Functional ENTPD1 Polymorphisms in African Americans With Diabetes and End-Stage Renal Disease

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Diabetes and its complications are enormous sources of mortality, morbidity, and cost in the U.S. African Americans are especially prone to diabetes and diabetic kidney disease (1). Genetic variation plays a major role in susceptibility to both development of diabetes and onset of diabetic renal injury (2,3). 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 (4). 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 (5–7). ENTPD1 is present in glomeruli, afferent arterioles, and larger vessels of the kidney (8,9). Mice null for ENTPD1 sustained markedly more severe renal injury than wild-type mice in models of type 1 diabetes (9) and ischemia reperfusion injury (10).

Previously, a locus for susceptibility to diabetic and nondiabetic renal disease was located on human chromosome 10q24 in African Americans (11). Subsequently, others have also found loci in this chromosomal region associated with renal dysfunction in Caucasians with type 2 diabetes (12). It appears likely that one or more genes with effects on nephropathy are located in this region. ENTPD1 is located close to the linkage peak in both of these studies. More recently, we have reported that ENTPD1-null mice also exhibit abnormalities of glucose homeostasis (13). The close functional relationship between ENTPD1 and another ectonucleotidase that influences metabolic syndrome traits in humans, ENPP1, (14) 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 control subjects, 326 patients with nondiabetic 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 nonfunctional 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.
<table>
<thead>
<tr>
<th>SNPs</th>
<th>Location on chromosome 10 (Mbp)</th>
<th>Location within ENTPD1</th>
<th>Major/minor allele (+) strand</th>
<th>Healthy control subjects (MAF)</th>
<th>Non-DM-ESRD case subjects (MAF)</th>
<th>Type 2 diabetic ESRD case subjects (MAF)</th>
<th>Control vs. non-DM-ESRD (P)</th>
<th>Odds ratio for non-DM-ESRD</th>
<th>Odds ratio for DM-ESRD</th>
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<td>rs3176894</td>
<td>97504440 Promoter</td>
<td>A/G</td>
<td>*</td>
<td>0.106</td>
<td>0.134</td>
<td>0.121</td>
<td>*</td>
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<td>0.325</td>
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<td>97541718 Intron</td>
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<td>0.238</td>
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<td>0.471</td>
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<td>0.450</td>
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<td>0.029</td>
<td>0.027</td>
<td>0.027</td>
<td>*</td>
<td>0.725</td>
<td>0.89 (0.48–1.67)</td>
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</table>

Individual SNP results for the association study comparing African American control subjects with type 2 diabetic ESRD and nondiabetic ESRD. *rs3176894 did not meet quality control criteria. †P < 0.05 (boldface). When the odds ratio is >1, the minor allele is the risk allele; when the odds ratio is <1, the minor allele is the protective allele. MAF, minor allele frequency.
Hardy-Weinburg equilibrium criteria as described in SNPs in the Yoruba (YRI) Hapmap population. Seventeen ENTPD1 that were predicted to cover 80% of the known athy (confer increased risk of diabetes and/or diabetic nephropathy (6.3%). The most common haplotype (AG) appeared to be associated with protection from diabetes and/or diabetic nephropathy (Table 2). We refer to the AG haplotype as the two-SNP risk haplotype, and the AA and TG haplotypes are referred to as the two-SNP protective haplotypes.

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 two-SNP risk haplotype, and the AA and TG haplotypes are referred to as the two-SNP protective haplotypes.

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

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, nonfasting glucose levels were measured at 3:00 p.m. For the non-db studies (aging), glucose levels were measured after an 8-h 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 (minor allele frequency >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 Hardy-Weinberg equilibrium criteria as described in Research Design and 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 control subjects and subjects with non–DM-ESRD were compared, one SNP (rs6584026) showed nominal significance (P = 0.027). Comparing control subjects 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; odds ratio 1.51) compared with the other two haplotypes, which were both nominally associated with protection from diabetes and/or diabetic nephropathy (P = 0.0027, P = 0.009 after permutation; odds ratio 1.51) compared with the other two haplotypes, which were both nominally associated with protection from diabetes and/or diabetic nephropathy (Table 2). We refer to the AG haplotype as the two-SNP risk haplotype, and the combined group of AA and TG haplotypes as the two-SNP protective haplotypes. No difference was seen with respect to these two-SNP risk or protective haplotypes when comparing control subjects 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 (odds ratio 0.34). This statistical significance of this protective haplotype remained unchanged when reduced to four SNPs (rs4918972, rs10748648, rs1342790, and rs3793744; GAGA in Table 2). After permutation analysis, the P value remained significant at 0.017. The GAGA haplotype is referred to as the four-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 control subjects 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 control subjects with all ESRD subjects (both DM-ESRD and non–DM-ESRD) and found much weaker associations for both the two-SNP and four-SNP haplotypes (supplementary Table 1 [available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db08-1214/DC1]). Then we compared non–DM-ESRD with DM-ESRD subjects: the two-SNP (AG) risk haplotype remained robust (P = 0.0038, odds ratio 1.53), while the four-SNP (GAGA) protective haplotype lost some statistical power (P = 0.024), though the odds ratio was unchanged (odds ratio 0.37) when non-ESRD subjects were substituted for control subjects. We also pooled healthy control subjects and non–DM-ESRD subjects into a nondiabetic group and compared them with DM-ESRD subjects. Here, the P value of the two-SNP (AG) risk haplotype improved to 0.0008 (odds ratio 1.51), while the four-SNP (GAGA) protective haplotype was similar to the control versus DM-ESRD comparison (P = 0.0056, odds ratio 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 two-SNP haplotype, we examined YRI Hapmap lymphoblasts for subjects who were homozygous for the three different haplotype groups. While many subjects in the YRI populations were homozygous for the AG risk haplotype, only two

### Table 2: Haplotypes

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>Control subjects</th>
<th>Case subjects</th>
<th>χ²</th>
<th>P</th>
<th>Odds ratio (95% CI)</th>
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<td>Two-SNP haplotypes</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>AG</td>
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<td>0.799</td>
<td>0.857</td>
<td>9.0</td>
<td>0.0027</td>
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<td>AA</td>
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<td>0.091</td>
<td>4.2</td>
<td>0.0404</td>
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<td>TG</td>
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<td>0.077</td>
<td>0.051</td>
<td>4.1</td>
<td>0.0431</td>
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<tr>
<td>Four-SNP haplotypes</td>
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<td>0.09</td>
<td>0.3259</td>
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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 two-SNP risk haplotype, and the AA and TG haplotypes are referred to as the two-SNP protective haplotypes.
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 $5.62 \pm 0.36$ vs. risk $7.81 \pm 0.36$; $P = 0.007$), 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 four-SNP protective haplotype (one 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. (18) generated from YRI HapMap cell lines, which allowed us to incorporate haplotype heterozygotes into the analysis. For consistency, we analyze the two- and four-SNP haplotypes in terms of number of protective alleles. We pooled subjects with one or two protective alleles for the two-SNP haplotype and compared them with subjects with no protective alleles (two risk alleles). Subjects with zero protective haplotypes ($n = 31$) expressed 16% more ENTPD1 than subjects with one or two protective haplotypes ($n = 29$; $P = 0.027$) (Fig. 1B). We then pooled subjects with one or two copies of the protective four-SNP allele ($n = 7$) and compared them with those with no copies ($n = 53$). While the difference was not significant ($P = 0.09$), those with no protective alleles expressed 16% more ENTPD1 than those with one or two protective alleles (Fig. 1C). These cell lines with at least one protective four-SNP allele had the lowest mRNA expression levels of any haplotype group, including the protective two-SNP haplotype. To account for the fact that we were comparing subjects with protective four-SNP alleles versus a pool that contained many more subjects with one or more protective two-SNP allele, we then compared subjects based on their total number of protective alleles (two-SNP protective alleles plus four-SNP protective alleles) to integrate the effects of two-SNP and four-SNP haplotypes on ENTPD1 expression. Those with no protective alleles ($n = 29$) expressed 18% more ENTPD1 than subjects with at least one protective two-SNP allele or one protective four-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 four-SNP allele prevented us from determining whether the effects of the two-SNP and four-SNP haplotypes were independent.

The protective four-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 with control subjects. We compared expression levels between cell lines with one or more copies of each of these haplotypes. Remarkably, the nonprotective (GTGA) four-SNP haplotype including the minor allele at rs3793744 expressed 45% more ENTPD1 mRNA than the protective (GAGA) four-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 four-SNP

![Graphs and figures](image-url)
haplotype rather than the amino acid substitution. We did note, though, that protein expression in the homozygotes for the protective four-SNP haplotype were surprisingly low compared with other cell lines (supplementary Fig. 1A and 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 and 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 8 months of age demonstrated that in the nondiabetic mice (db/−), ENTPD1 deletion caused mildly increased albuminuria (wild type = 37.2 ± 2.3 μg/mg vs. knockout = 54.7 ± 6.7 μg/mg; P = 0.028) and a trend toward increased glomerular sclerosis (Fig. 2A and 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. 2C–E).

Metabolic syndrome parameters revealed more dramatic differences. In nondiabetic mice, nonfasting (3:00 P.M.) glucose was lower in ENTPD1-null mice at both 8 weeks (wild type = 208.1 ± 4.8 mg/dl vs. knockout = 176.8 ± 7.8 mg/dl; P = 0.006) and 6 months (wild type = 179.2 ± 5.7 mg/dl vs. knockout = 161.9 ± 5.8 mg/dl; P = 0.039) (Fig. 3A and B). No differences in glucose levels were seen in the db/db groups at these time points with or without ENTPD1 (Fig. 3C and D).

While weights of nondiabetic mice were no different (Fig. 4A and 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 (wild type = 41.4 ± 1.0 g vs. knockout = 37.6 ± 1.4 g; P = 0.026) and 6 months (wild type = 62.4 ± 0.9 g vs. knockout = 52.0 ± 1.3 g; P < 0.000001) (Fig. 4C and D).

We also aged wild-type and ENTPD1-null mice (leptin receptor normal) to see if aging would cause similar

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**FIG. 2.** A: Albumin-to-creatinine ratio for db/− nondiabetic mice. n = 7 for ENTPD1 wild type and n = 12 for ENTPD1 knockout. B: Sclerosis index for db/− nondiabetic mice. n = 7 for ENTPD1 wild type and n = 12 for ENTPD1 knockout. C: Albumin-to-creatinine ratio for db/db mice. n = 13 for ENTPD1 wild type and n = 9 for ENTPD1 knockout. D: Sclerosis index for db/db mice. n = 13 for ENTPD1 wild type and n = 10 for ENTPD1 knockout. E: Glomerular area for db/db mice. n = 10 for ENTPD1 wild type and n = 8 for ENTPD1 knockout.
metabolic differences as we saw in the db/db model. At 2 years of age, ENTPD1-null mice had markedly lower fasting glucoses (wild type = 141.6 ± 5.1 mg/dl vs. knockout = 112.8 ± 3.3 mg/dl; P = 0.0009) and lower weights (wild type = 46.4 ± 2.7 g vs. knockout = 39.2 ± 0.7; P = 0.042) than wild type controls (Fig. 5A and B). Consistent with the db experiments, we did also note increased albuminuria in aged ENTPD1-null mice despite lower weights and blood glucoses (D.J.F., unpublished data).

**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 diabetic ESRD and nondiabetic 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 nondiabetic 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 four-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 bioinformatic analysis that showed similar results: zero alleles of the two-SNP protective haplotypes led to 16% greater ENTPD1 expression than one or two protective alleles. The protective four-SNP haplotype also showed a trend toward lower ENTPD1 expression levels, though this analysis was limited by fewer representative cell lines and was confounded by comparison with a group including many protective two-SNP haplotypes. In fact, the protective four-SNP haplotype showed the lowest ENTPD1 expression of any group. By using an index that took into account the total number of both the two- and four-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 four-SNP haplotype was associated with very low ENTPD1 levels and may suggest an effect beyond mRNA expression, though we did not have sufﬁ-
cient 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 with wild-type mice, 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 wild-type 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 wild-type 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 nondiabetic 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 two phosphate molecules. Low functional expression of either of these ectoenzymes could increase extracellular levels of ATP and decrease levels of the nucleoside adenosine.

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.

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