Brief Genetics Report

Association Between Variation in the Actin-Binding Gene Caldesmon and Diabetic Nephropathy in Type 1 Diabetes

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Dysfunction of the actin cytoskeleton is a key event in the pathogenesis of diabetic nephropathy. We previously reported that certain cytoskeletal genes are upregulated in mesangial cells exposed to a high extracellular glucose concentration. One such gene, caldesmon, lies on chromosome 7q35, a region linked to nephropathy in family studies, making it a candidate susceptibility gene for diabetic nephropathy. We screened all exons, untranslated regions, and a 5-kb region upstream of the gene for variation using denaturing high-performance liquid chromatography technology. An A>G single nucleotide polymorphism (SNP) at position −579 in the promoter region was associated with nephropathy in a case-control study using 393 type 1 diabetic patients from Northern Ireland (odds ratio [OR] 1.38, 95% CI 1.02–1.86, P = 0.03). A similar trend was found in an independent sample from a second center. When the sample groups were combined (n = 606), the association between the −579G allele and nephropathy remained significant (OR 1.35, 1.07–1.70, P = 0.01). The haplotype structure in the surrounding 7-kb region was determined. No single haplotype was more strongly associated with nephropathy than the −579A>G SNP. These results suggest a role for the caldesmon gene in susceptibility to diabetic nephropathy in type 1 diabetes. Diabetes 53:1162–1165, 2004

Diabetic nephropathy is the most common cause of end-stage renal failure in the western world (1). There is good evidence from epidemiological (2) and family (3–5) studies that a genetic predisposition to diabetic nephropathy exists, but as yet the causative genes remain elusive. To identify novel candidate genes for diabetic nephropathy, we previously employed suppression-subtractive hybridization and found >200 genes to be differentially expressed in mesangial cells exposed to a high extracellular glucose concentration (6,7). Prominent among the upregulated genes is a group coding for proteins that control regulation of the actin cytoskeleton. Both in vitro (8,9) and in vivo (9) studies suggest that there is disruption of the actin cytoskeletal structure in mesangial cells exposed to high glucose, and this is associated with glomerular hyporesponsiveness to vasopressor stimuli (8). Therefore, this may be partially responsible for the afferent arteriole vasodilation, intraglomerular hypertension, glomerular distention, and subsequent hyperfiltration seen in the early stages of diabetic nephropathy (10).

One of the upregulated actin-binding genes, caldesmon, is located on chromosome 7q35, a region previously linked to diabetic nephropathy in two independent family-based studies (11,12). Caldesmon is a cytoskeletal protein found in smooth muscle and modified smooth muscle cells, such as mesangial cells. It inhibits cell contractility (13) and promotes either assembly (14) or disassembly (13) of actin stress fibers. Phosphorylation of caldesmon is required for the formation of contractile rings during mitosis, and hence it may be involved in the regulation of cell proliferation (14). Furthermore, caldesmon may regulate exocytosis and hence matrix deposition (15). Thus, caldesmon is an excellent positional and biological candidate gene for diabetic nephropathy.

The caldesmon gene is large, comprising 16 exons and spanning over 180 kb of the genome (16). Screening for caldesmon gene variants included all exons, flanking intronic regions, the 5'- and 3'-untranslated regions (240 and 1001 bp in length, respectively), and 3 kb of promoter region upstream from the transcription start site (Fig. 1). We screened DNA samples from 30 type 1 diabetic patients from Northern Ireland (15 case and 15 control subjects, see RESEARCH DESIGN AND METHODS) for single nucleotide polymorphisms (SNPs) using WAVE (denaturing high-performance liquid chromatography) technology followed by DNA sequencing of samples for which the chromatograms differed from the wild type. In total, 12.5 kb were screened in each individual and a total of 20 SNPs identified, i.e., an average of 1 SNP for every 625 bp screened. Of

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*SNP, haplotype-tagged single nucleotide polymorphism; SNP, single nucleotide polymorphism.

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these, 12 (60%) had an estimated minor allele frequency of >5% (Fig. 1A).

Based on their frequency and potential effect on gene function, three SNPs were initially tested for association with nephropathy in a case-control study, which used 393 patients from Northern Ireland with type 1 diabetes: 155 patients with nephropathy and 238 nonnephropathic control subjects (17). We selected variants with a minor allele frequency of at least 20% because these would provide 95% power to detect a susceptibility allele with an odds ratio (OR) of 2.0 at the 5% level of significance, given the size of the Northern Ireland samples. A -579A>G SNP in the promoter region may affect gene expression, and a -834T>C SNP in the 3′ untranslated region may alter stability of the mRNA; therefore, both SNPs may influence the intracellular mRNA levels available for translation into protein. A +569G>A SNP results in a histidine-to-arginine substitution at position 397 in exon 6b.

The ex6b+569G>A and the 3′-untranslated region +834T>C SNPs showed no evidence of association with nephropathy (data not shown). However, the G allele of the -579A>G promoter polymorphism was significantly associated with nephropathy (OR 1.38, 95% CI 1.02–1.86, P = 0.03) (Table 1). For all three polymorphisms, the genotype distributions in the patient groups were in Hardy-Weinberg equilibrium. There was limited linkage disequilibrium between these three SNPs (Table 2).

In order to replicate the association between the -579G allele and diabetic nephropathy in an independent sample, a further 213 patients with type 1 diabetes from the Republic of Ireland (95 patients with nephropathy and 118 nonnephropathic control subjects) were genotyped for the -579A>G SNP. An increase in the frequency of the -579G allele, similar in magnitude to that seen in the Northern Ireland samples, was observed in patients with nephropathy, although this finding did not reach significance (Table 1).

When the nephropathic and nonnephropathic patients were combined, neither the frequency of the G allele (43.9 vs. 43.2%) nor the distribution of the GG, AG, and AA genotypes (19.3, 49.1, and 31.6 vs. 21.1, 44.1, and 34.7%, respectively) was significantly different between the North-
CALDESMON AND NEPHROPATHY IN TYPE 1 DIABETES

TABLE 1
Allele and genotype frequencies for the −579A>G SNP in the Northern Ireland, Republic of Ireland, and combined samples in diabetic patients with and without nephropathy

<table>
<thead>
<tr>
<th>SNP</th>
<th>Northern Ireland sample</th>
<th>Republic of Ireland sample</th>
<th>Combined sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetic</td>
<td>Nonnephrotic</td>
<td>Diabetic</td>
</tr>
<tr>
<td>Allele</td>
<td>155</td>
<td>238</td>
<td>95</td>
</tr>
<tr>
<td>G</td>
<td>151 (48.7)</td>
<td>194 (40.8)</td>
<td>89 (46.8)</td>
</tr>
<tr>
<td>A</td>
<td>159 (51.3)</td>
<td>282 (59.2)</td>
<td>101 (53.2)</td>
</tr>
<tr>
<td>P</td>
<td>0.03</td>
<td>0.17</td>
<td>1.38 (1.02–1.86)</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1.38 (1.02–1.86)</td>
<td>1.31 (0.87–1.96)</td>
<td>1.35 (1.07–1.71)</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>37 (23.9)</td>
<td>39 (16.4)</td>
<td>27 (28.4)</td>
</tr>
<tr>
<td>AG</td>
<td>77 (49.7)</td>
<td>116 (48.7)</td>
<td>35 (36.9)</td>
</tr>
<tr>
<td>AA</td>
<td>41 (26.4)</td>
<td>83 (34.9)</td>
<td>33 (34.7)</td>
</tr>
<tr>
<td>P</td>
<td>0.09</td>
<td>0.04</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data are n (%) unless noted otherwise.

ern Ireland and Republic of Ireland sample groups (P = 0.81 and 0.51, respectively). Therefore the data from the two samples were combined. This confirmed the association between the G allele and nephropathy (OR 1.35, 95% CI 1.07–1.71, P = 0.01) (Table 1).

To explore the possibility that the detected association with nephropathy was due to linkage disequilibrium between the −579A>G SNP and an adjacent unidentified causative SNP, a further 2 kb of upstream promoter and the first 2 kb of intron 1 were screened for polymorphisms using WAVE analysis in the same 30 individuals as before. A further eight SNPs with a minor allele frequency of >5% were identified (Fig. 1C). All of the common (>5% minor allele frequency) SNPs in this region were genotyped by DNA sequencing in 94 patients with type 1 diabetes (47 case and 47 control subjects) from Northern Ireland. The strong linkage disequilibrium between pairs of these four common SNPs confirms that they lie within a single haplotype block (SNPs 1–4; Table 2). From these data, we determined that within this block there were five common haplotypes, which could be identified using four haplotype-tagged SNPs (htSNPs) (Fig. 1D). The haplotype frequencies in the case and control subjects in the Northern Irish population were estimated and compared. This, however, was no more informative than the single SNP analysis (Table 3).

The −579A>G SNP is expected to cause the disruption of a ΔEF1 transcription factor–binding site and the creation of an AP2 binding site as predicted by Transfac (http://www.gene-regulation.com/pub/databases.html#transfac).

This may affect expression of the caldesmon gene and hence provide a mechanism by which the −579G allele contributes to the development of diabetic nephropathy. Functional studies would be required to confirm this hypothesis.

In conclusion, we have found that a −579A>G polymorphism in the promoter region of the caldesmon gene is associated with diabetic nephropathy in the Northern Ireland population and that a similar trend was observed in an independent sample from the Republic of Ireland. While we cannot rule out that the observed association is due to linkage disequilibrium with another SNP in the caldesmon gene, we have suggested a mechanism by which this polymorphism may influence the pathogenesis of the disease. This association needs to be replicated in additional large case-control studies in other populations. The possibility that a variant in the caldesmon gene predisposes to nephropathy in type 1 diabetic patients should stimulate further investigation into the role of the actin cytoskeleton in the pathogenesis of diabetic nephropathy.

RESEARCH DESIGN AND METHODS

All patients were Caucasians of Irish descent, had type 1 diabetes diagnosed before age 35 years, and required insulin therapy from the time of diagnosis. They attended diabetic or nephrology clinics in Northern Ireland or the Republic of Ireland. Patients with nephropathy (n = 250) had diabetes for at least 10 years before the onset of proteinuria (>0.5 g/24 h), had diabetic retinopathy, and had no clinical, serological, or radiological evidence of nondiabetic renal disease. The control patients (n = 356) had diabetes for at least 15 years, had no evidence of microalbuminuria on repeated testing, and

TABLE 2
Linkage disequilibrium between pairs of polymorphisms in the caldesmon gene in the Northern Ireland sample as quantified by values of Lewontin’s D’ (18)

<table>
<thead>
<tr>
<th>SNP</th>
<th>−2354T&gt;C</th>
<th>−579A&gt;G</th>
<th>IVS1+23T&gt;G</th>
<th>IVS1+893C&gt;A</th>
<th>Ex6b+569G&gt;A</th>
<th>3’UTR+834T&gt;C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. −2354T&gt;C</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2. −579A&gt;G</td>
<td>0.929</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3. IVS1+23T&gt;G</td>
<td>0.792</td>
<td>0.999</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4. IVS1+893C&gt;A</td>
<td>−0.857</td>
<td>−0.857</td>
<td>−0.782</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5. Ex6b+569G&gt;A</td>
<td>−0.288</td>
<td>−0.288</td>
<td>−0.244</td>
<td>−0.050</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6. 3’UTR+834T&gt;C</td>
<td>0.256</td>
<td>0.241</td>
<td>0.116</td>
<td>−0.407</td>
<td>−0.493</td>
<td>—</td>
</tr>
</tbody>
</table>

IVS, intervening sequence; UTR, untranslated region.
were not on antihypertensive medication (17). There was no significant difference in either the mean age of onset of diabetes or the mean duration of diabetes between the two groups. Informed consent was gained from all
patients, and the study had the approval of the ethics committees of both centers.

**Polymorphism screening.** Gene regions of interest were divided into ∼500-bp fragments and amplified by PCR using AmpliTaq Gold (Applied Biosystems, Foster City, CA). The PCR products were applied to a DNAsept column (Transgenomic, Omaha, NE) at temperatures corresponding to the melting points along the fragment as predicted by Wavemaker software (Transgenomic). Heterozygotes were identified on the basis of their different patterns of elution from the column, and the SNPs were then characterized by DNA sequencing. All sequencing was performed on an ABI 3100 Genetic Analyzer (Applied Biosystems), according to the manufacturer’s protocols.

**Genotyping.** All SNPs were genotyped using the Taqman 5′-nuclease assay, using allele-specific fluorescent MGB probes, on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The assays were designed by Primer Express software or by the Assay-on-Demand service (Applied Biosystems).

**Statistical analysis.** The $\chi^2$ test was used to compare genotype and allele frequencies between case and control subjects. The extent of linkage disequilibrium between pairs of SNPs was quantified using Lewontin’s D* value (18). htSNPs were determined using the htSNP2 package provided by David Clayton (http://www.gene.cimr.cam.ac.uk/clayton/software/stata [19]). Haplotype frequencies for individuals were inferred using Phase software (20). The frequencies of the common haplotypes in the Northern Ireland population were estimated by genotyping the samples at the htSNP loci and employing gene counting, a form of the EM algorithm as implemented in the Arlequin program (21). The estimated haplotype frequencies in the control subjects were then compared using a likelihood-ratio test statistic but with significance assessed empirically using the EHplus software (22). In this approach, an empirical $P$ value is obtained by comparing the observed test statistic with its permutation distribution, which is approximated by 1,000 random permutations of the case/control labels. This was considered preferable to assessing significance using the asymptotic $\chi^2$ approximation, which is known to give unreliable results in small numbers.

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