Diabetic Nephropathy Is Associated With AGT Polymorphism T235
Results of a Family-Based Study

John J. Rogus, Dariusz Moczulski, Maria Beatriz S. Freire, Yadong Yang, James H. Warram, Andrzej S. Krolewski

Abstract—Diabetic nephropathy is a serious and frequent complication of insulin-dependent diabetes mellitus (IDDM) that has a strong genetic component. Several case-control studies have reported conflicting results with regard to the role of angiotensinogen gene polymorphisms, specifically the M235T T allele, in the development of diabetic nephropathy. The primary limitation of the case-control approach is that bias may be introduced by unrecognized differences in the populations selected for cases and control subjects. In contrast, family-based approaches, such as the transmission/disequilibrium test, assess whether a particular variant, or allele, is transmitted preferentially from a parent having a single copy of that allele. Thus each family provides its own control, thereby eliminating spurious results caused by mismatched population samples. To take advantage of this study design for further investigation of M235T, we collected from the Joslin Diabetes Center in Boston 148 IDDM patients with diabetic nephropathy, 62 nephropathy-free patients with long-duration IDDM, and, very importantly, parents of all these individuals. We found that among males (but not females) the T allele of the M235T polymorphism was transmitted preferentially to those with nephropathy compared with IDDM patients without nephropathy ($P=0.05$). Moreover, the T allele was transmitted preferentially to patients with the most severe manifestation of nephropathy, end-stage renal disease ($P=0.04$). In conclusion, results obtained in our family-based study support a role of the angiotensinogen gene M235T polymorphism, and specifically the T allele, in the development of diabetic nephropathy in IDDM. (Hypertension. 1998;31:627-631.)

Key Words: angiotensinogen diabetic nephropathy, diabetic diabetes, insulin-dependent

Diabetic nephropathy is a serious complication of diabetes, which frequently results in hemodialysis, renal transplant, or death. Although common among all individuals with IDDM; (35% lifetime risk), DN is extremely common among IDDM patients with DN siblings (71% lifetime risk).\textsuperscript{1} Similar familial aggregation has been found in other studies.\textsuperscript{2,3} Because familial clustering of risk factors such as poor glycemic control is insufficient to explain such a high risk in the sibling of an affected patient, genetic factors are strongly suspected to play a large role in susceptibility to DN. However, little progress has been made in identifying genes relevant to this disease process.

About a decade ago, a clue about the genetic basis of DN surfaced: The genetics of DN and essential hypertension may overlap.\textsuperscript{4,5} Consequently, genes involved in blood pressure regulation are logical candidates for DN susceptibility. Although the genetic basis of hypertension is still unfolding, many studies have suggested putative hypertension susceptibility genes. One widely studied example is the angiotensinogen gene (AGT) on chromosome 1. The initial report implicating AGT in hypertension was made by Jeunemaitre et al\textsuperscript{6} and confirmed by Caulfield et al\textsuperscript{7} This finding was replicated by many but not all subsequent studies.\textsuperscript{8}

Several recent studies have tested AGT polymorphisms, most notably M235T (characterized by a threonine [T] to methionine [M] substitution at position 235) for a role in DN. For example, using study subjects from Northern Ireland, Fogarty et al\textsuperscript{9} found an excess of TT genotypes among 95 IDDM patients with DN compared with 100 nephropathy-free individuals with IDDM. Moreover, Marre et al\textsuperscript{10} found an interactive effect of AGT with the ACE gene. Other case-control studies\textsuperscript{11-14} have found no AGT effect.

Successful application of this type of case-control study design, however, is dependent on the ability to identify a group of cases and control subjects from the same gene pool. If allele frequencies of cases and control subjects differ for any reason not relevant to the disease process, such as population stratification, then false-positive or false-negative results may occur. While steps can be taken to minimize this problem (eg, drawing cases and control subjects from the same relatively homogeneous population as done in Fogarty et al\textsuperscript{9}, the only
Selected Abbreviations and Acronyms

ACE = angiotensin I-converting enzyme
AGT = angiotensinogen gene
DN = diabetic nephropathy
ESRD = end-stage renal disease
IDDM = insulin-dependent diabetes mellitus
M235T = methionine/threonine polymorphism (at position 235) of the AGT gene
TDT = transmission/disequilibrium test

foolproof way of eliminating potential bias is by application of family-based analytic methods.

We report in this article the results of a family-based study that investigates AGT for a possible role in DN. Specifically, we test the M235T T allele for evidence of association with DN by using the TDT. In addition to aggregate analysis, we also stratify by the presence or absence of ESRD, a measure of disease severity. Finally, in light of a recent report concluding that the AGT TT genotype causes blunted renal vascular response to angiotensin II in males but not in females, we also stratify by gender.

Methods

Study Population

Families for this study were selected from two rosters assembled as part of our ongoing research at the Joslin Diabetes Center. The first roster focuses on families with multiple cases of IDDM and includes 137 families (examined 1991 to 1996) having two or more siblings with 15+ years of IDDM. The second roster is based on a large-scale DN screening effort at the Joslin Diabetes Center. During 1991 to 1993, roughly half of Joslin’s IDDM patients who were 15 to 44 years old were screened for DN, resulting in 201 diagnoses of persistent proteinuria or more advanced stages of DN.

Examination of IDDM Patients and Parents

All IDDM patients and parents (if available) on these rosters had previously undergone physical examination including height, weight, and blood pressure measurement. They also provided demographic information and medical history (eg, IDDM diagnosis/treatment and late diabetic complications) through a standardized questionnaire. Each individual provided a random urine sample for urinalysis and for albumin/creatinine ratio determination and a blood sample for biochemical analysis and DNA isolation. All examinations were performed according to institutional guidelines by specially trained recruiters in either the subjects’ homes or the Joslin Diabetes Center. The protocol for the study was approved by the Human Subject Committee at the Joslin Diabetes Center. All subjects gave informed consent.

Definition of IDDM

Only white patients with IDDM were considered for the study. Diabetes was considered IDDM if it was diagnosed before age 30 years and treatment with insulin began within 1 year and continued thereafter.

Diagnosis of DN

IDDM patients were classified with regard to various stages of DN on the basis of questionnaires, medical records (from Joslin or other institutions), and measurements of albumin/creatinine ratio. Methods for albumin/creatinine ratio determination and classification have been described previously. IDDM patients without any history of DN having <17 (males) or <25 (females) µg albumin/mg creatinine were considered normoalbuminuric (DN−). IDDM patients with clear evidence of renal disease (renal transplant or dialysis), persistent proteinuria (Albustix 1+) or albumin/creatinine ratio 250+ [males] or 355+ [females] on two of three determinations), or persistent high microalbuminuria (albumin/creatinine ratio 65+ [males] or 92+ [females] on two of three determinations) were considered to have DN (DN+) if a review of all available medical information revealed no evidence of nondiabetic renal disease. IDDM patients who did not fit one of these categories were considered unclassifiable and were not included in this study.

Criteria for Family Inclusion

For this study, we included only families with at least one DN-classifiable IDDM patient whose parents had both been examined. From these families, we identified our final study population (summarized in Table 1) of 148 DN+ trios (DN+ child plus parents) and 62 DN− trios (DN− child with 15+ years of IDDM plus both parents).

Genetic Markers and Genotyping

The AGT exon 2 polymorphism M235T was genotyped according to the previously described denaturing gradient gel electrophoresis protocol.

Statistical Analysis

Because of the potential for bias in case-control studies, we chose to focus exclusively on family-based association testing. Consequently, instead of comparing distributions of alleles in cases and control subjects, our procedure tests for preferential transmission of a genetic variant, or allele, from a parent who has only one copy of that variant. If a variant is relevant to the disease process (or is a marker for a disease allele), it will be preferentially transmitted to affected children and not transmitted to unaffected children. Because parental DNA is crucial to avoid biased results, the relevant unit of analysis for this type of analysis is a family trio (ie, a child and both parents).

The cornerstone of our analytic strategy is the TDT. Typically, the TDT statistic is found by identifying all parents heterozygous for a particular variant and determining the number of times that variant is and is not transmitted to an affected child. The difference divided by the square root of the sum (ie, the square root of McNemar’s statistic) follows a standard normal distribution under the null hypothesis, and large values are indicative of allelic association. To apply this procedure to DN, we must account for the fact that a susceptibility gene will be transmitted in excess to DN+ children, whereas there will be deficient transmission to DN− children. Thus we divide our overall

![Table 1. Summary of Families Used for Family-Based Association Testing](http://hyper.ahajournals.org/lookup/doi/10.1161/01.HYP.0000059314.05487.84)
sample by DN status and carry out separate analyses conditional on renal status.

In addition to separate assessment of allele transmission in DN+ and DN− samples, we also compared the DN+ transmission rate with the DN− transmission rate by using a Pearson χ² statistic. This alternative statistic was proposed by Spielman et al.13 as a safeguard against false-positives caused by segregation distortion, a phenomenon characterized by preferential transmission of an allele to all children (in this context, both DN+ and DN−). However, aside from eliminating potential bias caused by segregation distortion, this statistic also allows us to consider all trios simultaneously, because searching for opposite transmission patterns in DN+ and DN− patients prevents IDDM loci from being detected (as they might be in a DN+ sample alone).

Allele transmission for both tests was determined by use of the publicly available computer program ANALYZE (ftp://linkage.cpmc.columbia.edu/software/analyze). To avoid asymptotic approximations, all corresponding P values were calculated with exact methods. For standard TDT, where transmission from each heterozygous parent may be viewed as a Bernoulli trial, exact P values were found by determination of the upper tail binomial probabilities with a spreadsheet. For the Pearson χ² statistic, P values reflecting the exact probability of obtaining the observed statistic or larger under the null hypothesis were calculated in STATXACT (CYTEL Software Corp).

### Results

Table 2 shows the clinical characteristics of the DN− (n=148) and DN+ (n=62) IDDM patients who, together with their parents, make up the family trios for this study. Both groups had similar age at examination, had diabetes diagnosed at a young age, and had long duration of IDDM. Twenty-five percent of patients with nephropathy had ESRD (8% on dialysis or 17% with kidney transplant).

Table 3 summarizes the results of the standard TDT analysis that we performed separately on DN+ and DN− samples. For each analysis, this table shows the number of informative parents (ie, parents having genotype MT as opposed to MM or TT), the number of times these heterozygous parents transmitted T versus M, and the exact P value corresponding to the probability of observing equal or greater transmission of T under the null hypothesis of random transmission. In aggregate, neither the 148 DN+ trios nor the 62 DN− trios demonstrated significant evidence for association of the M235T T allele with DN. In contrast, T was transmitted 35 times from 70 heterozygous parents (P=.17) and to DN− children 20 of 33 times to DN− males (P=.08). Thus among males, there was some suggestion that the T allele is associated with DN. In contrast, T was transmitted 35 of 70 times to DN− females (P=.55) and 20 of 37 times to DN− females (P=.74), suggesting random transmission.

This gender-dependent trend was even more pronounced when the transmission ratios in DN+ and DN− trios were compared directly by χ² analysis (Table 4). Now, instead of testing each sample individually for deviation from random transmission, the T/M transmission ratio in the DN− sample is compared with that in the DN+ sample. Among females, the DN− 35/35 ratio is not significantly different from the DN+ 20/17 ratio (P=.73). Among males, however, with both transmission ratios consistent with association (41/32 for DN+ and 12/21 for DN−), direct comparison of them is now statistically significant (P=.05).

Stratification of the DN+ sample by ESRD also revealed potential evidence for association of the T allele with DN. Specifically, while no transmission difference was observed in those without ESRD (P=.65), 38 heterozygous parents transmitted T 25 times to those with ESRD (P=.04).

Gender-specific analysis of the ESRD+ sample did not reveal any additional insight: The T allele was transmitted to 15 ESRD+ males from 22 heterozygous parents (68%) and to 10 ESRD+ females from 16 heterozygous parents (63%).

### Table 2. Clinical Characteristics of IDDM Patients Used for TDT According to Their Nephropathy Status

<table>
<thead>
<tr>
<th>Clinical Characteristic</th>
<th>Diabetic Nephropathy</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Present (DN+)</td>
<td>Absent (DN−)</td>
<td></td>
</tr>
<tr>
<td>Age at examination, y</td>
<td>37±7</td>
<td>32±7</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis of IDDM, y</td>
<td>10±6</td>
<td>11±6</td>
<td></td>
</tr>
<tr>
<td>Duration of IDDM, y</td>
<td>26±7</td>
<td>22±6</td>
<td></td>
</tr>
<tr>
<td>% On dialysis</td>
<td>8</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>% With kidney transplant</td>
<td>17</td>
<td>...</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Allele Transmission From M235T Heterozygous Parents to IDDM Offspring According to DN Status

<table>
<thead>
<tr>
<th>DN Status</th>
<th>No. of Informative Parents</th>
<th>Allele Transmitted</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>143 (100%)</td>
<td>76 (53%)</td>
<td>67 (47%)</td>
</tr>
<tr>
<td>DN+</td>
<td>70 (100%)</td>
<td>32 (46%)</td>
<td>38 (54%)</td>
</tr>
<tr>
<td>Females only</td>
<td>70 (100%)</td>
<td>35 (50%)</td>
<td>35 (50%)</td>
</tr>
<tr>
<td>DN+</td>
<td>37 (100%)</td>
<td>20 (54%)</td>
<td>17 (46%)</td>
</tr>
<tr>
<td>Males only</td>
<td>73 (100%)</td>
<td>41 (56%)</td>
<td>32 (44%)</td>
</tr>
<tr>
<td>DN−</td>
<td>33 (100%)</td>
<td>12 (36%)</td>
<td>21 (64%)</td>
</tr>
</tbody>
</table>

* One hundred fifty-three parents (DN+ group) and 54 parents (DN− group) were homozygous (MM or TT) and were not used in the analysis.

### Table 4. Allele Transmission (T/M) From M235T Heterozygous Parents to IDDM Offspring According to DN Status and P Values Based on Comparison of DN+ and DN− Samples

<table>
<thead>
<tr>
<th>DN Status</th>
<th>Total</th>
<th>Female</th>
<th>Male</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DN+</td>
<td>76/67</td>
<td>35/35</td>
<td>41/32</td>
<td></td>
</tr>
<tr>
<td>DN−</td>
<td>32/38</td>
<td>20/17</td>
<td>12/21</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>.19</td>
<td>.73</td>
<td>.05</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

Previously, investigations of AGT as a candidate gene for DN have been mixed. While several groups have published negative results, Fogarty et al found the M235T TT genotype to be associated with DN, and Marre et al observed an interactive effect of M235T and the ACE I/D polymorphism. Adding to this ambiguity, a previous study in our laboratory found that the T allele occurs more frequently among patients with diabetes and/or macroalbuminuria compared with those with microalbuminuria, but the level of evidence did not reach statistical significance.

To explore further the role of AGT, we identified family trios and tested for nonrandom allele transmission from parents to children. In our analysis, we considered two types of trios, those with a DN− child and those with a DN+ child. The addition of DN+ trios increases specificity because positive results using only DN− trios may be due to IDDM as well as DN genes or even another mechanism leading to segregation distortion that is independent of nephropathy status.

In aggregate, the M235T T allele displayed no significant evidence of association with DN. However, we did uncover an apparent association within certain subgroups. For example, we observed preferential transmission of the T allele to those with ESRD (P = .04). We can thus speculate that the T allele is important primarily in the most severe cases of DN. Alternatively, those without ESRD may simply tend to be more frequently misclassified with respect to DN.

We have also considered analysis stratified by gender. Recently, Hopkins et al found gender to be an important covariate in the relationship of AGT with blunted renal vascular response, a potential promoter of hypertension. Specifically, an effect due to a TT genotype was strongly evident for males but nonexistent for females. Thus our finding of preferential transmission of T to DN+ males compared with DN− males (P = .05) may be of particular interest. Furthermore, this finding is consistent with our investigation of patients with non–insulin-dependent diabetes mellitus, which revealed a strong relationship between the TT genotype and DN only in men and not in women.

Assuming that the M235T T allele is indeed associated with a subset of DN, it is still unclear whether the relationship is causative. If DN susceptibility is in fact related to blood pressure–driven alterations in hemodynamics, then causality would imply that the presence of T alleles could increase blood pressure directly. Although such a functional role has neither been definitively confirmed nor refuted, Jeunemaitre et al have recently conducted an extensive investigation of AGT diallelic polymorphisms in two large populations (French Caucasian and Japanese) and concluded that (1) the exception of G-6A, no other known diallelic polymorphism is consistently associated with hypertension and (2) the G-6A G and A alleles are virtually synonymous with the M235T M and T alleles, respectively. Notably, Inoue et al have shown that the defining nucleotide substitution of G-6A affects the basal transcription rate of AGT, which could potentially account for essential hypertension susceptibility. The only multiallelic marker reported to show strong association with hypertension is the AGT-GT polymorphism. For completeness, we also genotyped this marker but found no alleles to be either independently associated with DN or helpful in gaining further insight into transmission from M235T homozygous parents (data not shown).

Because we lacked systematic blood pressure measurements on parents and also on subjects at the time of diabetes, we cannot rule out the possibility that the M235T T allele (or the G-6A A allele) modifies renal hemodynamics directly (ie, independent of blood pressure) in the presence of IDDM. Further studies will therefore be necessary, not only to confirm our association results but also to investigate more fully the biology underlying renal complication of IDDM.

Acknowledgments

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References


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