Gender-Specific Association of M235T Polymorphism in Angiotensinogen Gene and Diabetic Nephropathy in NIDDM

Maria Beatriz S. Freire, Linong Ji, Tomio Onuma, Tihamer Orban, James H. Warram, Andrzej S. Krolewski

Abstract—This study examined the association between the development of nephropathy in non–insulin-dependent diabetes mellitus (NIDDM) patients and M235T polymorphism in the angiotensinogen gene. White NIDDM patients with diabetic nephropathy (case subjects, n=117) and patients without any evidence of nephropathy and ≥10 years of NIDDM (control subjects, n=125) were selected from among patients of the Joslin Diabetes Center and examined. In addition to a standardized examination, blood was drawn for DNA and determination of M235T genotypes at the angiotensinogen locus. For the angiotensinogen gene, the frequency of the genotype 235T/235T, known to be associated with essential hypertension, was higher among case subjects with nephropathy than in control subjects without this complication. This difference, expressed as the odds ratio for nephropathy among 235T/235T homozygotes in comparison with all other genotypes, was 2.2 (95% confidence interval, 1.1 to 4.4). The difference, however, was confined to men (odds ratio, 4.8; 95% confidence interval, 1.5 to 14.9), with the distribution of genotypes in case and control subjects being equal among women (odds ratio, 1.1). DNA polymorphism M235T in the angiotensinogen gene, which is associated with higher expression of this gene, contributes to the risk of diabetic nephropathy in NIDDM men but not in women. (Hypertension. 1998;31:896-899.)

Key Words: nephropathy ■ angiotensinogen ■ polymorphism ■ diabetes

Recent studies have provided evidence that the development of renal complications in diabetes may be determined by genetic factors. The evidence includes clustering of diabetic nephropathy in families, as well as an association between predisposition to essential hypertension and the development of diabetic nephropathy. It is hypothesized that DNA sequence differences in regulatory or structural parts of genes encoding for proteins of the RAS may contribute to essential hypertension in nondiabetic persons and to hypertension and renal damage in the presence of diabetes.

A molecular variant of the AGT gene (with a threonine substituted for methionine at amino acid residue 235) has been associated with elevated levels of serum angiotensinogen and essential hypertension in nondiabetics. Recently, this polymorphism has been found to be in complete linkage disequilibrium with a sequence difference at position 6 in the promoter region. The latter affects the expression of the AGT gene, with the highest tissue levels of angiotensinogen being found in homozygotes T235/T235.

Two case-control studies in IDDM patients have shown that the homozygotes 235T/235T had an elevated risk of developing diabetic nephropathy. Recently, this association has been confirmed in our family study using TDT. The 235T effect was found among men but not among women. Other case-control studies have not found any association with M235T polymorphism. A gender-specific effect of the AGT polymorphism, however, was not examined in those studies.

Because these studies were carried out mainly in IDDM patients, we conducted a case-control study to evaluate whether the M235T polymorphism in AGT gene plays a role in predisposition to diabetic nephropathy in NIDDM. We specifically tested the effect of this polymorphism according to gender.

Methods

Study Population

Among the patients of the Joslin Diabetes Center in 1991, there were 6600 Massachusetts residents whose diabetes was diagnosed at between ages 30 and 59 years. In 1993, a questionnaire regarding the family history of diabetes and presence or absence of diabetic complications was sent to a 33% random sample of this subset of the Joslin population. We obtained responses from 1429 patients (65% response rate). For all these patients, we retrieved results of urinalyses (albumin reagent strips as well as determinations of the albumin/creatinine ratio) obtained during routine clinic visits during the 3-year interval of January 1991 through December 1993.

Determination of the Presence or Absence of Nephropathy

During routine clinic visits to the Joslin Diabetes Center, a random specimen of a patient’s urine is examined with a reagent strip (Multistix, Ames Division, Miles Laboratories). Since January 1,
1991, urine samples with a Multistix reading <2+ have generally been examined for the albumin/creatinine ratio as well. For the present study, the Multistix and albumin/creatinine ratio results obtained during the 3-year interval were used to classify patients with regard to the presence or absence of diabetic nephropathy as described previously. If Multistix readings were ≥2+ in two of three samples, patients were considered to have overt proteinuria; otherwise, measurements of the albumin/creatinine ratio (micrograms per milligram) were used to classify patients. Two of three ratios ≥250 for men or ≥355 for women were considered indicative of overt proteinuria. Microalbuminuria was defined as a ratio of albumin to creatinine in the intermediate range, 17 to 249 for men and 25 to 354 for women. Patients with an albumin/creatinine ratio <17 in men and <25 in women were considered to be normoalbuminuric. In total, 617 individuals had normoalbuminuria, 232 had microalbuminuria, 236 had proteinuria, and 345 had insufficient measurements (Multistix or albumin/creatinine ratio) to determine diabetic nephropathy status.

Examination of Case and Control Subjects
All patients selected for this study were white. For the control group, we selected a 33% systematic sample of patients (n = 150) from among patients with normoalbuminuria and ≥10-year duration of diabetes (as of January 1994). For the case group, we selected a 66% systematic sample of patients with persistent proteinuria (n = 156). Between 1994 and 1997, we examined 125 of the controls (83%) and 117 of the cases (75%).

Examinations were performed by trained family recruiters, and the examinations took place at the Joslin Diabetes Center or in the home of the study participant. In addition to a standardized interview about the diagnosis of diabetes and history of its treatment, measurements of height, weight, and blood pressure were obtained. From all examinees, urine samples were obtained and blood was drawn for biochemical measurements and extraction of DNA. The protocol for this study was approved by the Human Subjects Committee of the Joslin Diabetes Center.

Determination of Clinical Characteristics
To evaluate what proportion of these patients might have IDDM, we determined the interval between the diagnosis of diabetes and the beginning of insulin treatment, as well as the level of IDDM-associated antibodies in the serum at the time of examination. Antibodies to GAD65Ab were measured by the GAD65Ab assay as previously described. IAAs were measured by the IA2-icAb assay as previously described. For both antibodies, the criterion for a positive assay was an index >0.1, which is >2 SD above the mean for normal control subjects. In the Combined Autoantibody Workshop 1995 (Denver, Colo), the GAD65Ab assay was 100% specific and 61% sensitive, and the IA2-icAb was 88% specific and 68% sensitive. The GAD65Ab was 91% specific and 89% sensitive in the Second International Glutamic Acid Decarboxylase Antibody Workshop.

Measurements of height and weight were obtained during the examination and used to calculate percent ideal body weight. Blood pressure was measured twice, 5 minutes apart, according to a standard protocol while the patient was resting in a sitting position. Hypertension was diagnosed if a patient was being treated with antihypertensive drugs at the time of examination or the average blood pressure obtained during the examination was ≥95 mm Hg diastolic or ≥160 mm Hg systolic.

Level of creatinine in serum and in urine was measured using alkaline picric colorimetry (modified Jaffe reaction) on an Astra7 (Beckman Instruments). Albumin concentration in urine was measured by immunonephelometry on a BN-100 with the N Albumin Kit (Behring) as described previously.

DNA Analysis
DNA was extracted from leukocytes according to standard protocols. For 7 control and 2 case subjects, insufficient blood was obtained for DNA extraction. Polymorphism M235T in the AGT gene was determined by a DGGE protocol that was developed in our laboratory and described previously. Briefly, the 3′ portion of exon 2 of the AGT gene was amplified by polymerase chain reaction with the same primers described by Jeunemaitre et al. To facilitate the analysis by a DGGE protocol, a 6-bp “GC-clamp” at the 5′ end was attached to the antisense primer. Polymerase chain reaction products (354 bp) were electrophoresed in 10% polyacrylamide gels with a linearly increasing concentration of denaturant from 35% to 70% (100% denaturant: 40% formamide, 7 mol/L urea, 1× TAE). The gels were electrophoresed for 600 volt-hours (150 V × 4 hours) in 1× TAE buffer at a constant temperature of 60°C. After staining with ethidium bromide, gels were exposed to UV light and photographed. The allele coding for a threonine (235T, codon ACG) stopped lower in the gel (higher concentration of denaturant, 48.5%) than the allele coding for a methionine (235M, codon ATG; concentration of denaturant, 48%). Homozygotes were detected as a single band at 48% or 48.5% of denaturant concentration, whereas heterozygotes were identified as two bands.

Statistical Analysis
The distribution of genotypes were compared between case and control subjects using χ² tests. The association between genotypes and outcomes was evaluated by computing ORs and 95% CIs. The homogeneity of ORs was tested using the Breslaw-Day test. Multiple logistic regression was used to estimate the effect of the AGT genotypes after adjustment for other variables.

Results
Clinical characteristics of the study groups are summarized in Table 1. We enrolled 117 diabetic patients with diabetic nephropathy (cases) and 125 individuals with long-duration diabetes and normoalbuminuria (controls). Both groups had similar average duration of diabetes and age at the time of examination. The proportion of each group with IDDM was similar regardless of whether IDDM was defined on the basis of clinical presentation (insulin required within 2 years of diagnosis and percent ideal body weight <130) or the presence of immunological markers of IDDM. Case subjects were significantly heavier, and the case group had a higher frequency of hypertension and a larger proportion of individuals treated for hypertension. Patients with nephropathy had higher serum creatinine than controls.

The genotypes and allele frequencies of the AGT M235T polymorphism are shown in Table 2 according to study group. The genotype distributions differed significantly (χ²=6.04, 2 df, P<.05), with the genotype 235T/235T being more frequent among cases with nephropathy than among controls. For these 235T homozygotes, the risk of nephrop-
TABLE 1. Characteristics of Patients With NIDDM According to Nephropathy Status

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control (n=125)</th>
<th>Case (n=117)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at examination, y</td>
<td>62±6</td>
<td>62±6</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Duration of diabetes, y</td>
<td>18±7</td>
<td>17±7</td>
<td></td>
</tr>
<tr>
<td>Type 1 presentation, %*</td>
<td>10</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Positive antibody titers, %†‡</td>
<td>18</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Treated with insulin, %</td>
<td>66</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Ideal body weight, %</td>
<td>128±28</td>
<td>140±32</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>With hypertension, %‡</td>
<td>36</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Anthyptensive therapy, %</td>
<td>30</td>
<td>60</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Serum creatinine, μmol/L</td>
<td>88.4±26</td>
<td>114.9±53</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SD.

*Insulin treatment required within 2 years of diagnosis and percent ideal body weight <130.
†Index >0.1 for GAD65Ab or IA2-icAb assay.‡Taking antihypertensive treatment or blood pressure (mm Hg) ≥95 diastolic or ≥160 systolic.

In contrast, a distinctive pattern of differences in the distributions of both genotypes and alleles emerged when the study groups were analyzed separately for men and women (Table 3). All of the difference between cases and controls was confined to men (OR, 4.8; 95% CI, 1.5 to 14.9), while the distribution of genotypes among women was quite similar for cases and controls (OR, 1.1; 95% CI, 0.4 to 3.0). The difference between the ORs in men and women was statistically significant (P = .05), indicating that the increased risk of nephropathy associated with 235T/235T homozygosity was specific to men. The pattern of OR was not changed when the analysis was carried out after excluding patients who could have adult-onset IDDM, ie, those with positive GAD65Ab or IAA antibodies or nonobese patients (ideal body weight <130%) treated with insulin within 2 years of the diagnosis of diabetes (data not shown).

TABLE 2. Distribution of M235T AGT Genotypes and Alleles in Study Groups

<table>
<thead>
<tr>
<th>M235T Genotypes</th>
<th>Control, n (%)</th>
<th>Case, n (%)</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>44 (37.3)</td>
<td>45 (39.1)</td>
<td>6.04</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>MT</td>
<td>60 (50.9)</td>
<td>44 (38.3)</td>
<td>11.19</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>TT</td>
<td>14 (11.9)</td>
<td>26 (22.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGT allele frequency</td>
<td>0.63/0.37</td>
<td>0.58/0.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

χ² for comparison of genotypes, 6.04 (2 df, P < .05); χ² for comparison of alleles, 0.64 (1 df, NS).

TABLE 3. Distribution of AGT Genotypes in Case and Control Subjects According to Gender

<table>
<thead>
<tr>
<th>M235T Genotypes</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Case</td>
<td>Control</td>
</tr>
<tr>
<td>MM/MT</td>
<td>57</td>
<td>54</td>
</tr>
<tr>
<td>TT</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>OR</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>1.5–14.9</td>
<td>0.4–3.0</td>
</tr>
</tbody>
</table>

Breslow-Day test for homogeneity of OR, P = .05.

To examine whether the effect of the 235T/235T homozygosity on the development of diabetic nephropathy is mediated through elevated systemic blood pressure, the analysis was carried out separately among individuals without and with hypertension. 235T/235T homozygotes (Table 4) did not have an increased risk of diabetic nephropathy if they were normotensive (OR, 1.2; 95% CI, 0.4 to 3.6), but they were at particularly high risk for diabetic nephropathy if they were hypertensive (OR, 3.4; 95% CI, 1.1 to 10.8). The difference between the two ORs, however, was not statistically significant (P = .21).

Finally, the effect of the M235T polymorphism was estimated by logistic regression models that included gender and type of diabetes and interactions between gender and the M235T polymorphism and type of diabetes. There was little difference in comparison with the results of univariate analyses. The relative OR for men homozygous for the 235T allele at the AGT locus increased to 6.9 compared with 4.8 in univariate analysis (Table 3), and as before, the interaction term for the difference between the relative OR in men and women had a value of P = .05.

Discussion

Recent studies have shown that the development of diabetic nephropathy may be associated with susceptibility to essential hypertension.1 Because the RAS plays a critical role in blood pressure homeostasis, DNA sequence differences in the regulatory or structural parts of genes encoding for the RAS proteins have been proposed as candidate genes for the development of essential hypertension in nondiabetics and of hypertension and renal damage in the presence of diabetes.19,10 The present study examined this hypothesis in white patients with NIDDM.

TABLE 4. Distribution of AGT Genotypes in Case and Control Subjects According to Hypertension Status

<table>
<thead>
<tr>
<th>M235T Genotypes</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Case</td>
<td>Control</td>
</tr>
<tr>
<td>MM/MT</td>
<td>64</td>
<td>37</td>
</tr>
<tr>
<td>TT</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>OR</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>0.4–3.6</td>
<td>1.1–10.8</td>
</tr>
</tbody>
</table>

Breslow-Day test for homogeneity of OR, P = .21.

*Defined as taking antihypertensive treatment or blood pressure (mm Hg) ≥95 diastolic or ≥160 systolic.
We found that a molecular variant of the AGT gene, arising from the substitution of C for T at nucleotide 714 and changing methionine to threonine (M235T), is associated with increased risk of the development of diabetic nephropathy in NIDDM. Nondiabetic individuals homozygous for this variant have elevated levels of angiotensinogen. It is postulated that the level of intrarenal angiotensin II is influenced mainly by the level of substrate, angiotensinogen, and is minimally affected by levels of angiotensin-converting enzyme. A recent study demonstrated that homozygotes for the T235 allele have a blunted renal vascular response to exogenous angiotensin II, a finding that could be explained by higher prevailing levels of angiotensin II in the kidney of these patients. This effect was evident specifically in men but not in women. It is interesting, therefore, that we found the risk of nephropathy in our study was associated with the T235 allele in men only.

In two recent studies of patients with IDDM, homozygotes for the T235 allele at the AGT locus had an elevated risk of the development of diabetic nephropathy, while in other studies this finding was not confirmed. None of these studies, however, analyzed the results in men and women separately. Interestingly, in our recent family-based study, we found evidence that the T235 allele was preferentially transmitted from heterozygous parents to individuals with nephropathy, but only among men.

The mechanisms underlying the association between T235 homozygosity and diabetic nephropathy are unknown. The association is restricted to hypertensive NIDDM patients, so the TT genotype by itself does not seem to be sufficient to increase susceptibility to diabetic nephropathy. However, in the presence of hypertension (only a minor portion of which is related to the M235T polymorphism), T235 homozygosity and its associated high levels of angiotensinogen in kidney may interact with the high blood pressure, possibly through a further increase of intraglomerular pressure.

Finally, some limitations of this study should be mentioned. In this case-control study, we enrolled patients who were being seen at a clinic. Most likely, this population was depleted of patients with coronary artery disease and case patients who progressed rapidly to end-stage renal disease and died. This might have reduced the differences in the distribution of the M235T alleles between case and control groups. Although the present study identified positive associations between the development of diabetic nephropathy and T235 homozygosity at the angiotensinogen locus, it is clear that the proportion of cases of nephropathy accounted for by this association is small, with the larger proportion being due, presumably, to as yet unidentified genes.

Acknowledgments

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