A leucine repeat in the carnosinase gene \( \textit{CNDP1} \) is associated with diabetic end-stage renal disease in European Americans

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Abstract

Background. Four linkage analyses have identified a region on chromosome 18q22-23 that appears to harbour a diabetic nephropathy (DN) susceptibility locus. A trinucleotide repeat sequence in exon 2 of the carnosinase gene (\( \textit{CNDP1} \)) residing on 18q22.3 was subsequently associated with DN in European Caucasians and Arabs.

Methods. We evaluated the role of the \( \textit{CNDP1} \) 5 leucine/5 leucine (5-5) polymorphism (\( \textit{CNDP1} \) Mannheim) in diabetic end-stage renal disease (ESRD) susceptibility in 858 European Americans: 294 with type 2 DN-associated ESRD (DN–ESRD), 258 with diabetes mellitus (DM) lacking nephropathy and 306 healthy controls.

Results. Subjects with DM lacking nephropathy were significantly more likely to be homozygous for the 5-leucine repeat \( \textit{CNDP1} \) genotype (5-5), compared with those with DN–ESRD (\( P = 0.02 \)). Healthy controls were also more likely to be homozygous for the 5-5 genotype, compared with those with DN–ESRD (\( P = 0.008 \)). No significant difference in 5-5 genotype frequency was observed between healthy controls and DM cases without nephropathy (\( P = 0.74 \)).

Conclusion. European Americans homozygous for the 5-5 leucine repeat polymorphism in the \( \textit{CNDP1} \) gene are at significantly reduced risk for developing diabetic ESRD. This replicates the \( \textit{CNDP1} \) gene association with DN that was initially detected in European Caucasians and in Arabs, and further demonstrates that the \( \textit{CNDP1} \) gene and carnosine pathway appear to play a role in susceptibility to DN.

Keywords: carnosinase; carnosine; diabetic nephropathy; end-stage renal disease; European Americans; genetics

Introduction

The emerging worldwide epidemic of type 2 diabetes mellitus (DM) has fueled recent increases in diabetic complications, including atherosclerotic cardiovascular disease and kidney failure. Diabetic nephropathy (DN) is now the most common cause of renal failure in developed nations. Epidemiological evidence supports the contribution of genetic factors to nephropathy susceptibility in type 1 and type 2 DM [1,2]. Linkage of polymorphic markers on human chromosome 18q22-3q23 to type 2 DN[McD2] has been observed in Turkish and Pima Indian families [3] as well as in African-American families [4] and European American families [5].

An initial evaluation of positional candidate genes for DN under the chromosome 18q peak excluded a Kruppel-like zinc finger gene, \( \textit{ZNF236} \) [6] Janssen et al. [7] reported that a tri-nucleotide repeat in exon 2 of the carnosinase dipeptidase 1 (metallopeptidase M20 family) gene (\( \textit{CNDP1} \)) was significantly associated with DN in highly selected cohorts. The observed association was with the number of leucine repeats in the leader peptide of \( \textit{CNDP1} \). Janssen et al. [7] reported that the allelic form containing 5 leucine repeats, termed \( \textit{CNDP1} \) Mannheim, was more prevalent in diabetic controls lacking DN despite long durations of diabetes. \( \textit{CNDP1} \) Mannheim appeared to be protective and was associated with lower serum carnosinase concentrations. The evidence for protection from DN was only observed when the 5-5 homozygote class of genotypes was compared with all others. In other genotypic and allelic analyses, no evidence for DN association was observed suggesting...
that the 5-leucine allele has a recessive effect, protecting only homozygous cases. Yard and colleagues [6] recently cloned the 5, 6 and 7 leucine CNDP1 variants in a pCS2 vector system containing a myc epitope (personal communication). The constructs were expressed in Cos-7 cells and the cell lysates and supernatants were assessed for CNDP1 expression using western blot (% secreted CNDP1 = [CNDP1 intensity in supernatant]/CNDP1 intensity in supernatant and cell lysate) × 100]. Three antibodies were used to ensure that the bands identified were CNDP1 [a commercially available polyclonal anti-CNDP1 (R&D), an in-house synthesized polyclonal anti-CNDP1, and a myc monoclonal antibody]. All three antibodies identified the same bands in cell lysates and supernatants. These analyses revealed that the 5L expressing Cos-7 cells secreted 22% of CNDP1, compared with 45% and 52%, respectively, in the 6L and 7L expressing Cos-7 cells. These data clearly demonstrate that the hydrophobic leucine stretch is of critical importance for this particular signal-peptide function as was postulated by Janssen et al. [7].

Previously, we reported evidence of linkage between chromosome 18q markers and type 2 DN-associated end-stage renal diseases (DN–ESRD) in African-American families [4] and more recently with DN in European American families from the Family Investigation of Nephropathy and Diabetes (FIND) [5]. The current report investigated whether we could replicate whether the CNDP1 Mannheim allele was also associated with a decreased risk for type 2 DN–ESRD in European Americans.

**Methods**

**Patient populations**

Unrelated European American cases with type 2 DM lacking evidence for DN were identified from participants in the Diabetes Heart Study at the Wake Forest University School of Medicine [8]. A diagnosis of DM without nephropathy was based upon the presence of a spot urine albumin:creatinine ratio (ACR) <30 mg/g and a serum creatinine concentration ≤1.6 mg/dl in men or ≤1.4 mg/dl in women after >5 years diabetes duration. DNA samples were also collected from unrelated European American individuals from the Family Investigation of Nephropathy and Diabetes (FIND) [5].

The number of leucine repeat segments in the CNDP1 gene was confirmed by DNA sequence analysis in all participants. For molecular quality control purposes, 60 duplicate samples were run. Of these duplicate samples, 98.3% (59/60) were concordant.

**Genotyping**

DNA extraction was performed using the Pure-Gene System (Gentra Systems, Minneapolis, MN). The CNDP1 exon 2 trinucleotide repeat polymorphism was genotyped by fragment length analysis on an ABI Prism DNA Analyzer 3700 (Applied Biosystems Incorporated, Foster City, CA) in a manner similar to that described by Janssen et al. [7]. The number of leucine repeat segments in the CNDP1 gene was identified protective 5-leucine allele (5-5, also termed CNDP1 Mannheim). The percentage of individuals homozygous for the previously identified protective 5-leucine allele (5-5, also termed CNDP1 Mannheim) was determined in all subjects. A power analysis demonstrated that at the P = 0.01 level of significance, we had 80% power to detect an odds ratio (OR) = 1.66 for the dominant model, OR = 1.42 for the additive model, and OR = 1.92 for the recessive model. Comparisons of allelic and genotypic frequencies between groups were performed using the statistical analysis program CLUMP (http://www.mds.qmw.ac.uk/statgen/dcurnis/software.html) [9]. CLUMP is designed to assess the significance of departure of observed values in a contingency table from the expected values, conditional on the marginal totals using a Monte Carlo approach. CLUMP performs repeated simulations to generate tables having the same marginal totals as the one observed and counts the number of times that a chi-squared value associated with the real table is achieved by the randomly simulated data (a permutation test). The a priori hypothesis was to contrast the 5-leucine allele homozygotes (5-5) vs all others.

**Results**

The study group consisted of 858 European American subjects: 306 healthy controls, 165 felt to have definite diabetic retinopathy documented prior to the start of renal replacement therapy and if the diagnosis of diabetes mellitus was made >5 years prior to the onset of ESRD. Diabetic cases with shorter diabetes durations prior to dialysis and/or lacking ophthalmological data were classified as ‘presumptive DN–ESRD’.

Unrelated, healthy population-based European American controls ≥35 years of age were also recruited. Controls denied having diabetes mellitus, kidney disease or first-degree relatives with kidney disease. All ESRD and diabetic cases and healthy controls were born in North Carolina, South Carolina, Georgia, Tennessee or Virginia, and provided written informed consent. Ethnicity was self-reported by all participants. The study protocol was approved by the Institutional Review Board (IRB) at the Wake Forest University School of Medicine. The IRB also required total anonymity among healthy European American controls to maintain privacy; hence only gender was collected in the healthy control population.
DN-ESRD, 129 felt to have presumptive DN-ESRD and 258 cases with DM lacking nephropathy. Table 1 contains demographic data on the cases with DM and ESRD. We did not collect information on blood pressure, body mass index or lipid levels in the DN-ESRD cases since uraemia clearly impacts these variables and more than 85% of our ESRD patients have high blood pressure. Among the healthy European American non-diabetic controls, 30% were males and no other identifying or laboratory data were collected due to IRB restrictions.

Diabetic control subjects who lacked nephropathy had a mean \( \pm SD \) (median) age of 62.2 \( \pm 8.9 \) years (62.6), age at diabetes onset 51.6 \( \pm 9.7 \) years (51.0), body mass index 32.7 \( \pm 6.8 \) kg/m\(^2\) (31.2), systolic blood pressure 138.0 \( \pm 18.6 \) mmHg (136.0), diastolic blood pressure 71.7 \( \pm 10.1 \) mmHg (71.0), 51% were taking lipid-lowering medications, 59% were former or current smokers and 59% were prescribed angiotensin converting enzyme inhibitors or angiotension II receptor blockers.

Among the diabetic subjects without nephropathy, mean \( \pm SD \) (median) ACR was 10.5 \( \pm 7.90 \) mg/g (7.60), hemoglobin A1c 7.42 \( \pm 1.44\% \) (7.1), MDRD glomerular filtration rate 69.8 \( \pm 16.7 \) ml/min (67.1), and serum creatinine concentration 1.03 \( \pm 0.22 \) mg/dl (1.0).

Table 2 contains the results of the \textit{CNDP1} association analyses. Based upon the number of leucine repeats, four alleles (4L, 5L, 6L and 7L) were observed in the European American population with relative frequencies of 0.001, 0.57, 0.37 and 0.06. All genotype frequencies were consistent with Hardy–Weinberg equilibrium expectations.

A significant reduction in the frequency of the 5 leucine/5 leucine repeat genotype (5-5) was observed in subjects with DN–ESRD, compared with all other genotypes (Table 3). The homozygous 5-5 genotype was detected significantly more often in healthy controls (38.6%), compared with definite DN–ESRD cases \[26.1%; \text{OR (95\% CI)} \equiv 1.78 \quad (1.17–2.70), \quad P = 0.008\] and all DN–ESRD cases (29.3%; \text{OR} \equiv 1.52 \quad (0.99–2.01), \quad P = 0.059). Significant differences were not observed in 5-5 genotype frequency between healthy controls and DM cases lacking nephropathy \(P = 0.74\) (Table 3).

Most coding sequences in our genome are part of conserved haplotype blocks. However, the \textit{CNDP2} exon 2 containing the leucine repeat was not part of any conserved haplotype block in independent analyses conducted at the Janssen and Freedman labs. It was in very low linkage disequilibrium with
Table 3. Frequency of CNDP1 5 leucine/5 leucine (5-5) repeat genotypes

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype</th>
<th>Ratio</th>
<th>Empiric P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-5</td>
<td>All other genotypes</td>
<td>5-5 Frequency</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>118</td>
<td>188</td>
<td>0.386</td>
</tr>
<tr>
<td>Definite DN–ESRD</td>
<td>43</td>
<td>122</td>
<td>0.261</td>
</tr>
<tr>
<td>All DN–ESRD</td>
<td>86</td>
<td>208</td>
<td>0.293</td>
</tr>
<tr>
<td>DM (non-nephropathy)</td>
<td>95</td>
<td>163</td>
<td>0.368</td>
</tr>
</tbody>
</table>

Empiric P-value based on 1000 simulations maintaining marginal totals as described in methods.

Discussion

Linkage analyses in Turkish, Pima-Indian, European American and African-American families suggest that a gene(s) modifying susceptibility to type 2 DN resides on human chromosome 18q22.3-q23. The carnosinase gene, CNDP1, residing in the 18q linkage region, was first associated with DN in a small cohort (135 DN cases and 107 diabetic non-nephropathy controls) of carefully selected Europeans from the Czech Republic, Germany and the Netherlands, and Arabs from Qatar [7]. We confirm that polymorphisms in the CNDP1 gene are associated with susceptibility to type 2 DN–ESRD in European Americans. In contrast to the highly selected DN cases in the original report, our cases were representative of typical DN–ESRD in the US.

The 5-5 CNDP1 Mannheim allele is a short form of the carnosinase-1-precursor gene that is observed in Caucasian populations. Individuals homozygous for the 5 leucine repeat allele have reduced serum carnosinase concentrations [7]. This is expected to result in higher renal concentrations of the protective dipeptide carnosine (β-alanyl-L-histidine). Carnosine has been reported to have natural ACE-inhibitor activity [10,11] as well as free radical scavenger [12] and cleave advanced glycation end-products [13]. Addition of carnosine markedly reduces scavenger [12] and cleave advanced glycation end-products [13]. Expression of CNDP1 is significantly greater in podocytes and renal parietal epithelial cells from subjects with DN, compared with healthy renal tissue [7]. These data strongly implicate carnosine and the CNDP1 gene as playing a role in susceptibility to DN.

We performed a preliminary analysis of the CNDP1 gene in 442 African-American subjects with definite type 2 diabetes-associated ESRD and 637 reportedly healthy controls. No association was detected as the 5-5 CNDP1 frequencies were 35.9% in healthy controls and 36.9% in the definite DN–ESRD cases (P = 0.806). It is possible, that African-American individuals have a slightly different carnosine metabolism with higher carnosine levels, making them less susceptible to differences in carnosinase activity. It is also possible that an additional gene(s) on chromosome 18q is associated with susceptibility to DN–ESRD in African Americans [4]. Of particular interest are the ongoing studies of adjacent carnosinase 2 gene (CNDP2) on 18q (13 kilobases distant from CNDP1). With respect to this, it is of interest that the leucine repeat is not part of any conserved haplotype block and therefore not in linkage disequilibrium with other CNDP1 or CNDP2 sequence variants (www.hapmap.org/index.html).

An important issue in case-control genetic association studies is potential population stratification and cryptic relatedness. This is an area of research that is a current focus of our group and others. It should be noted that our results clearly replicate the findings reported in other populations by a single group (Netherlands, Germany, Czech Republic and Qatar), suggesting that this is a ‘true’ effect and not due to population differences. Furthermore, we recognize that while some admixture and substructure may occur in the European American population, the effect of substructure would be expected to be a larger problem in more heavily admixed populations.

Although we evaluated a more severely renal diseased cohort relative to the original report, the frequencies of the 5-5 genotype were remarkably similar. Among the European Americans in our report, 38.6% of healthy controls, 36.8% of DM cases without nephropathy and 26.1% of definite DN–ESRD cases, respectively, were CNDP1 5-5 homozygotes. In the original European and Arabic cohort, 43% of diabetic non-nephropathy controls and 27% of DN cases had the 5-5 genotype [7]. As was stated in the initial report, individuals who are homozygous for the 5-5 CNDP1 allele occasionally develop DN. The roles of conventional nephropathy risk factors, including hyperglycaemia, smoking, hypertension and hyperlipidaemia need to be evaluated in genetically similar cohorts.

The frequency of the 5-5 genotype was not significantly different among European American healthy controls and diabetic cases lacking nephropathy.
The 5-5 genotype was also present in significantly higher frequency among our diabetic non-nephropathy cases compared with DN-ESRD cases. These results clearly demonstrate that the 5-5 CNDP1 allele was protective from diabetic kidney disease, and not involved in susceptibility to DM per se. The results observed using the stringent study design employed by Janssen and colleagues [7] also support this finding.

We conclude that the CNDP1 gene plays a role in susceptibility to advanced DN in Eastern and Western Europeans and Arabs and to DN-associated ESRD in European Americans. Carnosine and the genetic association between CNDP1 Mannheim with protection from DN shed light on a novel and potentially important pathway in development of DN. It will be important to evaluate whether the administration of carnosine or agents that inhibit carnosinase activity will protect susceptible diabetic individuals from the development of progressive nephropathy.

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Conflict of interest statement. None declared.

References

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