabetic mice. N=7 for ENTPD1 WT and 12 for ENTPD1 KO. (B.) Sclerosis Index for db/- non-diabetic mice. N=7 for ENTPD1 WT and 12 for ENTPD1 KO. (C.) Albumin:creatinine ratio for db/db mice. N=13 for ENTPD1 WT and 9 for ENTPD1 KO. (D.) Sclerosis Index for db/db mice. N=13 for ENTPD1 WT and 10 for ENTPD1 KO. (E.) Glomerular area for db/db mice. N=10 for ENTPD1 WT and 8 for ENTPD1 KO.
Fig 3. (A. and B.) Blood glucose levels for db/- non-diabetic mice at (A.) 8 weeks and (B.) 6 months. N=9 for ENTPD1 WT and 12 for ENTPD1 KO. (C. and D.) Blood glucose levels for db/db mice at (C.) 8 weeks and (D.) 6 months. N=19-20 for ENTPD1 WT and 12-13 for ENTPD1 KO.
Fig 4. (A. and B.) Weights for db/- non-diabetic mice at (A.) 8 weeks and (B.) 6 months. N=9 for ENTPD1 WT and 12 for ENTPD1 KO. (C. and D.) Weights for db/db mice at (C.) 8 weeks and (D.) 6 months. N=19-20 for ENTPD1 WT and 13-15 for ENTPD1 KO.
Fig 5. (A.) Fasting glucose levels in mg/dL for 2-year old WT (n=8) and ENTPD1-null (n=6) mice. (B.) Weight at 2 years in WT (n=7) and ENTPD1-null (n=11) mice.
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<td>0.142</td>
<td>0.79 (0.57-1.08)</td>
<td>0.694</td>
</tr>
<tr>
<td>rs10748648</td>
<td>97576857</td>
<td>intron</td>
<td>T/A</td>
<td>0.457</td>
<td>0.471</td>
<td>0.450</td>
<td>0.613</td>
<td>1.06 (0.85-1.31)</td>
<td>0.805</td>
</tr>
<tr>
<td>rs12763743</td>
<td>97582146</td>
<td>intron</td>
<td>A/T</td>
<td>0.077</td>
<td>0.097</td>
<td>0.051</td>
<td>0.192</td>
<td>1.29 (0.88-1.90)</td>
<td><strong>0.041</strong></td>
</tr>
<tr>
<td>rs1342790</td>
<td>97590529</td>
<td>intron</td>
<td>A/G</td>
<td>0.474</td>
<td>0.484</td>
<td>0.478</td>
<td>0.703</td>
<td><strong>0.84 (0.68-1.05)</strong></td>
<td>0.873</td>
</tr>
<tr>
<td>rs3176883</td>
<td>97594931</td>
<td>intron</td>
<td>T/C</td>
<td>0.247</td>
<td>0.287</td>
<td>0.288</td>
<td>0.104</td>
<td>1.23 (0.96-1.57)</td>
<td>0.087</td>
</tr>
<tr>
<td>rs12244606</td>
<td>97596321</td>
<td>intron</td>
<td>C/T</td>
<td>0.068</td>
<td>0.092</td>
<td>0.084</td>
<td>0.094</td>
<td>1.40 (0.94-2.08)</td>
<td>0.239</td>
</tr>
<tr>
<td>rs3181123</td>
<td>97597065</td>
<td>intron</td>
<td>C/T</td>
<td>0.349</td>
<td>0.377</td>
<td>0.370</td>
<td>0.280</td>
<td>1.13 (0.91-1.41)</td>
<td>0.395</td>
</tr>
<tr>
<td>rs3793744</td>
<td>97597256</td>
<td>exon 7 (V-I)</td>
<td>G/A</td>
<td>0.043</td>
<td>0.050</td>
<td>0.029</td>
<td>0.600</td>
<td>1.15 (0.69-1.92)</td>
<td>0.146</td>
</tr>
<tr>
<td>rs3897983</td>
<td>97608187</td>
<td>intron</td>
<td>G/A</td>
<td>0.125</td>
<td>0.104</td>
<td>0.091</td>
<td>0.223</td>
<td>0.81 (0.58-1.14)</td>
<td><strong>0.037</strong></td>
</tr>
<tr>
<td>rs3181112</td>
<td>97608570</td>
<td>intron</td>
<td>T/C</td>
<td>0.096</td>
<td>0.088</td>
<td>0.086</td>
<td>0.612</td>
<td>0.90 (0.63-1.32)</td>
<td>0.504</td>
</tr>
<tr>
<td>rs11598475</td>
<td>97611256</td>
<td>intron</td>
<td>G/A</td>
<td>0.132</td>
<td>0.119</td>
<td>0.135</td>
<td>0.448</td>
<td>0.88 (0.64-1.22)</td>
<td>0.897</td>
</tr>
<tr>
<td>rs3181118</td>
<td>97613366</td>
<td>intron</td>
<td>C/T</td>
<td>0.032</td>
<td>0.029</td>
<td>0.027</td>
<td>0.725</td>
<td>0.89 (0.48-1.67)</td>
<td>0.555</td>
</tr>
</tbody>
</table>

Table 1. Single nucleotide polymorphism analysis.
Individual SNP results for the association study comparing African-American controls with DM2 ESRD and Non-DM ESRD. ***rs3176894 did not meet quality control criteria. # denotes nominal significance (p< 0.05). MAF (Minor Allele Frequency). When the odds ratio > 1, the minor allele is the risk allele; when the odds ratio is < 1, the minor allele is the protective allele.
**2a.**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>Controls</th>
<th>Cases</th>
<th>$\chi^2$</th>
<th>p-value</th>
<th>OR (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG</td>
<td>0.829</td>
<td>0.799</td>
<td>0.857</td>
<td>9.0</td>
<td>0.0027</td>
<td>1.51 (1.15-1.99)</td>
</tr>
<tr>
<td>AA</td>
<td>0.108</td>
<td>0.124</td>
<td>0.091</td>
<td>4.2</td>
<td>0.0404</td>
<td>0.70 (0.51-0.98)</td>
</tr>
<tr>
<td>TG</td>
<td>0.063</td>
<td>0.077</td>
<td>0.051</td>
<td>4.1</td>
<td>0.0431</td>
<td>0.64 (0.42-0.98)</td>
</tr>
</tbody>
</table>

**2b.**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>Controls</th>
<th>Cases</th>
<th>$\chi^2$</th>
<th>p-value</th>
<th>OR (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAGG</td>
<td>0.432</td>
<td>0.428</td>
<td>0.436</td>
<td>0.10</td>
<td>0.7495</td>
<td>1.03 (0.84-1.27)</td>
</tr>
<tr>
<td>GTAG</td>
<td>0.393</td>
<td>0.386</td>
<td>0.398</td>
<td>0.23</td>
<td>0.6323</td>
<td>1.05 (0.85-1.30)</td>
</tr>
<tr>
<td>ATAG</td>
<td>0.139</td>
<td>0.141</td>
<td>0.138</td>
<td>0.04</td>
<td>0.8336</td>
<td>0.97 (0.72-1.30)</td>
</tr>
<tr>
<td>GAGA</td>
<td>0.023</td>
<td>0.035</td>
<td>0.012</td>
<td>8.26</td>
<td>0.0041</td>
<td>0.34 (0.16-0.73)</td>
</tr>
<tr>
<td>GTGA</td>
<td>0.013</td>
<td>0.010</td>
<td>0.016</td>
<td>0.09</td>
<td>0.3259</td>
<td>1.67 (0.65-4.26)</td>
</tr>
</tbody>
</table>

**Table 2. Haplotype analysis**

(2a.) Association of DM-ESRD with two-SNP haplotypes consisting of the two nominally significant SNPs rs12763743 and rs3897983. The AG haplotype is referred to as the "2-SNP risk haplotype," and the AA and TG haplotypes are referred to as the "2-SNP protective haplotypes."

(2b.) Association of DM-ESRD with four-SNP haplotypes consisting of rs4918972, rs10748648, rs1342790, and rs3793744. The GAGA haplotype is referred to as the "4-SNP protective haplotype."