# Genetic Variants of the Protein Kinase C-β 1 Gene and Development of End-Stage Renal Disease in Patients With Type 2 Diabetes

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**Context** Protein kinase C-β (PKC-β) is a cell-signaling intermediate implicated in development of diabetic complications.

**Objective** To examine the risk association of PKC-β 1 gene (*PRKCB1*) polymorphisms and end-stage renal disease (ESRD) in an 8-year prospective cohort of Chinese patients with type 2 diabetes.

**Design, Setting, and Participants** We genotyped 18 common tag single-nucleotide polymorphisms (SNPs) that span the *PRKCB1* gene ($r^2$=0.80) in 1172 Chinese patients (recruited 1995-1998) without renal disease at baseline. A validation cohort included an additional 1049 patients with early-onset diabetes who were free of renal disease at baseline and were recruited after 1998.

**Main Outcome Measures** Associations of *PRKCB1* polymorphisms under additive, dominant, and recessive genetic models with new onset of ESRD (defined as estimated glomerular filtration rate <15 mL/min/1.73 $m^2$ or dialysis or renal-related death) were assessed by Cox proportional hazard regression, adjusted for all conventional risk factors including use of medications.

**Results** After a mean (SD) of 7.9 (1.9) years, 90 patients (7.7%) progressed to ESRD. Four common SNPs were associated with ESRD ($P<.05$). The closely linked T allele at rs3760106 and G allele rs2575390 ($r^2$=0.98) showed the strongest association with ESRD (hazard ratio [HR], 2.25; 95% confidence interval [CI], 1.31-3.87; $P=.003$, and HR, 2.26; 95% CI, 1.31-3.88; $P=.003$, respectively). Four common variants predicted ESRD in separate models. The HR for ESRD increased with increasing number of risk alleles ($P<.001$) in the joint effect analysis. The adjusted risk for ESRD was 6.04 (95% CI, 2.00-18.31) for patients with 4 risk alleles compared with patients with 0 or 1 risk allele. Incidence was 4.4 per 1000 person-years (95% CI, 0.5-8.2) among individuals with 0 or 1 risk allele compared with 20.0 per 1000 person-years (95% CI, 8.8-31.1) in those carrying 4 risk alleles (6.9% of the cohort). These results were validated in a separate prospective cohort of young-onset diabetic patients. Of 1049 patients in the validation cohort, 151 (14.3%) developed chronic kidney disease (CKD) during follow-up, and there were significant associations between both the T allele of rs3760106 and the G allele of rs2575390 and development of CKD (HR, 1.68; 95% CI, 1.10-2.57; $P=.02$, and HR, 1.62; 95% CI, 1.07-2.47; $P=.02$, respectively).

**Conclusion** Genetic variants in the *PRKCB1* gene were independently associated with development of ESRD in Chinese patients with type 2 diabetes.

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Asia is in the midst of an epidemic of type 2 diabetes.[1-3] A recent large-scale epidemiology survey estimated that there are 94.2 million adults affected by diabetes in China, with another 148.2 million with prediabetes.[4] Renal failure is an important cause of mortality among patients with type 2 diabetes.[5,6] Asian populations appear to be particularly at risk of diabetic kidney disease (DKD). In the Microalbuminuria Prevalence (MAP) Study, microalbuminuria was present in 40% and macroalbuminuria in 20% of Asian patients with type 2 diabetes and hypertension.[7] Compared with white individuals, Asian patients have higher risk of end-stage renal disease (ESRD).[8,9] Using a prospective survey, 1% to 3% of Chinese patients with type 2 diabetes developed DKD including ESRD annually depending on baseline risk factors.[10,11] The pathogenesis of DKD is complex and involves hemodynamic, inflammatory,

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and growth factor pathways.[12] Previous studies have identified a strong genetic component to the susceptibility to DKD, although only a few genetic loci have been associated with this complication.[13]

Protein kinase C-β (PKC-β) is an important molecule involved in cell signaling and has been implicated in the development of diabetic microvascular complications as well as diabetic cardiomyopathy.[14-17] Pharmacological inhibition of PKC-β was found to be effective in normalizing hemodynamic changes, extracellular matrix accumulation, and histological features of glomerular damage as well as reducing albuminuria in diabetic animal models.[18-20] Mice that lack PKC-β are protected from glomerular hypertrophy, oxidative stress, and albuminuria when exposed to hyperglycemia.[21] In randomized clinical trials, PKC-β inhibitors decreased loss in glomerular filtration rate (GFR) and proteinuria in diabetic patients who were optimally treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.[22] These data support an important role of PKC-β in the pathogenesis of DKD. Both isoforms of PKC-β, PKC-βI and PKC-βII, are encoded by the PKC-β 1 gene (*PRKCB1*) on the short arm of chromosome 16. Patients with type 1 diabetes carrying 2 single-nucleotide polymorphisms (SNPs) in the promoter region of *PRKCB1* had increased risk of nephropathy,[23] while Japanese patients with type 2 diabetes carrying these variants had increased risk of deterioration in renal function.[24]

Given the increasing recognition of interethnic differences in both distribution and frequency of SNPs for the same gene,[1,25] as well as the high risk for DKD in the Chinese population,[26] we used the HapMap database specific to the Han Chinese population (CHB) to examine the gene structure in Chinese individuals and examined whether polymorphisms in *PRKCB1* are associated with risk of new-onset ESRD in a large prospective cohort of Chinese patients with type 2 diabetes.

## METHODS

Our cohort consisted of 1338 unrelated patients with type 2 diabetes from the Hong Kong Diabetes Registry (HKDR) enrolled between 1995 and 1998. All patients were of southern Han Chinese ancestry and resided in Hong Kong. Patients with classic type 1 diabetes (defined as acute presentation with diabetic ketoacidosis, heavy ketonuria, or continuous requirement of insulin within 1 year of diagnosis) were excluded. In addition, patients were excluded if they had incomplete clinical information (n=4), follow-up less than 3 years (n=145), dialysis requirements at baseline (n=1), or ESRD defined as estimated GFR (eGFR) less than 15 mL/min/1.73 $m^2$ at enrollment (n=16). The study design, ascertainment, inclusion criteria, and phenotyping of the study patients have been described.[6,27]

The validation cohort consisted of additional patients recruited from the HKDR. Patients in the replication cohort were recruited into the registry in an identical manner to the original cohort but were recruited from 1998 onwards. Patients with chronic kidney disease (CKD) at enrollment were excluded in the validation cohort. For the cross-sectional study on association between *PRKCB1* genotype (RefSeq NM_002738) and quantitative measures of renal function, we recruited 1892 unrelated Chinese individuals with type 2 diabetes from the inpatient database of the Shanghai Diabetes Institute.[28] This study was approved by the clinical research ethics committee of the Chinese University of Hong Kong and the ethics committee of the Shanghai Jiaotong University. Written informed consent was obtained from all participants.

### Clinical Studies and Outcomes

All study participants were examined in the morning after an overnight fast. Anthropometric parameters including body weight and height, waist circumference, and blood pressure were measured. Fasting blood samples were collected for measurement of plasma glucose, hemoglobin $A_{1c}$ ($HbA_{1c}$), and lipid profiles (levels of total cholesterol, triglycerides, and both high-density and low-density lipoprotein cholesterol). Hypertension was defined as blood pressure at or above 130/85 mm Hg, use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, or use of other antihypertensive medications. A timed urine collection (4- or 24-hour) was used for measurement of urinary albumin excretion rate (AER). For eGFR, we used the abbreviated formula developed by the Modification of Diet in Renal Disease study group further adjusted for the Chinese ethnicity: $186 \times [S_{CR} \times 0.011]^{-1.154} \times [\text{age in years}]^{-0.203} \times [0.742 \text{ if female}] \times [1.233 \text{ if Chinese}]$ where $S_{CR}$ is serum creatinine expressed as μmol/L and 1.233 is the adjusting coefficient for Chinese population.[29] Use of medications, including oral blood glucose–lowering agents and insulin, was also recorded for all study patients. Antihypertensive medications included all classes that are indicated for hypertension, other than angiotensin-converting enzyme inhibitors and angiotensin receptor blockers. Lipid-lowering medications included statins and fibrates.

All clinical end points, including hospital admissions and mortality, were censored on July 30, 2005, according to the databases from the Hospital Authority Central Computer System, which records admissions to all public hospitals. These databases, including the Hong Kong Death Registry, were matched by a unique identification number, the Hong Kong Identity Card number, which is compulsory for all residents in Hong Kong and used by all government departments and major organizations. Using codes from the *International Classification of Diseases, Ninth Revision*, ESRD was defined as death due to diabetes with renal manifestations (250.4), chronic renal failure (585), or unspecified renal failure (586); nonfatal chronic renal failure (585) or unspecified renal failure (586); dialysis (39.95) or peritoneal dialysis (54.98); or follow-up eGFR less than 15 mL/min/1.73 $m^2$.

In the validation cohort, we defined CKD as death due to diabetes with renal manifestations (250.4), chronic renal failure (585), or unspecified renal failure (586) (principal diagnosis only); nonfatal chronic renal failure (585) or unspecified renal failure (586); dialysis (39.95) or peritoneal dialysis (54.98); or follow-up eGFR less than 60 mL/min/1.73 $m^2$. For the validation cohort, all clinical end points, including hospital admissions and mortality, were censored on December 31, 2008, according to the databases from the Hospital Authority Central Computer System, in identical manner as described earlier for the original cohort.

## Genotyping

Based on the phase III HapMap CHB database (HapMap release 27, NCBI build 36; dbSNP b126), we selected 18 SNPs from a 388-kilobase (kb) region (chromosome 16: 23 752 823 to 24 141 063 bp) spanning 2 kb upstream and downstream of *PRKCB1*. Among these SNPs, 12 tagging SNPs (rs7404928, rs3785394, rs3785391, rs120908, rs4788423, rs4787733, rs198198, rs380932, rs429342, rs1015408, rs3785380, rs3785378) with $r^2 < 0.80$ and minor allele frequency (MAF) 0.05 or greater were selected using the Tagger algorithm in Haploview (version 4.1; Broad Institute of MIT and Harvard, Cambridge, Massachusetts).[30] Under the common disease–common variant hypothesis, common diseases have common causal variants (those with MAF $\geq 0.05$), so we focused on investigating the effects of common genetic variants in this study. Also, SNPs with $r^2 \geq 0.80$ are considered redundant because 80% of their information overlaps, so selecting just 1 of them would be more cost-effective. In addition, we selected 6 SNPs (rs3760106, rs2575390, rs3900007, rs3900008, rs432998, and rs3729904) with suggestive evidence of association from previous studies,[23,24] so a total of 18 SNPs were genotyped in all study participants in 2009 using genomic DNA.

Genotyping was performed at the McGill University and Genome Quebec Innovation Centre using primer extension of multiplex products with detection by MALDI-TOF mass spectroscopy on a MassARRAY platform (Sequenom, San Diego, California). All SNPs were in Hardy-Weinberg equilibrium ($P > .05$), as assessed by the exact test of PLINK.[31] The overall genotype call rate was 99.3%. Genotyping accuracy was demonstrated by showing a greater than 99.6% overall concordance rate in 14 blinded duplicate samples. Samples from Shanghai were genotyped using a MassARRAY iPLEX system (MassARRAY Compact Analyzer, Sequenom).

## Bioinformatic Analyses

To annotate the possible underlying functions of the genotyped loci within *PRKCB1*, the data of chromatin structure, evolutionary conserved sequence, CpG islands, and cis-regulatory elements were assessed using the following bioinformatics tools. The genomic locations (Ensembl build 40, NBCI v36, hg18) of those SNPs were mapped from dbSNP130 and tracked on the University of California, Santa Cruz, human genome browser. The data of CpG islands, UW histone modification region,[32] and *PRKCB1* coding region were selected on the genome browser. The conserved sequence in the promoter region was identified by the cisRED database.[33] The transcriptional regulatory modules within the gene were predicted by the PReMOD database.[34]

## Statistical Analysis

All data are expressed as percentages, means and standard deviations, or medians and interquartile ranges (IQRs) as appropriate. Triglycerides and AER were natural log-transformed because of skewed distributions. Between-group comparisons were performed by $\chi^2$ test for categorical variables and unpaired *t* test or the Wilcoxon rank sum test for continuous variables.

The relationships between *PRKCB1* polymorphisms under additive, dominant, and recessive genetic models and ESRD were tested by Cox proportional hazard regression model with adjustment for conventional risk factors at baseline, including sex, age, duration of diabetes, systolic and diastolic blood pressure, $HbA_{1c}$ level, total cholesterol level, natural logarithms of triglycerides and AER, eGFR, retinopathy (present/absent), and use of medications (yes/no). An additive model assumes that the causal allele exerts an additive effect so that carriers with 0, 1, or 2 risk alleles would have no, some, and the most causal effect, respectively. A dominant model assumes that all carriers with the causal allele (both homozygote and heterozygote) would have a causal effect, while a recessive model assumes that only carriers with 2 causal alleles (homozygote) would have a causal effect. Hazard ratios (HRs) with 95% confidence intervals (CIs) are presented. We corrected for multiple comparisons of SNPs under additive, dominant, and recessive genetic models using the permutation method rather than the false discovery rate approach. The largest test statistic obtained from the 3 models was chosen (MAX statistic).[35] Because the distribution of the MAX statistic under the null hypothesis is unknown, experiment-wise significance was estimated from the empirical distribution of the MAX statistic after performing 10 000 permutations of genotypes for all 18 SNPs.

Pairwise linkage disequilibrium measures were computed in all samples using Haploview. Haplotype frequencies between patients with and without new-onset ESRD were compared using a haplotype-specific test implemented in Haploview. Hazard ratios with 95% CIs were calculated for risk association of ESRD with various haplotypes. To assess interaction effects between pairwise SNPs on outcome variables, a Cox proportional hazard regression model was applied including the main and interaction effects of SNPs with covariates.

The joint effect of the 3 *PRKCB1* polymorphisms (rs3760106 with the dominant model, rs7404928 with the recessive model, and rs4787733 with the additive model) for ESRD risks were

**Table 1.** Clinical Characteristics and Biochemical Profile at Baseline Stratified According to the Progression to End-Stage Renal Disease in Chinese Patients With Type 2 Diabetes

| | No ESRD (n = 1082) | ESRD (n = 90) | *P* Value |
|---|---|---|---|
| Male/female sex, No. | 437/645 | 47/43 | .03 |
| Age, mean (SD), y | 55.7 (11.9) | 60.4 (10.7) | <.001 |
| Age at onset, mean (SD), y | 47.2 (12) | 51.6 (11.3) | .001 |
| Duration of diabetes, mean (SD), y | 8.5 (6.8) | 8.8 (6.3) | .63 |
| BMI, mean (SD)[a] | 24.8 (3.7) | 24.9 (4) | .77 |
| Waist circumference, mean (SD), cm | | | |
| Male | 87.5 (9.0) | 89.3 (10.3) | .21 |
| Female | 83.0 (9.7) | 83.4 (11.1) | .78 |
| Hemoglobin $A_{1c}$, mean (SD), % | 7.9 (1.8) | 8.6 (2.2) | .002 |
| Total cholesterol, mean (SD), mg/dL | 208.5 (42.5) | 235.5 (65.6) | .001 |
| Triglycerides, median (IQR), mg/dL | 115.0 (79.7-177.0) | 150.4 (106.2-221.2) | <.001 |
| HDL cholesterol, mean (SD), mg/dL | 50.2 (15.4) | 46.3 (15.4) | .21 |
| LDL cholesterol, mean (SD), mg/dL | 131.3 (37.1) | 146.7 (50.2) | .02 |
| Systolic BP, mean (SD), mm Hg | 134.9 (20.6) | 152.1 (26.6) | <.001 |
| Diastolic BP, mean (SD), mm Hg | 78.3 (10.8) | 83.8 (12.7) | <.001 |
| Hypertension, No. (%) | 682 (63) | 79 (88) | <.001 |
| Retinopathy, No. (%) | 260 (24) | 56 (62) | <.001 |
| AER, median (IQR), μg/min | 14.3 (8.0-62.4) | 1008 (269.4-2033.1) | <.001 |
| eGFR, mean (SD), min/mL/1.73 $m^2$ | 114.8 (33.9) | 70.4 (32.6) | <.001 |
| Treatment, No. (%) | | | |
| Lipid-lowering | 65 (6) | 11 (12) | .01 |
| Antihypertensive | 238 (22) | 43 (48) | <.001 |
| ACE inhibitor | 87 (8) | 18 (20) | <.001 |
| Oral glucose-lowering | 541 (50) | 47 (52) | .66 |
| Insulin | 162 (15) | 24 (27) | .006 |

Abbreviations: ACE, angiotensin-converting enzyme; AER, albumin excretion rate; BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

SI conversion factors: To convert HDL, LDL, and total cholesterol to mmol/L, multiply by 0.0259; to convert triglycerides to mmol/L, multiply by 0.0113.

[a] Calculated as weight in kilograms divided by height in meters squared.

shown by Kaplan-Meier curves adjusted for conventional risk factors at baseline, for which the cumulative probability of new-onset events was estimated according to number of risk allele. The significance of the trend was tested by Cox regression using the categories of risk allele carried as independent variables.

All statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, North Carolina) or SPSS for Windows version 15 (SPSS, Chicago, Illinois) unless specified otherwise. A 2-tailed $P < .05$ was considered statistically significant. We estimated the posterior study power using Genetic Power Calculator.[36] Assuming dominant models with MAFs of 0.07, our sample size had 95% power to detect a minimal HR of 2.25 for ESRD, at $\alpha = .05$.

## RESULTS

We genotyped 18 SNPs spanning *PRKCB1* in 1172 patients with type 2 diabetes, including 4 SNPs in the promoter region, 2 SNPs in the coding exons, and 6 SNPs in the noncoding intron region. The gene structure and location of SNPs are shown in the eFigure (available at http://www.jama.com). All SNPs were in modest linkage disequilibrium with $r^2 < 0.80$ in our population except for 4 pairs of SNPs (rs3760106 and rs2575390, rs3900007 and rs3900008, rs3785394 and rs3785391, rs3785380 and rs3785378). The latter 2 pairs of SNPs demonstrated stronger linkage disequilibrium in our population as compared with the HapMap CHB population (for rs3785394 and rs3785391, $r^2 = 0.81$ in the present study and 0.78 in HapMap CHB data; for rs3785380 and rs3785378, $r^2 = 0.94$ in the present study and 0.78 in HapMap CHB data).

### Characteristics of Participants

For this analysis, a total of 1172 unrelated patients with type 2 diabetes were included (mean [SD] age, 56.0 [11.9] years; 41.3% male; disease duration, 8.5 [6.7] years). During the study period, 90 patients developed ESRD. The mean (SD) follow-up period for ESRD was 7.9 (1.9) years. Patients with new-onset ESRD were older and had higher levels of $HbA_{1c}$, total cholesterol, triglycerides, and low-density lipoprotein cholesterol; higher systolic and diastolic blood pressure; higher AER; and higher rates of hypertension and retinopathy and were more likely to be treated with insulin, angiotensin-converting enzyme inhibitors, and lipid-lowering medications at baseline than those without. In addition, patients with new-onset ESRD had lower eGFR (TABLE 1).

### Variants of *PRKCB1* and ESRD

The frequencies of the minor T allele of rs3760106 and G allele of rs2575390 ($r^2 = 0.99$) were markedly different between patients with (12.2%) and without (7%) incident ESRD.

In the Cox regression model after adjustment for conventional risk factors, 4 SNPs were significantly associated with ESRD ($P < .05$) (TABLE 2). The T allele at rs3760106 and the G allele at the closely linked SNP rs2575390 ($r^2 = 0.98$) were strongly associated with increased risk for ESRD (HR, 2.25; 95% CI, 1.31-3.87; $P = .003$ for rs3760106 in dominant model; HR, 2.26; 95% CI, 1.31-3.88; $P = .003$ for rs2575390 in dominant model). We also observed nominal associations for ESRD with the common TT genotype of rs7404928 (HR, 1.59; 95% CI, 1.01-2.52; $P = .05$ in recessive model) and the major A allele of rs4787733 (HR, 1.78; 95% CI, 1.03-3.09; $P = .04$ in additive model). The 2 SNPs rs3760106 ($P = .03$) and rs2575390 ($P = .03$) remained significant after correction for multiple comparisons.

Haplotype analyses of the 3 significant and independent SNPs ($r^2 < 0.80$) (rs3760106, rs7404928, and rs4787733)

**Table 2.** Genotype Distributions of *PRKCB1* SNPs and HRs of *PRKCB1* Polymorphisms for Risk of ESRD in Chinese Patients With Type 2 Diabetes[a]

| SNP | Location | R/N | ESRD: RR/RN/NN Genotype Frequencies | No ESRD: RR/RN/NN Genotype Frequencies | Additive | | Dominant | | Recessive | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | HR (95% CI) | *P* Value | HR (95% CI) | *P* Value | HR (95% CI) | *P* Value |
| rs3760106 | Promoter | T/C | 0.000/0.244/0.756 | 0.004/0.132/0.865 | 2.22 (1.30-3.80) | .004 | 2.25 (1.31-3.87) | .003[b,c] | NA | .99 |
| rs2575390 | Promoter | G/C | 0.000/0.244/0.756 | 0.004/0.132/0.865 | 2.23 (1.30-3.81) | .003 | 2.26 (1.31-3.88) | .003[b,c] | NA | .99 |
| rs3900007 | Promoter | C/T | 0.111/0.544/0.344 | 0.111/0.449/0.439 | 1.03 (0.74-1.45) | .85 | 1.21 (0.76-1.93) | .43 | 0.73 (0.34-1.58) | .43[c] |
| rs3900008 | Promoter | C/T | 0.111/0.544/0.344 | 0.113/0.449/0.438 | 1.03 (0.74-1.45) | .85 | 1.21 (0.76-1.93) | .43 | 0.73 (0.34-1.57) | .42[c] |
| rs7404928 | Intron | T/C | 0.461/0.393/0.146 | 0.437/0.451/0.112 | 1.27 (0.89-1.80) | .19 | 0.93 (0.48-1.78) | .82 | 1.59 (1.01-2.52) | .047[c] |
| rs3785394 | Intron | T/C | 0.382/0.472/0.146 | 0.397/0.474/0.130 | 1.13 (0.80-1.58) | .50 | 1.04 (0.53-2.04) | .92 | 1.23 (0.78-1.94) | .38[c] |
| rs3785391 | Intron | T/C | 0.467/0.433/0.100 | 0.456/0.443/0.102 | 1.10 (0.78-1.55) | .58 | 1.36 (0.60-3.10) | .46[c] | 1.07 (0.69-1.67) | .77 |
| rs120908 | Intron | C/A | 0.400/0.444/0.156 | 0.314/0.490/0.196 | 1.21 (0.89-1.64) | .23 | 1.66 (0.87-3.15) | .12[c] | 1.14 (0.72-1.79) | .58 |
| rs4788423 | Intron | C/T | 0.303/0.416/0.281 | 0.215/0.499/0.286 | 1.20 (0.87-1.66) | .27 | 0.99 (0.59-1.64) | .95 | 1.63 (0.99-2.67) | .05[c] |
| rs4787733 | Intron | A/G | 0.811/0.189/0.000 | 0.769/0.221/0.010 | 1.78 (1.03-3.09) | .04[c] | NA | .98 | 1.74 (0.98-3.12) | .06 |
| rs198198 | Intron | T/A | 0.116/0.488/0.395 | 0.168/0.494/0.338 | 1.02 (0.73-1.43) | .90 | 1.14 (0.71-1.83) | .60[c] | 0.84 (0.42-1.68) | .62 |
| rs380932 | Intron | G/T | 0.044/0.567/0.389 | 0.102/0.452/0.446 | 1.16 (0.80-1.68) | .44 | 1.35 (0.84-2.17) | .22[c] | 0.73 (0.26-2.07) | .56 |
| rs432998 | Exon | G/A | 0.100/0.556/0.344 | 0.121/0.456/0.423 | 1.09 (0.76-1.55) | .65 | 1.22 (0.76-1.97) | .41[c] | 0.86 (0.39-1.90) | .71 |
| rs429342 | Intron | G/C | 0.022/0.200/0.778 | 0.009/0.206/0.785 | 1.41 (0.87-2.28) | .17 | 1.63 (0.93-2.87) | .09[c] | 0.72 (0.09-5.84) | .75 |
| rs3729904 | Exon | A/G | 0.000/0.156/0.844 | 0.009/0.153/0.837 | 1.56 (0.87-2.78) | .14 | 1.74 (0.93-3.25) | .08[c] | NA | .98 |
| rs1015408 | Intron | T/A | 0.618/0.348/0.034 | 0.625/0.333/0.043 | 1.01 (0.68-1.50) | .97 | 1.08 (0.33-3.60) | .90[c] | 1.00 (0.62-1.60) | .99 |
| rs3785380 | Intron | T/C | 0.056/0.289/0.656 | 0.030/0.263/0.708 | 1.16 (0.76-1.76) | .50 | 1.12 (0.69-1.80) | .65 | 1.76 (0.54-5.82) | .35[c] |
| rs3785378 | Intron | C/T | 0.056/0.292/0.652 | 0.033/0.268/0.699 | 1.13 (0.75-1.72) | .55 | 1.10 (0.68-1.77) | .69 | 1.63 (0.50-5.37) | .42[c] |

Abbreviations: CI, confidence interval; ESRD, end-stage renal disease; HR, hazard ratio; N, nonrisk allele; NA, not applicable; R, risk allele; SNP, single-nucleotide polymorphism.
[a] Hazard ratios refer to the risk-conferring alleles. *P* values were calculated from Cox proportional hazard regression adjusted for conventional risk factors at baseline: sex, age, duration of diabetes, systolic and diastolic blood pressure, hemoglobin $A_{1c}$, total cholesterol, natural logarithm of triglycerides, estimated glomerular filtration rate, natural logarithm of albumin excretion rate, retinopathy (present/absent), and use of medications (yes/no).
[b] Significant SNP after corrected for multiple comparisons.
[c] Refers to the genetic model considered in MAX statistics.

revealed similar effect size as compared with the single SNP associations (TABLE 3). Only haplotypes constructed from the risk-conferring (TTA) and protective (CCG) alleles conferred increased trends (HR, 1.89; 95% CI, 1.12-3.21; *P* = .02) and decreased trends (HR, 0.38; 95% CI, 0.13-1.16; *P* = .08), respectively, for ESRD.

**Table 3.** Association Between Haplotype Consisting of 3 Significant SNPs in *PRKCB1* Region and ESRD End Point in Chinese Patients With Type 2 Diabetes[a]

| rs3760106 | rs7404928 | rs4787733 | Haplotype Frequencies: ESRD | Haplotype Frequencies: No ESRD | OR (95% CI) | *P* Value |
|---|---|---|---|---|---|---|
| C | T | A | 0.485 | 0.535 | 0.82 (0.60-1.11) | .20 |
| C | C | A | 0.304 | 0.280 | 1.12 (0.81-1.57) | .49 |
| C | T | G | 0.070 | 0.069 | 1.02 (0.56-1.84) | .96 |
| T | T | A | 0.097 | 0.054 | 1.89 (1.12-3.21) | .02 |
| C | C | G | 0.018 | 0.046 | 0.38 (0.13-1.16) | .08 |
| T | C | A | 0.019 | 0.011 | 1.78 (0.57-5.51) | .33 |

Abbreviations: CI, confidence interval; ESRD, end-stage renal disease; OR, odds ratio; SNPs, single-nucleotide polymorphisms.
[a] Haplotypes were compared between patients with and without new-onset ESRD by haplotype-specific tests (1 *df*) using Haploview. TTA and CCG are constructed from the risk and protective alleles for rs3760106, rs7404928, and rs4787733, respectively.

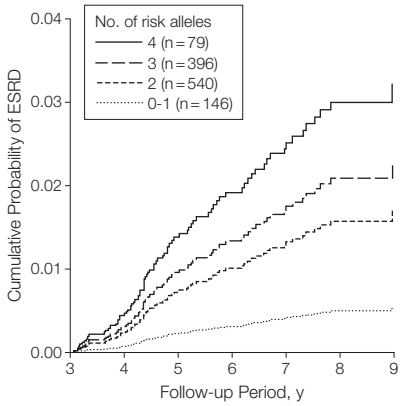
### Additive Effects of *PRKCB1*

We further examined possible 2-way epistasis of *PRKCB1* polymorphisms on ESRD but failed to observe any evidence of interaction. However, there were significantly increased risks for ESRD with increasing number of risk alleles ($P < .001$ for ESRD) in the joint effect analysis of 3 significant and independent ($r^2 < 0.80$) SNPs (rs3760106 with the dominant model, rs7404928 with the recessive, and rs4787733 with the additive) (FIGURE). The adjusted risk for ESRD was 6.04 (95% CI, 2.00-18.31) in patients with 4 risk alleles compared with patients with 0 or 1 risk allele. Apart from presence of risk variants of *PRKCB1*, other independent risk factors identified for development of ESRD were elevated $HbA_{1c}$, decrease in eGFR, elevated log-transformed AER, and presence of retinopathy (TABLE 4). Among our cohort of patients who were free of ESRD at baseline, new ESRD events occurred in 90 patients (7.7%) during a maximum 9-year follow-up, giving an annualized incidence of 9.7 per 1000 person-years (95% CI, 7.7-11.7). The incidence was 4.4 per 1000 person-years (95% CI, 0.5-8.2) for patients carrying 0 or 1 risk alleles (12.3% of the cohort), compared with 20.0 per 1000 person-years (95% CI, 8.8-31.1) in those carrying 4 risk alleles (6.9% of the cohort).

### *PRKCB1* and Development of DKD

To validate the association between *PRKCB1* polymorphisms and DKD, we examined the associations of variants in *PRKCB1* in additional patients from the HKDR. All patients in the validation cohort were recruited subsequent to the initial discovery cohort. The baseline

**Figure**. Cumulative Probability of New-Onset ESRD According to Number of Risk Alleles, Adjusted for Conventional Risk Factors




No. at risk by allele count

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4 | 79 | 79 | 74 | 63 | 54 | 46 | 34 |
| 3 | 396 | 394 | 376 | 340 | 309 | 265 | 182 |
| 2 | 540 | 535 | 510 | 468 | 415 | 349 | 245 |
| 0-1 | 146 | 144 | 138 | 124 | 109 | 94 | 66 |

Adjusted for mean values of sex, age, duration of diabetes, systolic and diastolic blood pressure, hemoglobin $A_{1c}$, total cholesterol, natural logarithm of triglycerides, estimated glomerular filtration rate, natural logarithm of albumin excretion rate, retinopathy (present/absent), and use of medications (yes/no). Three significant and independent single-nucleotide polymorphisms with $r^2 < 0.80$ (rs3760106 with the dominant model, rs7404928 with the recessive model, and rs4787733 with the additive model) were selected to calculate the number of risk alleles for end-stage renal disease (ESRD).

characteristics of patients in the validation cohort are summarized in eTable 1. Compared with the original cohort, patients in the validation cohort were older with significantly shorter duration of diabetes and follow-up and significantly greater use of angiotensin-converting enzyme inhibitors. As a result, a smaller proportion of patients in this cohort developed ESRD during follow-up (253/3677, or 6.9%) compared with the original cohort. To examine the effects of variants of *PRKCB1* on development of DKD in this cohort, we selected patients from this cohort with early-onset disease (age at onset, <45 years) who were free of CKD at baseline and identified patients who progressed to CKD during the follow-up period. The characteristics of this cohort of patients genotyped are summarized in eTable 1. Of 1049 patients, 151 (14.3%) developed CKD during the follow-up period. Both the T allele of the rs3760106 variant and the G allele of the rs2575390 variant were found to be significantly associated with development of CKD in this cohort, with HRs of 1.68 (95% CI, 1.10-2.57; $P=.02$) and 1.62 (95% CI, 1.07-2.47; $P=.02$), respectively (eTable 2). Although the rs7404928 and rs4787733 variants did not reach statistical significance for association with CKD, the at-risk variants increased risk of developing renal dysfunction in the same direction using the same genetic model as in the original discovery cohort.

### Variants of *PRKCB1* and eGFR

To better understand the relationship between genetic variants of *PRKCB1* and development of renal dysfunction, we further examined the relationship between genetic variants in *PRKCB1* and baseline renal function in an independent cross-sectional cohort of Chinese patients with type 2 diabetes recruited in Shanghai (eTable 3). We did not detect association between variants of *PRKCB1* and baseline eGFR ($P=.66$ to $.82$) or CKD (defined as eGFR <60 mL/min/1.73 $m^2$, n=195; $P=.27$ to $.53$) after adjustment for age, sex, and body mass index. This is similar to an analysis of all cross-sectional data from baseline eGFR in our genotyped patients.

### Functional Annotation of *PRKCB1*

We performed extensive bioinformatics analyses on the functional significance of the genetic variants identified. One of these variants, rs3760106, is known to lie in potential binding sites for the transcription factor Sp1, suggesting possible interaction between DNA and binding proteins.[23] Further bioinformatics analysis suggests that rs3760106, rs2575390, and rs3900007 all lie within the conserved sequence of the *PRKCB1* promoter, while rs2575390 and rs3900007 both lie within histone modification sites (H3K27me3) and may thereby alter transcriptional activity of the gene. The rs7404928 and rs4787733 variants likewise lie within DNA regions with predicted functional significance, which may alter the binding of regulatory factors and thereby lead to altered gene transcription (eTable 4).

## COMMENT

In this study of Chinese patients with type 2 diabetes followed up for 8 years, we found that genetic variants of the *PRKCB1* gene were associated with development of incident ESRD independent of other known risk factors, with joint effects among the risk-conferring alleles. These associations persist despite correction for retinopathy, albuminuria, renal function, risk factor control, and use of medications including angiotensin-converting enzyme inhibitors at baseline. Strengths of our study include the relatively long follow-up, detailed documentation of clinical parameters and medication use, and the use of ESRD as an outcome measure for DKD. In addition, we obtained further supporting evidence of the role of genetic variants in the *PRKCB1* gene in development of CKD in an additional cohort of Chinese patients with type 2 diabetes with a comparatively shorter period of follow-up. Our consistent results thus suggest that genetic variation in the *PRKCB1* gene is an important determinant for the risk of developing DKD in Chinese patients with type 2 diabetes.

Diabetes is the most common cause of ESRD worldwide.[2,5] Hyperglycemia is a major causal factor that can activate signaling pathways such as the protein kinase C signaling cascade, leading to glomerular damage and mesangial expansion.[14] Hyperglycemia also induces allosteric modification, increases protein kinase C activity, and increases renal PKC-β expression.[19,37] In diabetic nephropathy, PKC-β gene expression is up-regulated, which is closely correlated with $HbA_{1c}$ levels and may explain the relationship between glycemic control and progression of diabetic nephropathy.[38] In support of this notion, we found close associations between DKD with polymorphisms in the *PRKCB1* gene, which encodes PKC-βI and PKC-βII.

In this prospective analysis, we found high linkage disequilibrium ($r^2$=0.98) between the T allele at rs3760106 and G allele at rs2575390, which are associated with transition to ESRD. Although we did not examine the function of these genetic variants, both variants have been associated with type 1 and type 2 diabetic nephropathy.[23,24] Because these variants are situated in the promoter region, they are likely to be implicated in *PRKCB1* transcription. Additional bioinformatics analyses have provided supporting evidence of the functional significance of several other variants examined and included in the at-risk and protective haplotypes. Our novel finding of an additive effect of increasing number of *PRKCB1* variants in predicting DKD is unexpected, especially given that several of the variants were in noncoding regions. This may reflect effects of these variants on binding of other transcription factors, or other epigenetic effects, as predicted by our bioinformatics analyses. Further functional studies are warranted for better understanding of the biological functions of these variants.

In this analysis, we used decline in eGFR as a marker for progression of DKD instead of albuminuria because of the confounding effects of increasing use of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers. Furthermore, many type 2 diabetic patients with DKD did not have albuminuria or retinopathy,[39,40] because of heterogeneity of renal pathology in these patients.[41] Classic diabetic glomerulosclerosis, mainly due to hyperglycemia, is characterized by increased basement membrane thickness, diffuse mesangial sclerosis with nodular formation, and hyalinosis. These pathological changes, often seen in type 1 diabetes, are closely associated with albuminuria but are only found in a minority of renal biopsies in type 2 diabetes.[41,42] In the latter patients, other common histological features of renal injury include tubular atrophy, interstitial fibrosis, tubular basement membrane thickening, and advanced glomerular arteriolar hyalinosis.[41] Several studies have also shown that inhibition of PKC-β attenuated glomerulosclerosis and tubulointerstitial fibrosis in nondiabetic kidney disease.[43] In this regard, our results indicate that the risk associations of the *PRKCB1* polymorphisms with ESRD were independent of glycemic control, suggesting that other nonglycemic pathways may be implicated.

In support of this notion, we have reported the predictive power of metabolic syndrome traits on new-onset DKD[26] and that control of glycemia, blood pressure, and lipids as well as inhibition of the renin-angiotensin system reduced renal events and death in type 2 diabetes.[44] Other factors that may determine progression of renal injury in type 2 diabetes include obesity, insulin resistance, and increased oxidative stress.[45-47] Here, the NAD(P)H oxidase, which is a cytosolic enzyme complex and an important mediator of diabetes-induced reactive oxygen species (ROS) production, is a possible candidate.[48-51] For example, the Nox4 subunit of NAD(P)H oxidase is the major source of ROS in diabetic kidneys and responsible for inducing renal hypertrophy and increased fibronectin expression.[50] Interestingly, mice that lack PKC-β have significantly less activation of NAD(P)H oxidase subunits by hyperglycemia and are protected from diabetes-induced oxidative stress, renal dysfunction, and fibrosis.[21] Other sources of oxidative stress in the kidney include glycolysis, the polyol pathway, and advanced glycation products.[47] Taken together, our findings suggest that the risk association of renal disease with *PRKCB1* may be mediated by interacting pathways, including but not limited to hyperglycemia, and increased oxidative stress causing tubular damage and interstitial fibrosis.

**Table 4.** Cox-Regression Analysis With HRs of Predictors for ESRD in Type 2 Diabetes[a]

| | ESRD | |
|---|---|---|
| | HR (95% CI) | *P* Value |
| Male/female sex, per 1% | 0.82 (0.49-1.35) | .43 |
| Age, per y | 0.99 (0.97-1.02) | .64 |
| Duration of diabetes, per y | 1.01 (0.96-1.05) | .77 |
| Systolic BP, per 1 mm Hg | 1.00 (0.98-1.01) | .56 |
| Diastolic BP, per 1 mm Hg | 1.02 (0.99-1.05) | .21 |
| Total cholesterol, per 1 mg/dL | 1.06 (0.87-1.29) | .57 |
| Log-transformed triglycerides, per 1 mg/dL | 0.70 (0.47-1.05) | .08 |
| Hemoglobin $A_{1c}$, per 1% | 1.23 (1.11-1.37) | <.001 |
| eGFR, per 1 min/mL/1.73 $m^2$ | 0.97 (0.96-0.98) | $4.42 \times 10^{-9}$ |
| Log-transformed AER, per 1 µg/min | 1.81 (1.52-2.15) | $1.72 \times 10^{-11}$ |
| Retinopathy, per 1% | 1.87 (1.14-3.08) | .01 |
| Treatment, per 1% | | |
| Lipid-lowering | 1.15 (0.57-2.34) | .69 |
| Antihypertensive | 1.04 (0.61-1.79) | .88 |
| ACE inhibitor | 0.91 (0.48-1.73) | .77 |
| Oral glucose-lowering | 1.31 (0.76-2.27) | .33 |
| Insulin | 1.13 (0.65-1.99) | .66 |
| No. of risk alleles | | |
| 0-1 | 1 [Reference] | |
| 2 | 3.18 (1.21-8.35) | .02 |
| 3 | 4.21 (1.55-11.42) | .005 |
| 4 | 6.04 (2.00-18.31) | .002 |

Abbreviations: ACE, angiotensin-converting enzyme; AER, albumin excretion rate; BP, blood pressure; CI, confidence interval; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; HR, hazard ratio; SNPs, single-nucleotide polymorphisms.

[a] Three significant and independent SNPs with $r^2 < 0.80$ were selected (rs3760106 with the dominant model, rs7404928 with the recessive model, and rs4787733 with the additive model) to calculate the number of risk alleles for ESRD.

Despite the consistent association of variants in *PRKCB1* with development of ESRD and CKD in our Chinese patients, we did not detect association between variants in *PRKCB1* and baseline renal function. Several genetic variants have recently been identified via genome-wide association studies to be associated with kidney function and CKD.[52-54] Although *PRKCB1* was not identified as a variant associated with renal function or CKD at genome-wide significance level in these studies, none of the studies were conducted specifically in patients with diabetes, and most of the association studies were cross-sectional by design. Our study was designed to examine the association between variants of *PRKCB1* and development of renal dysfunction. Furthermore, given our biological understanding of the interaction between hyperglycemia and activation of PKC-β, one may expect the impact of variants of *PRKCB1* to be specific for patients with diabetes. It is also important to note that different genetic factors may be associated with renal function measured as a quantitative trait, compared with progression to renal dysfunction such as CKD and ESRD using a specific cutoff, as recently seen in the case of different genetic variants being found to be associated with fasting plasma glucose, 2-hour plasma glucose, and type 2 diabetes.[55] In this light, it has been argued that multiple mechanisms, each of which may contain several components, can be implicated in development of complex diseases and that for the disease to become clinically manifest, all components within a mechanism need to be present.[56] Despite the heterogeneity of risk factors, complications, care processes, medications, and clinical outcomes in our patients, the overall consistency of our results in prospective and bioinformatic analysis thus supports the causal role of this gene in DKD.

Given the important role of PKC-β in the pathogenesis of DKD and other diabetic vascular complications, a selective inhibitor of PKC-β, ruboxistaurin, has been developed. This medication normalized hemodynamic changes, extracellular matrix accumulation, and histological features of glomerular damage associated with diabetes in several animal models.[18-20] In a phase 2 clinical trial involving 123 patients with type 2 diabetes and DKD treated optimally with angiotensin receptor blockers or angiotensin-converting enzyme inhibitors, treatment with ruboxistaurin further attenuated urinary transforming growth factor–β levels, decreased rate of loss in GFR, and reduced proteinuria.[22,57] Preliminary analysis of markers of renal disease in patients with diabetic peripheral neuropathy participating in clinical trials of ruboxistaurin also suggests improved renal parameters during the study period.[58,59] Given the phenotypic and genetic heterogeneity of complex diseases such as DKD, it would be of interest to examine the interaction between *PRKCB1* genotype and clinical response to ruboxistaurin, as recently demonstrated in the case for clinical response to angiotensin receptor blockers in patients with the angiotensin-converting enzyme insertion/deletion polymorphism.[60]

Our study has several limitations. Although a previous smaller cohort study in Japan[24] suggested an association between polymorphisms in *PRCKB1* promoter region with renal dysfunction, we do not know of any other similar large prospective Chinese cohorts with sufficiently long follow-up to replicate our findings. For ethical reasons, we did not perform renal biopsy in the majority of patients, although patients with renal impairment had all undergone renal tract ultrasound scan to exclude urinary tract obstruction or parenchymal disease. Furthermore, the majority of patients who subsequently progressed to ESRD had evidence of diabetic retinopathy at baseline. We used only 1 measurement to estimate GFR at baseline, although all patients had GFR measured during follow-up to determine its progression.

In summary, using 2 large prospective cohorts and a cross-sectional cohort of Chinese patients with type 2 diabetes, we found that genetic variants of the *PRKCB1* gene were associated with incident ESRD, independent of confounders such as albuminuria, glycemia, retinopathy, and other risk factor control.

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**Author Contributions:** Dr Ma had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
*Study concept and design:* Ma, Wang, Ng, So, J. Chan.
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*Drafting of the manuscript:* Ma, Tam, Lam, A. Chan.
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*Obtained funding:* Ma, J. Chan.
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*Study supervision:* Ma, Ng, So, J. Chan.
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