Polymorphisms of the Protein Kinase C-β Gene (PRKCB1) Accelerate Kidney Disease in Type 2 Diabetes Without Overt Proteinuria

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OBJECTIVE — We investigated the contribution of PKC-β gene (PRKCB1) polymorphisms to diabetic kidney disease in a prospective observational follow-up study.

RESEARCH DESIGN AND METHODS — A total of 364 Japanese subjects with type 2 diabetes without overt proteinuria were enrolled during 1996–1998 and followed until 2004. Five single nucleotide polymorphisms (−1504C/T, −546C/G, −348A/G, −278C/T, and −238C/G) in the promoter region of PRKCB1 were genotyped. The end points were transition from stage to stage of diabetic nephropathy as a time-to-event outcome and the annual decline rate of estimated glomerular filtration rate (eGFR) as a slope-based outcome.

RESULTS — During the study (median 6 years), 34 of 364 subjects (9.3%) progressed. Kaplan-Meier estimation revealed that subjects with both T allele at −1054 C/T and G allele at −546 C/G polymorphisms frequently showed transition to advanced stages of diabetic nephropathy (P = 0.015). The annual change rate in eGFR in the subjects with both alleles was also significantly higher than in others (−2.96 ± 0.62 vs. −1.63 ± 0.15 ml/min per 1.73 m²/year, P = 0.02). The estimated frequency of this risk T-G haplotype was significantly higher in the progressors than in the nonprogressors (5%) (odds ratio 2.3 [95% CI 1.0–5.2]), and it was also higher in those with accelerated decline of the ΔeGFR (≥3 ml/min per 1.73 m²/year) than in those without (2.1 [1.1–3.9]).

CONCLUSIONS — Our study indicates that PRKCB1 is a predictor for worsening of kidney disease in Japanese subjects with type 2 diabetes.

Diabetic nephropathy associated with type 2 diabetes is a leading cause of end-stage renal disease. While clinical studies clearly show that prolonged hyperglycemia is an important risk factor for this microvascular complication (1,2), epidemiological and familial studies (3–6) suggest that genetic susceptibility also plays a pivotal role.

An abnormal activation of protein kinase C (PKC)-β under diabetic condition is proposed as a putative mechanism in the pathogenesis of diabetic nephropathy (7). Thus, the gene (PRKCB1) encoding this protein is considered to be a candidate gene for susceptibility to diabetic nephropathy. Recently, we first reported the association between the PRKCB1 polymorphisms and diabetic nephropathy in type 1 diabetic patients by using two independent methods, a case-control study and a family-based study (8). In our previous study (8), a T-G haplotype consisting of two single nucleotide polymorphisms (−1504C/T and −546C/G) in the promoter region was associated with twofold increased risk of diabetic nephropathy. Although our report provided the first evidence that the PRKCB1 polymorphisms may contribute to genetic susceptibility to diabetic nephropathy (8), several questions remain unclear: whether this relevance extrapolates to type 2 diabetic subjects, whether the risk haplotype is associated with early diabetic nephropathy, and whether this risk haplotype of PRKCB1 influences kidney function.

Therefore, in the present study, we investigated the effect of the risk haplotype of PRKCB1 on diabetic nephropathy by assessing two renal outcomes: transition from any given stage to the more advanced stage of diabetic nephropathy defined by albumin excretion rate (AER) as a time-to-event outcome and kidney function defined by the annual decline rate of estimated glomerular filtration rate (eGFR) as a slope-based outcome. To this purpose, we carried out a prospective observational follow-up study in Japanese subjects with type 2 diabetes without overt proteinuria.

RESEARCH DESIGN AND METHODS — The subjects were recruited from among the participants at the outpatient clinic of the Department of Medicine, Shiga University of Medical Science (9). During 1996–1998, patients clinically diagnosed as having type 2 diabetes in accordance with World Health Organization criteria were examined with multiple measurements of urinary AER and estimated GFR in 24-h urine sample collection in the initial 2 years (baseline period). On the basis of multiple measurements (average of 2.7 times [range 2–9]), patients (n = 518) were classified (regardless of diabetes duration) as having normoalbuminuria, microalbuminuria, or overt proteinuria. Only patients with normoalbuminuria or microalbu-
minuria in the baseline period were enrolled in this study. Patients with overt proteinuria (n = 72) or with intermittent microalbuminuria (n = 22) were excluded. As other exclusive criteria, diabetic patients with complicating cancer, liver disease, or non diabetic kidney disease confirmed by renal biopsy were excluded (n = 28). Each individual provided a blood sample for biochemical measurements and DNA extraction. The diagnosis of diabetic retinopathy was made by ophthalmologists. Diabetic retinopathy was defined as simple retinopathy or more. At the beginning of the study, 396 patients were eligible for enrollment in this study. The participants, during the follow-up periods, underwent the standardized physical examination, biochemical measurements, and a measurement of AER in 24-h urine collection at least once a year. The follow-up periods lasted at least 3 years, until the end of 2004 or death. All participants received treatment based on the standard strategies for diabetes, hypertension, and hyperlipidemia during the follow-up periods. Of 396 participants, 19 patients were excluded from the analysis because the follow-up periods were <3 years. Thirteen patients were not included in the analysis because they had undergone treatment for cancer detected during the follow-up periods. Thus, data for 364 patients (261 with normoalbuminuria and 103 with microalbuminuria) were used in the analysis. The study protocol and informed consent procedure were approved by the ethics committee of Shiga University of Medical Science.

**Definition of outcomes**

The diabetic nephropathy stage of each patient was determined based on the degree of the AER measured by immunoturbidimetry assay (HITACHI 7070E; Hitachi High-Technologies, Tokyo, Japan) in 24-h urine samples after confirming the absence of pyuria and hematuria and the exclusion of other diseases that can increase albuminuria. Patients were designated as being in the stage of normoalbuminuria if AER was <20 μg/min, microalbuminuria if 20 ≤ AER <200 μg/min, and overt proteinuria if AER ≥200 μg/min and estimated GFR (eGFR) (ml/min per 1.73 m²) = 60 (stage 1 or 2 according to the Kidney Disease Outcomes Quality Initiative clinical practice guidelines [10]) in two consecutive measurements. The outcome of diabetic nephropathy stage was defined as transition from any given stage to the more advanced stage of diabetic nephropathy. The progressors included both cases that developed microalbuminuria and those that progressed from microalbuminuria to overt proteinuria. Subjects who showed intermittent transition to advanced stages were not counted among the progressors.

The eGFR was estimated with one of the equations developed in the Modification of Diet in Renal Disease (MDRD) study (11). In the present study, the individual data for the daily urea excretion from a 24-h urine collection was available. Thus, we used the MDRD equation with demographic, serum, and urine variables, which were reported to be the most precise in the original MDRD study (11): eGFR = 198 × (Pcr)−0.293 × (Age)−0.037 × (0.822 if patient is female) × (SUN)−0.018 × (UUN)1.016 where Pcr is serum creatinine concentration (mg/dl), SUN is serum urea nitrogen concentration (mg/dl), and UUN is urinary nitrogen excretion (g/day). The rate of annual decline in eGFR (Δ eGFR) over the course of the study was determined from the slope of the plot of all measurements of eGFR for each individual (median 8 times [range 4–13]) calculated with linear regression analysis and was expressed as ml/min per 1.73 m²/year. Also, we dichotomized the annual decline in eGFR as accelerated decline (Δ eGFR ≥3 ml/min per 1.73 m²/year) on the basis of previous studies (12,13).

**Genotyping of the PRKCB1 polymorphism**

Genomic DNA was extracted from peripheral leukocytes with a DNA purification kit (QIAamp blood kit, Qiagen, Chatsworth, CA) from the five polymorphic sites in the promoter region of PRKCB1 (−1504C/T, −546G/C, −348A/G, −278C/T, and −238C/G) were amplified by the PCR method according to a previously described method (8). Genotyping for all polymorphisms was performed by hybridization with allele-specific oligonucleotide probes (14).

**Statistical analysis**

Comparisons between frequencies in the study groups were made by χ² tests with Fisher’s exact test. Comparisons between groups were performed by using an unpaired Student’s t test for normally distributed variables or a Mann-Whitney U test for nonnormally distributed variables. For a time-to-event analysis, the association

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**Table 1—Clinical characteristics at baseline and allele frequencies at each polymorphic site of PRKCB1 in subgroups according to the progression of diabetic nephropathy**

<table>
<thead>
<tr>
<th></th>
<th>Nonprogressors</th>
<th>Progressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>330</td>
<td>34</td>
</tr>
<tr>
<td>Clinical characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>162/168</td>
<td>18/16</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60 ± 9</td>
<td>61 ± 10</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>13 ± 7</td>
<td>13 ± 8</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.2 ± 0.9</td>
<td>7.3 ± 1.1</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>213 ± 30</td>
<td>210 ± 32</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>110 ± 60</td>
<td>136 ± 60*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>133 ± 16</td>
<td>137 ± 15</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>76 ± 9</td>
<td>77 ± 10</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>48</td>
<td>65</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23 ± 3</td>
<td>24 ± 3*</td>
</tr>
<tr>
<td>Retinopathy (%)</td>
<td>80</td>
<td>94</td>
</tr>
<tr>
<td>AER (μg/min)</td>
<td>20 ± 24</td>
<td>38 ± 43*</td>
</tr>
<tr>
<td>eGFR (min/ml per 1.73 m²)</td>
<td>105 ± 20</td>
<td>100 ± 20</td>
</tr>
<tr>
<td>Diabetes treatment (diet/oral agents/insulin)</td>
<td>64/191/75</td>
<td>9/13/12</td>
</tr>
<tr>
<td>Allele frequencies (%)†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−1054 C/T</td>
<td>94.4/5.6</td>
<td>88.8/11.2*</td>
</tr>
<tr>
<td>−546 C/G</td>
<td>94.9/6.1</td>
<td>88.8/11.2</td>
</tr>
<tr>
<td>−348 A/G</td>
<td>66.7/33.3</td>
<td>61.8/38.2</td>
</tr>
<tr>
<td>−287 C/T</td>
<td>66.4/33.6</td>
<td>60.3/39.7</td>
</tr>
<tr>
<td>−238 C/G</td>
<td>74.5/25.5</td>
<td>79.6/20.6</td>
</tr>
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</table>

Data are means ± SD. *P < 0.05 vs. nonprogressors. †Data are expressed as percent of chromosomes for each allele.
between the PRKCB1 polymorphisms and the transition of stage of diabetic nephropathy was estimated using Kaplan-Meier procedure and was compared by the log-rank test. Follow-up time was censored if the development or progression of microalbuminuria occurred or if the patient was unavailable for follow-up. To adjust the influence of conventional risk factors for the transition, a multivariate Cox proportional hazard regression model was applied. The independent variables used as conventional risk factors were sex, duration of diabetes, HbA1c, total cholesterol, triglycerides, hypertension (present/absent), the use of renin-angiotensin system blockade drugs (yes/no), retinopathy (present/absent), BMI, AER, and eGFR at baseline. Hypertension was defined as blood pressure ≥140/90 mmHg or on the use of antihypertensive drugs. Multiple logistic regression analysis was used to assess the prognostic effect for ΔeGFR and to adjust the influence of conventional risk factors. The linkage disequilibrium coefficients (the correlation coefficient Δ) and estimated haplotype frequencies were calculated by a statistical method described previously (15). All data were analyzed using the SPSS software package (Version 11; SPSS, Chicago, IL). A value of P < 0.05 was taken to be statistically significant.

RESULTS — The mean follow-up period after the baseline period was 6.0 ± 0.9 years (median 6 years [range 3–7]). During the study period, 34 of 364 subjects showed transition to advanced stages of diabetic nephropathy; they comprised 18 of 261 with normoalbuminuria and 16 of 103 with microalbuminuria. The rates of annual change in eGFR for patients grouped according to the genotypes at −1054 C/T and −546 C/G sites of the PRKCB1 polymorphisms. Log-rank test: \( \chi^2 = 5.92, P = 0.015 \). Solid line, subjects with both the T allele at −1054 C/T (CT or TT) and the G allele at −546 C/G (CG or GG) (n = 43). Broken line, subjects with other patterns (n = 321).

Clinical characteristics of the nonprogressors and the progressors at baseline are shown in Table 1. BMI, levels of triglycerides, and AER at the baseline were significantly higher in the progressors than in the nonprogressors. The allele frequencies at the −1054 C/T polymorphic site were significantly different in the progressors and the nonprogressors (\( \chi^2 = 4.03, P = 0.043 \)). The G allele frequencies at −546 C/G were weakly but not significantly different in the two groups (\( \chi^2 = 3.26, P = 0.071 \)). The frequencies of the alleles at the other three polymorphic sites were similar in the two groups.

For the time-to-event analysis, being a carrier of the T allele at the −1054 C/T polymorphic site (n = 44) was significantly associated with transition to advanced stages of diabetic nephropathy as examined with the Kaplan-Meier method (log-rank test: P = 0.021). The adjusted risk of the T allele carrier was 2.9 (95% CI 1.3–6.5) after adjustment for conventional risk factors in the multiple Cox model. Also, being a carrier of the G allele at −546 C/G (n = 47) was associated with the transition to advanced stages (P = 0.037), and the adjusted risk was 2.7 (1.2–6.2). No association between the other three polymorphic sites and transition to advanced stages was found. Interestingly, comparison for the frequency of transition to advanced stages in the subjects carrying both risk alleles, which were previously reported to be associated with diabetic nephropathy (8), revealed that 19% of 43 subjects with both the T allele (CT or TT) at −1054 C/T and the G allele (CG or GG) at −546 C/G polymorphisms showed transition to advanced stages of diabetic nephropathy, in contrast to 8% of 321 subjects with other patterns. For the time-to-event analysis, the subjects having both the T allele at −1054 C/T and the G allele at −546 C/G showed more rapid transition to advanced stages than those without them (P = 0.015) (Fig. 1). The adjusted risk of these subjects was 3.0 (1.3–6.7).

Next, we examined the role of the risk T-G haplotype in affecting the kidney function evaluated by the rate of annual change in eGFR. In the subjects with both the T allele at −1054 C/T (CT or TT) and the G allele at −546 C/G (CG or GG), the rate of annual change in eGFR was significantly higher than in subjects with other patterns (means ± SE) −2.96 ± 0.62 vs. −1.63 ± 0.13 ml/min per 1.73 m²/year, P = 0.02 by Mann-Whitney U test) (Fig. 2). Eighteen (41.9%) of 43 subjects with
both the T allele at −1054 C/T and the G allele at −546 C/G had an accelerated decline in eGFR (Δ eGFR ≥3 m/min per 1.73 m²/year), in contrast to 81 (25.6%) of 317 subjects with other patterns (χ² = 5.1, P = 0.02). The risk for the Δ eGFR in those with both risk alleles was 2.1 (95% CI 1.1–4.0). Similarly, the risk for the Δ eGFR in the multiple logistic regression analysis was twofold after adjustment for risk factors (adjusted odds ratio 2.2 [95% CI 1.1–4.5]).

Finally, we analyzed the structure and estimated frequencies of haplotypes using the expectation maximization algorithm (Table 2). The degree of linkage disequilibrium of the G-T haplotype at −1054 C/T and −546 C/G was very strong, with a value of 0.94 for the correlation coefficient Δ (15). The estimated frequency of the T-G haplotype was significantly higher in the progressors (12%) than in the nonprogressors (5%) (χ² = 4.32, P = 0.038). The odds ratio of the T-G haplotype was 2.3 (95% CI 1.0–5.2). Regarding the Δ eGFR, the estimated frequencies of the T-G haplotype were also different in the two groups (χ² = 5.8, P = 0.02). The risk associated with the T-G haplotype for the Δ eGFR was 2.1 (95% CI 1.1–3.9).

### CONCLUSIONS

The present study provides evidence that PRKCB1 associates with susceptibility to the worsening of diabetic kidney disease in Japanese subjects with type 2 diabetes. In this study, we used both time-to-event and slope-based renal outcomes to evaluate the effect of PRKCB1 on diabetic kidney disease. The subjects having the T allele at −1054 and the G allele at −546 polymorphisms of PRKCB1 showed transition from any given stage to the more advanced stage of diabetic nephropathy defined by AER. Furthermore, those subjects at risk showed an accelerated rate of annual decline in eGFR.

Our finding is consistent with the previous results (8) in Caucasian subjects with type 1 diabetes. Although the previous results provided the first evidence of an association between the PRKCB1 polymorphisms and diabetic nephropathy, the results could not resolve the question of whether subjects with the risk haplotype frequently develop diabetic nephropathy and progress to more advanced stages. In the present study, the subjects with both risk alleles showed more rapid transition to advanced stages of diabetic nephropathy than those without them, and the risk was twice as great. This result from the time-to-event analysis strongly supports the notion of the association between the PRKCB1 polymorphisms and diabetic nephropathy.

One strength of the present study is its investigation of the influence of PRKCB1 on the annual change in eGFR. It is now well-appreciated that the majority of the subjects who progress to end-stage renal disease have a chronic and progressive decline of renal function over the years. This process may continue even when no initial renal damage is present. In the diabetic patients, increased urinary albumin excretion has been considered to reflect the initial renal damage and to be a strong predictor of the progression (16). However, albuminuria does not always reflect the change of the renal function. In fact, several studies (17,18) showed that some diabetic subjects with normoalbuminuria have low GFR. Also, Rudberg and Osterby (19) reported that the decline rate in GFR, although still within the normal range, was positively correlated with glomerular structural changes. Here, we provide evidence that the subjects with the T-G haplotype of the PRKCB1 polymorphisms show accelerated annual decline in eGFR. Thus, the T-G haplotype of PRKCB1 may be one of the valuable predictors for the rapid deterioration of renal function in type 2 diabetic patients.

In the previous study, the association between the risk haplotype of PRKCB1 and diabetic nephropathy was particularly strong in subjects with a short duration of diabetes (8). However, in the present study, we observed no such effect of diabetes duration on our outcomes (data not shown). This may have resulted from the difficulty of identifying the exact diabetes duration in type 2 diabetes.

A limitation of the present study is that we did not perform direct measurement of GFR, such as inulin clearance. Also, it is debatable whether the MDRD equation can be applied to Japanese patients because ethnicity is a factor that might influence results of the tested GFR-estimating equations (11). We realize that more precise methods for the measurement of GFR will be needed to confirm our results and that careful adjustment of MDRD equations is necessary in Japanese populations with chronic kidney disease. However, the comparison of serum creatinine- and iothalamate-based measurements of GFR was reported to give similar time-to-event and slope-based renal outcome results in the African-American Study of Kidney Disease and Hypertension (20). Therefore, in serial measurements of eGFR, the difference between the estimation and the real measurement of GFR is unlikely to change the main conclusion. Also, the mechanism by which the risk haplotype of PRKCB1 influences renal function remains unclear. We previously demonstrated that PKC-β1 and PKC-β2 mRNA levels in lymphoblasts cultured under high-glucose conditions were influenced by the presence of the T-G haplotype (8). However, the different expression of PKC-β or the functional significance according to the PRKCB1 polymorphisms has not been clarified. Further study regarding the functional role is required to verify this effect.

In conclusion, our study indicates that the T-G haplotype of the PRKCB1 polymorphisms is a predictor for worsening of kidney disease in Japanese patients with type 2 diabetes. Furthermore, this risk haplotype may also be a risk factor for cardiovascular disease because an elevated AER and a reduced GFR were identified as independent risk factors for it (21,22).
PRKCB1 polymorphisms and nephropathy

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References