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Short Leukocyte Telomere Length Predicts Albuminuria Progression in Individuals With Type 2 Diabetes

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Introduction: Telomere length, a marker for biological aging, is implicated with diabetic kidney disease (DKD); however, the association between telomere length and albuminuria progression among Asian patients with type 2 diabetes (T2D) is not well understood. Here, we aim to study whether leukocyte telomere length (LTL) may independently predict albuminuria progression in patients with T2D with preserved renal filtration function (estimated GFR >60 ml/min per 1.73 m^2 and urine albumin-to-creatinine ratio [uACR] <300 mg/g).

Methods: The baseline LTL was measured by real-time polymerase chain reaction in the SMART2D cohort ($n = 691$) with a median follow-up of 3 years. Albuminuria progression was defined as a change in albuminuria category to a higher category and at least 30% increase in uACR from baseline in 3 years.

Results: Progressors ($n = 123$) had significantly shorter median LTL compared with nonprogressors ($n = 568$) (0.58 [0.38–0.79] vs. 0.62 [0.45–0.88], $P = 0.039$). Compared with subjects with longer LTL (fourth quartile), subjects with shorter LTL (first quartile) had 1.93-fold (1.04–3.60, $P = 0.038$) increased risk for albuminuria progression after adjustment for traditional risk factors. The association of LTL with microalbuminuria to macroalbuminuria progression was stronger than its association with normoalbuminuria to microalbuminuria (odds ratio [OR]: 1.54; 95% confidence interval [CI]: 1.02–2.32; $P = 0.042$ vs. OR: 1.13; 95% CI: 0.91–1.40; $P = 0.263$ per 1-SD decrement in natural log-transformed LTL).

Conclusion: Therefore, our results demonstrated that in patients with T2D with preserved renal filtration function, LTL predicts albuminuria progression beyond traditional risk factors, suggesting LTL may be novel biomarker for DKD progression.

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Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease and cardiovascular mortality.[1,2] DKD is characterized by the presence of albuminuria and/or decline in glomerular filtration rate (GFR).[3] Although microalbuminuria is clinically the first indicator of incipient DKD, DKD is a heterogeneous disease, both in terms of its clinical manifestations and rate of decline in renal function.[4–8] Despite improved management of known risk factors, the residual risk for DKD still remains high, suggesting our incomplete understanding in the pathophysiology of DKD.[9]

Telomeres are tandem repeats made up of TTAGGG sequence and proteins found at the ends of chromosomes, and protects chromosomal stability.[10] Telomeres naturally shorten with each cell division, as conventional DNA replicative enzymes cannot fully replicate telomere ends. Critically short telomere length, termed the HayFlick limit, triggers cell senescence and apoptosis.[11–13] Several epidemiological studies have shown that short telomeres are associated with age-related disorders, such as cardiovascular and neurodegenerative disease, cancer,[14–16] and metabolic syndrome.[17] Therefore, decreased telomere length is suggested to be an indicator of biological age and has been extensively studied as a potential marker for disease risk and progression.[18–21] Accumulating evidence has begun to demonstrate the link between telomeres and pathogenesis of diabetes and renal disease. Experimental data from mouse models and humans have shown that short telomeres lead to beta cell dysfunction and defects in the insulin signaling pathway, increasing the susceptibility to and more severe diabetes than

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controls.[22,23] A series of prospective studies mainly in non-Asian cohorts have confirmed a clear association between short telomere length and incident type 2 diabetes (T2D).[24–26] Moreover, a recent study in Europeans found that short leukocyte telomere length (LTL) is also associated with increased risk for all-cause mortality in patients with T2D.[27]

Telomere shortening is also accelerated by deregulated renin-angiotensin system, inflammation, and chronic exposure to hyperglycemia,[28–30] which are all risk factors associated with DKD.[31] Several lines of evidence have demonstrated the association of telomere length with renal disease.[32,33] Betjes *et al*.[34] found that telomeres in T cells were shorter in patients with end-stage renal disease compared with healthy individuals in a cross-sectional study. Increased expression of cellular senescence markers was also observed in renal tissue from patients with T2D with renal disease.[30] In agreement, a cross-sectional study found that telomere lengths were significantly shorter in patients with T2D with microalbuminuria as compared with patients with T2D with normal albuminuria.[35] Prospective association between LTL and renal disease progression in a European population with type 1 diabetes (T1D) by Fyhrquist *et al*.[36] demonstrated that short telomeres are associated with increased risk for albuminuria progression, whereas Raschenberger *et al*.[37] showed that association between short LTL and chronic kidney disease progression was stronger in individuals with diabetes. However, the latter study included individuals with impaired renal function.

The natural course of DKD is less understood in patients with T2D as compared with T1D, as diagnosis is delayed by many years, and the prevalence of DKD is approximately twofold to threefold higher in Asian compared with Caucasian individuals.[38] Given the roles of telomeres in aging, diabetes, and renal function, we hypothesized that LTL may be involved in deterioration of renal function in Asian patients with T2D with preserved renal function. Therefore, using a diverse sample with T2D and preserved renal filtration function, we investigated the association of LTL with DKD progression as measured by albuminuria progression in a prospective cohort in Asian patients with T2D.

METHODS

Study Population

This prospective study is nested within the Singapore Study of Macro-angiopathy and Micro-Vascular Reactivity in Type 2 Diabetes (SMART2D). Briefly, 2058 adult participants (21 years and older) with T2D were recruited from a regional hospital and a community medical center in the northern part of Singapore from August 2011 to March 2014. Three years after enrollment date, participants were recalled consecutively for the planned 3-year follow-up study. Given that this was an ongoing study, at the time the study commenced, we had 1066 patients' follow-up (censored at July 2016 for this study) (Supplementary Figure S1). The exclusion criteria included the following: T1D, pregnant subjects, subjects with active inflammation (e.g., systemic lupus erythematosus) and cancer, subjects taking nonsteroidal anti-inflammation drugs on the same day of clinical/vascular/biomedical assessment, or subjects on oral steroids equivalent to >5 mg/d prednisolone, as described previously.[39,40] Subjects would be excluded when involvement of other causes of renal disease was suspected. The SMART2D study and the follow-up of patients complies with Helsinki Declaration, has been approved by our domain-specific ethical review board, and written informed consent was obtained from all participants.

Clinical and Biochemical Assessments

Detailed information on collection of baseline data and biological samples has been described previously.[40] Briefly, blood pressure was measured by a sphygmomanometer and average of 3 readings was used. Blood and urine (spot urine collected in the morning void) specimens were collected after overnight fast. HbA1c was measured by point-of-care immunoassay analyzer (DCA Vantage Analyzer; Siemens AG, Erlangen, Germany). Creatinine, triglycerides, and high-density lipoprotein and low-density lipoprotein cholesterol were quantified by enzymatic methods (Roche/Hitachi Cobas C System; Roche Diagnostic GmbH, Basel, Switzerland). Urinary albumin was measured based on a solid-phase competitive chemiluminescent enzymatic immunoassay (Immulite; Diagnostic Products Corp, Gwynedd, UK). Albuminuria level was expressed as albumin-to-creatinine ratio (ACR, mg/g). The estimated GFR (eGFR) was calculated based on Chronic Kidney Disease Epidemiology Collaboration equation. Baseline and follow-up biochemical measurements were performed in the same clinical laboratory, which is accredited by the College of American Pathologists.

Definitions of Albuminuria Progression

Albuminuria status was categorized according to American Diabetes Association criteria as normal to mildly elevated (ACR <30 mg/g, "normoalbuminuria"), moderately elevated (ACR 30–299 mg/g, "microalbuminuria") or severely elevated (ACR >300 mg/g, "macroalbuminuria").[3] Progression of albuminuria was defined as changes from normoalbuminuria to microalbuminuria or macroalbuminuria or from microalbuminuria to macroalbuminuria[36] and more than 30%

increase in urine ACR (uACR) from baseline as used in the CANVAS study.[41] Regression was defined as micro- to normoalbuminuria transition. Nonprogressors was defined as no change or regression in albuminuria levels. Only participants with eGFR level of >60 ml/min per 1.73 m^2 or greater and uACR <300 mg/g at baseline and followed-up for 2 years or longer were included for the current study ($n = 691$) (Supplementary Figure S1).

Measurement of LTL

Telomere length was measured at baseline and compared as telomere to single-copy gene ratio (T/S) using quantitative polymerase chain reaction (PCR), as described earlier.[42] Telomeric (T) DNA and β-globin (S), used as internal control to normalize the amount of DNA loaded, was amplified from each sample. For standard, 1 reference DNA sample (commercially available human G304A DNA) was serially diluted in water by twofold dilution to produce 6 DNA concentrations from 40 to 1.25 ng in 5 μl. To reduce interassay variability, telomere and β-globin were analyzed on the same plate. Additionally, HepG2 DNA (experimental control) was run in every plate, and the average normalizing factor was used to correct the participant DNA samples to obtain the final T/S ratio (adjusted T/S ratio). All samples, standards, and controls were run in duplicate and the average values were used for the analyses. A standard curve derived from reference sample was used to transform the cycle threshold into nanograms of DNA. The amount of telomeric DNA (T) was divided by the amount of single-copy control gene DNA (S), producing a relative measurement of the telomere length (T/S ratio). The coefficients of variation within duplicates of the telomere and single-gene assay were less than 2%. The reported interassay coefficient of variation for the quantitative PCR across was less than 12%. All quantitative PCR experiments were performed by the same individual and were blinded for the phenotype of the participants.

Statistical Analysis

Continuous variables with normal distribution were expressed as the mean ± SD, whereas non-normally distributed variables were presented as median and interquartile ranges (IQR). Categorical data were expressed as proportions. LTL, triglycerides, and uACR were natural logarithmically transformed due to their skewed distribution. Comparisons between groups were performed by independent-sample *t*-test for normally distributed variables, Mann-Whitney *U* test for nonparametric distributions, and χ^2 test for categorical variables (Table 1). Participants were also divided into quartiles based on LTL levels to visualize clinical and biochemical characteristics. One-way analysis of variance or χ^2 test was applied to analyze the differences in continuous or categorical variables across quartiles (Table 2). The association between LTL quartiles and the outcomes were analyzed using binary logistic regression models and adjusting for traditional risk factors, such as age, gender, ethnicity, duration of diabetes, body mass index (BMI), systolic blood pressure, HbA1c, lipid profiles, baseline eGFR and uACR, the key risk factors for DKD progression, as well as usage of insulin and renin-angiotensin system antagonists. Highest LTL quartile (longer LTL) was taken as reference category (Table 3).

Given that albuminuria progression included transition from normoalbuminuria to microalbuminuria, or from normoalbuminuria or microalbuminuria to macroalbuminuria, and macroalbuminuria is a risk factor for chronic kidney disease and end-stage renal disease, we further performed subgroup analysis to examine the association of 1-SD decrement of natural log-transformed LTL with risk for progression from normo- to microalbuminuria and from normo- or micro- to macroalbuminuria (Table 4). Additionally, participants with albuminuria regression ($n = 57$) were excluded from the control group in albuminuria progression analysis.

Next, we calculated the area under the receiver-operating characteristic curve to evaluate the predictive performance of the models after adding LTL. Given that area under the receiver-operating characteristic curve assesses risk rankings instead of clinically important relative differences in risk magnitude, we used integrated discrimination improvement to measure the separation between progressors and nonprogressors in terms of average predicted risk.[43] As the risk category for renal progression in T2D was unknown, we used the category-free (continuous) net reclassification improvement to calculate the sum of the net percentages of the subjects with and without rapid renal progression being correctly assigned a different predicted risk.[44]

All analyses were performed using SPSS version 22 (SPSS Inc, IBM Corporation, Chicago, IL) and R software (version 3.3.2). A 2-sided $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristic Stratified by Albuminuria Progression Status

Table 1 illustrates the baseline characteristics of the participants in this study. There were 123 progressors and 568 nonprogressors identified and their median follow-up was 3.1 (IQR 3.0–3.2) years and 3.2 (IQR 3.1–3.3) years, respectively. At baseline, progressors had higher BMI, systolic blood pressure, longer duration of

Table 1. Baseline characteristics of patients with type 2 diabetes stratified by albuminuria progression status

Variables	All patients (n = 691)	Nonprogressor (n = 568)	Progressor (n = 123)	P
LTL (T/S ratio)	**0.61 (0.44–0.86)**	**0.62 (0.45–0.88)**	**0.58 (0.38–0.79)**	**0.039**
Age (yr)	56.2 ± 9.9	56.1 ± 9.9	56.7 ± 10.0	0.528
Gender (female) (%)	52.0	51.4	54.5	0.538
Ethnicity (%)				**0.034**
Chinese	**55.7**	**56.2**	**53.7**	
Malay	**19.8**	**18.1**	**27.6**	
Indian	**24.5**	**25.7**	**18.7**	
Body weight (kg)	**71.4 ± 15.0**	**70.8 ± 14.6**	**74.4 ± 16.0**	**0.014**
BMI (kg/m^2)	**27.6 ± 5.0**	**27.3 ± 4.9**	**28.7 ± 5.6**	**0.005**
Current smokers (%)	8.0	7.8	8.9	0.661
CVD history (%)	12.2	12.7	9.5	0.561
HbA1c (%)	7.62 ± 1.23	7.61 ± 1.20	7.66 ± 1.33	0.653
FPG (%)	7.85 ± 2.42	7.84 ± 2.37	7.91 ± 2.61	0.762
Diabetes duration (yr)	**10.0 ± 8.2**	**9.54 ± 8.0**	**11.7 ± 9.0**	**0.016**
Systolic BP (mm Hg)	**136 ± 16.0**	**136 ± 15.7**	**139 ± 17.1**	**0.022**
Diastolic BP (mm Hg)	78.8 ± 9.2	78.5 ± 9.1	79.9 ± 9.4	0.127
Total cholesterol (mM)	4.39 ± 0.91	4.38 ± 0.89	4.45 ± 1.01	0.464
HDL-C (mM)	1.30 ± 0.37	1.31 ± 0.38	1.29 ± 0.36	0.720
LDL-C (mM)	2.75 ± 0.80	2.74 ± 0.79	2.79 ± 0.88	0.531
Triglycerides (mM)	1.35 (1.0–1.85)	1.33 (1.01–1.84)	1.40 (1.06–1.91)	0.280
Renal function				
eGFR (ml/min per 1.73 m^2)	**96.8 ± 15.4**	**97.3 ± 15.3**	**94.2 ± 15.8**	**0.042**
uACR (μg/mg)	**13 (5–38)**	**11 (4–37)**	**21 (10–45)**	**<0.0001**
Usage of medication (%)				
Metformin	85.5	84.6	89.4	0.168
Insulin	**21.3**	**19.3**	**30.1**	**0.008**
Statin	81.3	80.2	86.2	0.122
RAS antagonist	52.5	50.6	61.0	**0.037**

Data are described as mean ± SD or median (25th, 75th interval) for skewed variables or proportion of participants (%) where appropriate. Bold values represent statistically significant data between progressors and nonprogressors.
BMI, body mass index; BP, blood pressure; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LTL, leukocyte telomere length; RAS, renin-angiotensin system; T/S ratio, telomere to single copy gene ratio; uACR, urine albumin creatinine ratio.

diabetes, and were more likely to be Malay as compared with nonprogressors. No differences between progressors and nonprogressors were observed regarding age, gender, HbA1c level, lipid profiles, smoking status, and cardiovascular disease history. The eGFR levels were compatible between the groups, but uACR level was significantly higher in progressors as compared with nonprogressors (21 [IQR 10–45] and 11 [IQR 4–37] μg/mg). Notably, LTL was significantly shorter in progressors as compared with nonprogressors (0.58 [IQR 0.38–0.79] vs. 0.62 [IQR 0.49–0.88], $P = 0.039$) (Table 1).

Baseline Characteristics of Participants Stratified by LTL

We further divided participants into quartiles according to LTL to visualize the relationship between LTL and clinical characteristics at baseline (Table 2). As expected, there was a progressive decline in LTL with increasing age. Participants with shorter LTLs were older as compared with those with longer LTLs (57.8 ± 9.8 and 54.1 ± 10.1 years, $P = 0.001$). Compared with participants with longer LTL (highest quartile), those with shorter LTL had a lower body weight ($P = 0.089$), higher uACR ($P = 0.014$), and were more likely to be Chinese ($P < 0.0001$). No significant differences in HbA1c levels, diabetes duration, and lipid profiles were observed among participants across different LTL quartiles.

LTL was inversely correlated with age (rho: −0.174; $P < 0.0001$), diabetes duration (rho: −0.105; $P = 0.006$), and uACR (rho: −0.117; $P = 0.002$) and positively correlated with BMI (rho: 0.087; $P = 0.022$) and low-density lipoprotein cholesterol (rho: 0.078; $P = 0.040$) (Supplementary Table S1). When adjusted LTL for age, the observed correlation between LTL and clinical variables were attenuated. Multivariable linear regression analysis showed that LTL was associated with age (β: −0.011; $P < 0.0001$) and uACR (β: −0.045; $P = 0.002$) independently after adjusting for age, gender, HbA1c, diabetes duration, BMI, systolic blood pressure, lipid profiles, and eGFR (Supplementary Table S2).

Baseline LTL Independently Predicted Albuminuria Progression

In primary analysis, we combined participants with stable albuminuria ($n = 511$) and albumin regression ($n = 57$) as controls. Logistic regression showed that

Table 2. Baseline characteristics of patients with type 2 diabetes according to leukocyte telomere length quartile

Variables	Quartile 1 (n = 169)	Quartile 2 (n = 171)	Quartile 3 (n = 177)	Quartile 4 (n = 174)	P
LTL (T/S ratio)	**0.33 ± 0.09**	**0.52 ± 0.04**	**0.72 ± 0.08**	**1.16 ± 0.34**	**<0.0001**
Age (yr)	**57.8 ± 9.8**	**57.7 ± 9.7**	**55.4 ± 9.5**	**54.1 ± 10.1**	**0.001**
Female (%)	52.1	47.4	55.4	52.9	0.507
Ethnicity (%)					**<0.0001**
Chinese	**68.0**	**57.9**	**49.2**	**48.3**	
Malay	**11.2**	**15.8**	**23.2**	**28.7**	
Indian	**20.7**	**26.3**	**27.7**	**23.0**	
Body weight (kg)	69.5 ± 14.1	70.7 ± 15.2	71.8 ± 14.2	73.6 ± 16.0	0.089
BMI (kg/m^2)	27.0 ± 4.9	27.4 ± 5.1	27.8 ± 4.7	28.1 ± 5.5	0.218
Current smokers (%)	9.5	5.8	8.0	8.6	0.644
CVD history (%)	8.8	17.3	11.4	12.0	0.587
HbA1c (%)	7.69 ± 1.18	7.59 ± 1.24	7.55 ± 1.16	7.63 ± 1.32	0.740
FPG (%)	7.99 ± 2.53	7.67 ± 2.51	7.97 ± 2.25	7.79 ± 2.40	0.557
Diabetes duration (yr)	10.7 ± 8.7	10.1 ± 7.8	9.6 ± 7.5	9.4 ± 8.8	0.437
Systolic BP (mm Hg)	137 ± 18	138 ± 16	136 ± 16	134 ± 14	0.214
Diastolic BP (mm Hg)	78.8 ± 8.6	78.8 ± 9.4	80.0 ± 9	78 ± 9	0.486
Total cholesterol (mM)	4.36 ± 0.85	4.29 ± 0.85	4.50 ± 0.98	4.41 ± 0.95	0.202
HDL-C (mM)	1.34 ± 0.39	1.33 ± 0.46	1.26 ± 0.31	1.29 ± 0.30	0.180
LDL-C (mM)	**2.69 ± 0.72**	**2.63 ± 0.75**	**2.87 ± 0.85**	**2.79 ± 0.87**	**0.023**
Triglycerides (mM)	1.37 (1.06–1.86)	1.28 (0.94–1.81)	1.45 (1.08–1.91)	1.23 (0.95–1.84)	0.104
Renal function					
uACR (μg/mg)	**19 (7–48)**	**13 (5–38)**	**13 (5–36)**	**10 (3–29)**	**0.014**
eGFR (ml/min per 1.73 m^2)	96.6 ± 14.9	95.6 ± 15.0	96.2 ± 16.2	98.6 ± 15.6	0.301
Usage of medication (%)					
Metformin	87.5	83.5	89.8	80.9	0.084
Insulin	23.2	17.2	25.4	19.1	0.221
Statin	84.5	81.8	82.5	76.3	0.244
RAS antagonist	58.4	50.6	46.0	55.2	0.108

Data are described as mean ± SD or median (25th, 75th interval) for skewed variables or proportion of participants (%) where appropriate. Bold values represent statistically significant data.
BMI, body mass index; BP, blood pressure; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LTL, leukocyte telomere length; RAS, renin-angiotensin system; T/S ratio, telomere to single copy gene ratio; uACR, urine albumin creatinine ratio.

individuals with LTL in the lowest quartile (shorter LTL) were associated with an increased risk for albuminuria progression (odds ratio [OR]: 2.10; 95% confidence interval [CI]: 1.15–3.83; $P = 0.016$; Model 1 adjusted for age, gender, ethnicity, HbA1c, and diabetes duration) compared with those with LTL in the highest quartile (longer LTL). Further adjustment for BMI, systolic blood pressure, and triglyceride level did not attenuate the association (Model 2) (Table 3). The association between LTL and risk for albuminuria progression remained significant after additional adjustment for baseline eGFR and uACR (OR: 1.93; 95% CI; 1.04–3.60; $P = 0.038$). Usages of medications did not materially alter the independent association of short LTL and increased risk for albuminuria progression (OR: 1.90; 95% CI: 1.02–3.54; $P = 0.044$; Model 4); however, individuals in second and third quartiles did not show significantly increased risk for albuminuria progression as compared with those in the fourth quartile.

Next, we assessed the improvement in risk discrimination after adding LTL into the model with traditional risk factors, such as age, gender, ethnicity, diabetes condition, blood pressure, BMI, triglycerides, baseline renal function, and medications usage (Model 4). Inclusion of LTL in the model 4 did not significantly improve area under the receiver-operating characteristic curve (from 0.675 to 0.687, $P = 0.201$) (Supplementary Table S3). Given that area under the receiver-operating characteristic curve is an insensitive indicator of added value of biomarker, we calculated the integrated discrimination improvement and category-free net reclassification improvement. Including LTL significantly improved the predictive performance of albuminuria as reflected by an increased category-free net reclassification improvement of 0.428 (95% CI: 0.243–0.613; $P = 1e\text{-}5$) and integrated discrimination improvement of 0.063 (95% CI: 0.63–0.124; $P < 0.001$).

Sensitivity Analysis

Exclusion of participants with albuminuria regression ($n = 57$) in controls did not alter the association of LTL and albuminuria progression (quartile 1 vs. quartile 4) (OR: 1.93; 95% CI: 1.02–3.68; $P = 0.045$ vs. OR: 1.54; 95% CI: 10.02–2.32; $P = 0.042$) in primary analysis. In subgroup analysis, we examined the association between LTL and transition from normoalbuminuria to

Table 3. Logistic regression analysis for association of leukocyte telomere length with albuminuria progression

Variables	Model 1 OR (95%CI)	Model 2 OR (95%CI)	Model 3 OR (95%CI)	Model 4 OR (95%CI)
LTL Quartile 1	**2.10 (1.15–3.83)**	**2.12 (1.15–3.89)**	**1.93 (1.04–3.60)**	**1.90 (1.02–3.54)**
Quartile 2	1.64 (0.89–3.02)	1.62 (0.87–3.00)	1.52 (0.81–2.86)	1.50 (0.80–2.85)
Quartile 3	**1.81 (1.00–3.26)**	1.73 (0.95–3.15)	1.61 (0.87–2.95)	1.56 (0.85–2.88)
Quartile 4	-	-	-	-
Age (yr)	1.00 (0.98–1.02)	1.00 (0.97–1.02)	0.98 (0.95–1.01)	0.98 (0.95–1.01)
Female	1.13 (0.76–1.69)	1.03 (0.68–1.56)	1.25 (0.80–1.97)	1.26 (0.80–1.98)
Male[a]	-	-	-	-
Malay	**1.82 (1.11–2.97)**	1.61 (0.97–2.68)	1.37 (0.81–2.30)	1.34 (0.79–2.26)
Indian	0.79 (0.47–1.33)	0.77 (0.45–1.31)	0.76 (0.45- 1.31)	0.75 (0.44–1.29)
Chinese[a]	-	-	-	-
HbA1c (%)	0.98 (0.82–1.16)	0.95 (0.80–1.14)	0.94 (0.78–1.13)	0.94 (0.78–1.13)
Diabetes duration (yr)	**1.03 (1.01–1.06)**	**1.03 (1.01–1.06)**	1.03 (1.00–1.05)	1.02 (1.00–1.05)
BMI (kg/m^2)		**1.06 (1.02–1.10)**	1.04 (1.00–1.09)	1.04 (1.00–1.09)
SBP (mm Hg)		1.01 (1.00–1.03)	1.01 (0.99–1.02)	1.01 (0.99–1.02)
Triglycerides (mM)[b]		1.06 (0.67–1.68)	0.93 (0.58–1.47)	0.93 (0.58–1.50)
uACR (μg/mg)[b]			**1.31 (1.12–1.52)**	**1.31 (1.11–1.53)**
eGFR (ml/min per 1.73 m^2)			0.99 (0.97–1.00)	0.99 (0.97–1.00)
RAS antagonists[c]				0.97 (0.62–1.52)
Statin[c]				1.29 (0.71–2.33)

Data are ORs (95% CIs) estimated for continuous and categorical variables in different models. LTL was divided into quartiles and quartile 4 (longest LTL) was used as reference. Bold values represent statistically significant data ($P < 0.05$).
BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; LTL, leukocyte telomere length; OR, odds ratio; RAS, renin-angiotensin system; SBP, systolic blood pressure; uACR, urine albumin creatinine ratio.
[a]Male gender and Chinese ethnicity were references (dashes).
[b]Natural log transformed value was used for analysis.
[c]No usage of medicine was reference.

microalbuminuria and from normo- or micro- to macroalbuminuria separately (Tables S4 and S5). The association of LTL with progression from normo- or micro- to macroalbuminuria ($n = 35$) was stronger than normo- to- microalbuminuria ($n = 88$) (OR: 1.54; 95% CI: 1.02–2.32; $P = 0.042$ vs. OR: 1.13; 95% CI: 0.91–1.40; $P = 0.263$) (Table 4).

DISCUSSION

In this prospective cohort of multiethnic Asian population, we found that LTL independently predicted the progression of albuminuria in T2D with preserved renal filtration function (eGFR >60 ml/min per 1.73 m^2 and uACR <300 μg/mg). Participants with shorter LTL (lowest quartile) were at twofold increased risk for albuminuria progression as compared with those with longer LTL (highest quartile). Furthermore, the association of LTL and albuminuria progression is independent of traditional risk factors, including hypertension, hyperglycemia, long diabetes duration, and dyslipidemia, as well as existing kidney function impairment, suggesting short LTL may be a novel biomarker for DKD progression.

Previously, in a prospective study involving patients with T1D, shorter LTL also predicted the risk for albuminuria progression.[36] Although short telomeres are also associated with decline in renal function in nondiabetic conditions, in a recent study that included patients with T1D or T2D with mild to severe chronic kidney disease, association of LTL with chronic kidney disease progression was the strongest in patients with diabetes.[37] Our study not only provided the necessary evidence of association of short LTL and albuminuria progression in Asian patients with T2D, it also demonstrated the consistency of short LTL as a potential marker for increased risk for renal disease across different ethnic groups. Furthermore, the prognostic significance of LTL in albuminuria progression was independent of known risk factors, suggesting that it may influence DKD risk through biological pathways beyond established risk factors.

In our study, compared with individuals in the fourth quartile, only individuals in the first quartile (shortest LTL) had significantly increased risk of albuminuria

Table 4. Subgroup univariate and multivariate association between baseline LTL and progression of albuminuria

Progression	Univariate OR (95% CI)	*P value*	Multivariate OR (95% CI)	*P*
Normo- to microalbuminuria				
LTL (1-SD decrement)	1.13 (0.91–1.40)	0.263	1.17 (0.93–1.47)	0.185
Normo- or micro- to macroalbuminuria				
LTL (1-SD decrement)	**1.39 (1.03–1.87)**	**0.032**	**1.54 (1.02–2.32)**	**0.042**

Data are ORs (95% CIs) estimated as per effect of 1-SD decrement in natural log LTL in univariate or multivariate model adjusted for age, gender, ethnicity, HbA1c, diabetes duration, body mass index, triglycerides, systolic blood pressure, urine albumin creatinine ratio, estimated glomerular filtration rate, and usage of renin-angiotensin system antagonist and statins. Bold values represents significant data.
CI, confidence interval; LTL, leukocyte telomere length; OR, odds ratio.

progression. In contrast, although there was a relative 50% increased risk in albuminuria progression in both second and third quartiles as compared with the fourth quartile, these differences were nonsignificant, indicating a nonlinear relation between LTL and albuminuria progression in the Asian population. This is consistent with pervious observations demonstrating the nonlinear association of telomere length with incident diabetes, coronary heart disease, and cancer.[26,45,46] This is in line with the Hayflick limit hypothesis of telomeres.[13] In our study, there appears to be a critical threshold value between first and second quartiles beyond which replicative senescence and consequently negative cascade of biological events may lead to propensity of DKD progression.

In the largest single telomere length assay performed in the Genetic Epidemiology Research on Adult Health and Aging (GERA) cohort, it was shown that LTL variation increases with age, demonstrating the influence of nongenetic factors.[47] A series of studies have demonstrated that oxidative stress plays a major role in telomere attrition.[29] Furthermore, telomere repeats are more sensitive to oxidative stress-induced damage as compared with nontelomeric regions.[48] A recent study by Sun *et al.*,[49] using a novel technique that measures oxidative damage at telomere real-time, showed that oxidative telomere damage leads to telomere shortening, providing another clue as to how oxidative damage may contribute significantly to telomeric erosion. Biologically, data from animal models demonstrated that telomere dysfunction represses expression of peroxisome proliferator–activated receptor gamma, coactivator 1 alpha and beta, leading to impaired mitochondria function, decreased gluconeogenesis, and increase in reactive oxygen species level.[50] A recent study revealed that patients with diabetic nephropathy have reduced mitochondrial DNA content in the circulation, and reduced maximal respiration and reserve capacity compared with diabetic patients without diabetic nephropathy, suggesting that systemic mitochondrial dysfunction initiated by hyperglycemia-induced mitochondrial DNA damage may be involved in the development of diabetic nephropathy.[51] Although we do not have evidence on the oxidative stress level in our study cohort, it is known that renal cells are subjected to ongoing replicative stress, which accelerates telomere shortening and reduces proliferation capacity, resulting in less efficient regeneration and repair of tissue.[32,52] Therefore, it is possible that during the early stages of DKD, short telomeres may predispose patients with T2D to be more susceptible to adverse metabolic milieu and therefore accelerated DKD progression. In a subgroup analysis, we found a significant association between shorter LTL and progression from normo- or micro- to macroalbuminuria, but not with progression from normo- to microalbuminuria, further supporting the hypothesis that LTL also represents environmental impact on renal function. However, given the small sample size in this subgroup analysis, this warrants further investigation in a larger cohort with longer follow-up.

The inverse correlation between LTL and age has been well documented in clinical studies, including this study. Besides age, other clinical variables have been shown to be associated with LTL. Albuminuria levels were independently associated with LTL at cross-sectional levels, further supporting the close relationship between early renal impairment and short telomeres. A recent clinical study in American Indian individuals showed that short LTL was associated with obesity.[53] In contrast, we found a positive correlation between LTL and BMI, which was attenuated when adjusted for age. Interestingly, we found that BMI independently predicted progression from normo- to microalbuminuria beyond traditional risk factors.

The strengths of the current study include a prospective design, a relatively large sample size of multiethnic individuals in Singapore, and detailed clinical phenotyping. In our analysis, we also examined the added value of LTL beyond albuminuria and traditional clinical risk factors for DKD, including commonly prescribed medications such as renin-angiotensin system antagonists. This is also essential given that the deregulated renin-angiotensin-aldosterone system is suggested to be a contributor to telomere shortening.[28] This study has limitations that should be acknowledged. First, we measured mean LTL using a PCR-based assay that does not quantify absolute telomere length as compared with Southern blotting. LTL was also measured in blood leukocytes and not the target organs, such as kidney, because widespread renal biopsies are neither practical nor ethnical to carry out. However, it has been shown that telomere length measurement with quantitative PCR and Southern blotting have strong concordance,[54] and telomere length in different tissues may be highly correlated.[55] Second, we used a single measurement of uACR at baseline and follow-up from spot urine samples, which may raise concern with some misclassification, given that there is considerable intraindividual variation in albuminuria, which may affect classification of DKD progression.[3] Moreover, a higher random ACR and eGFR measurement variation may reduce the statistical power and tend to lead the outcome to false negative. In this regard, the 1-time measurement may not be a major concern for the positive findings in our current study. Lambers Heerspink *et al.*[56] previously demonstrated that uACR from spot urine samples

correlates with 24-hour urine protein and is clinically relevant for predicting progression. Furthermore, in addition to increase in uACR categories, we included 30% increase in uACR from baseline to define our progressors. Last, and more importantly, because of the nature of the study design, we cannot elucidate the mechanisms underlying the linkage between shortened telomeres to the progression of renal disease. A larger cohort with longer follow-up is warranted to demonstrate the association of LTL with DKD progression, including end-stage renal disease.

In conclusion, our results indicate that LTL is independently associated with albuminuria progression in patients with T2D with early-stage DKD (eGFR >60 ml/min per 1.73 m^2) in Asian individuals. We propose that a shorter LTL may be a biomarker for propensity of renal disease progression in patients with diabetes, although the biological linkage between shorter leucocyte LTL and propensity for renal impairment in the kidney remains to be elucidated.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

RLG designed the study, research the data, and wrote the manuscript; YM measured the LTL and researched the data. SL and J-JL reviewed the data, edited the manuscript, and contributed to the critical discussion of the work. SCL critically reviewed and edited the manuscripts, is the guarantor of this work, and takes responsibility for the integrity of the data.

SUPPLEMENTARY MATERIAL

Figure S1. Participant recruitment in this study.

Table S1. Values of Spearman correlation coefficient between leukocyte telomere length and clinical variables.

Table S2. Association of clinical variables with leukocyte telomere length in multivariable linear regression model.

Table S3. Odds ratio (OR) and area under the receiver-operating characteristic curve by logistic regression models for prediction of outcome by leukocyte telomer length (LTL) and albuminuria as categorical or continuous variable.

Table S4. Logistic regression analysis for association between per SD in leukocyte telomer length (LTL) (natural log transformed) and albuminuria progression (normo- to microalbuminuria).

Table S5. Logistic regression analysis for association between per SD in leukocyte telomer length (LTL) (natural log transformed) and albuminuria progression (normo- or micro- to macroalbuminuria).

Supplementary material is linked to the online version of the paper at http://www.kireports.org/.

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