Shortened Relative Leukocyte Telomere Length Is Associated With Prevalent and Incident Cardiovascular Complications in Type 2 Diabetes: Analysis From the Hong Kong Diabetes Register

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OBJECTIVE

Several studies support potential links between relative leukocyte telomere length (rLTL), a biomarker of biological aging, and type 2 diabetes. This study investigates relationships between rLTL and incident cardiovascular disease (CVD) in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Consecutive Chinese patients with type 2 diabetes ($N = 5,349$) from the Hong Kong Diabetes Register for whom DNA obtained at baseline was stored and follow-up data were available were studied. rLTL was measured by using quantitative PCR. CVD was diagnosed on the basis of ICD-9 code.

RESULTS

Mean follow-up was 13.4 years (SD 5.5 years). rLTL was correlated inversely with age, diabetes duration, blood pressure, HbA_{1c}, and urine albumin-to-creatinine ratio (ACR), and positively with estimated glomerular filtration rate (eGFR) (all $P < 0.001$). Subjects with CVD at baseline had a shorter rLTL (4.3 ± 1.2 ΔΔCt) than did subjects without CVD (4.6 ± 1.2 ΔΔCt) ($P < 0.001$). Of the 4,541 CVD-free subjects at baseline, the 1,140 who developed CVD during follow-up had a shorter rLTL (4.3 ± 1.2 ΔΔCt) than those who remained CVD-free after adjusting for age, sex, smoking, and albuminuria status (4.7 ± 1.2 ΔΔCt) ($P < 0.001$). In Cox regression models, shorter rLTL was associated with higher risk of incident CVD (for each unit decrease, hazard ratio 1.252 [95% CI 1.195–1.311], $P < 0.001$), which remained significant after adjusting for age, sex, BMI, systolic blood pressure, LDL cholesterol, HbA_{1c}, eGFR, and ACR (hazard ratio 1.141 [95% CI 1.084–1.200], $P < 0.001$).

CONCLUSIONS

rLTL is significantly shorter in patients with type 2 diabetes and CVD, is associated with cardiometabolic risk factors, and is independently associated with incident CVD. Telomere length may be a useful biomarker for CVD risk in patients with type 2 diabetes.

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Diabetes and cardiovascular disease (CVD) are leading causes of morbidity and mortality worldwide. People with diabetes have two to four times the risk of CVD compared with those without diabetes (1). A large Hong Kong hospital-based cohort study reported that ~15% of patients with type 2 diabetes already have a diagnosis of CVD at baseline (2), with another 15.1% developing CVD after a mean of 6.7 years of follow-up (3). Elevated glucose level, blood pressure, and lipid levels, and obesity, smoking, and renal dysfunction are well-recognized risk factors for CVD in people with diabetes and in the general population (4,5).

Telomeres, the (TTAGGG) repeat sequences at the end of each DNA strand, protect chromosomal DNA from deteriorating or fusing with other chromosomes during cell division. Telomeres shorten with every cell division, and hence telomeric repeat shortening is associated with "cellular aging" and age-related diseases including diabetes and CVD (6). Previous studies have shown that relative leukocyte telomere length (rLTL) was shorter in patients with type 2 diabetes, myocardial infarction (MI), ischemic heart disease, or CVD (6–10). rLTL was also associated with traditional vascular risk factors including aging, obesity, smoking, elevated blood pressure and lipids, and glycemia (6,11). Possible mechanisms linking diabetes and rLTL shortening include glucose-induced oxidative stress and proinflammatory conditioning, which are integral in the development of CVD (12,13).

rLTL has been associated with prevalent diabetes and CVD, as well as incident diabetes and CVD, in the general population (6,14,15). However, the relationship between rLTL and incident CVD in patients with type 2 diabetes has not been examined thoroughly. Here we evaluate the role of rLTL in predicting incident CVD in the Hong Kong Diabetes Register (HKDR), composed of a large cohort of patients with type 2 diabetes with moderate follow-up duration. In addition, we explore associations between rLTL and traditional CVD risk factors.

RESEARCH DESIGN AND METHODS

Subjects

Consecutive patients with type 2 diabetes for whom DNA and clinical data were available ($N = 5,506$) were selected from the HKDR (16). The methods for enrollment and assessment have been described previously (16). Briefly, the HKDR was established at the Prince of Wales Hospital in 1995. Every week, 30–50 patients with known type 2 diabetes were referred to the Diabetes Mellitus and Endocrine Centre for a comprehensive assessment of metabolic control and a screening for complications based on the European DIABCARE protocol (17,18). Once enrolled, patient outcomes were tracked until death by using a territory-wide electronic medical record or the latest data collection date of 30 June 2017, whichever came first. Upon enrollment, each subject provided written informed consent and agreed to additional blood collection for genetic and biomedical analyses. Ethical approval was obtained from the Clinical Research Ethics Committee of the Chinese University of Hong Kong.

Clinical and Biochemical Parameters

The comprehensive assessment included an interview by a trained diabetes nurse, anthropometric measurements, blood and urine tests, fundoscopy, and a podiatry assessment. Each patient's blood pressure (in millimeters of mercury), weight (in kilograms), and height (in meters) were measured. BMI was calculated as weight in kilograms divided by height in meters squared.

Blood was collected from each patient after an overnight fast (≥8 h) in order to measure glycated hemoglobin (HbA_{1c}), fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and estimated glomerular filtration rate (eGFR) (calculated by using the Chronic Kidney Disease Epidemiology Collaboration formula) (19). Urine was collected with a spot urine test for quantifying the urinary albumin-to-creatinine ratio (ACR). All laboratory analyses were performed by using standard methods in the Prince of Wales Hospital Department of Chemical Pathology, which is accredited by the Australian National Association of Testing Authorities.

Clinical End Points

CVD end points were defined as MI, coronary heart disease (CHD), congestive heart failure (CHF), cerebral vascular accident (CVA), and peripheral vascular disease (PVD), and were based on ICD-9 codes (18). The MI end point was defined as ICD-9 code 410, the CHD end point, codes 410–414; CHF, as nonfatal heart failure (code 428); CVA, as nonfatal or fatal ischemic stroke (codes 432–438), subarachnoid hemorrhage (code 430), and intracerebral hemorrhage(code 431). A transient ischemic attack was excluded. PVD was defined as peripheral circulatory disorders (code 250.7), gangrene (code 785.4), angiopathy in diseases classified elsewhere (code 443.81), PVD unspecified (code 443.9), other peripheral vascular shunt or bypass (procedure code 39.29), insertion of non-drug-eluting peripheral vessel stents (procedure code 39.90), or amputation of lower limb (procedure code 84.1) without a traumatic amputation diagnosis (codes 895–897).

Telomere Length

rLTL was measured in peripheral blood leukocyte DNA by using an updated method for quantitative PCR (20,21). The details of rLTL measurement are described in the Supplementary Material and our recently published protocol (21). The telomere and single copy gene human β-globin (HBG) were measured in order to obtain the ΔΔCt values which determined the rLTL. A no-template control (NTC; water) and a reference human sample (for quality control [QC]) were included in all plates in order to adjust for plate-to-plate variability and to act as the reference for calculating ΔΔCt. There is currently no universally accepted approach to normalization, and calculations based on QC or NTC are considered acceptable in most studies. We compared the results obtained using the two different methods of normalization in order to further examine the consistency of our results. The coefficient of variation (CV) of each sample was <2.5%, and the interplate CVs of the telomere and HBG assays were 2.9% and 1.2%, respectively. The overall intraplate CV was 1.2% for telomere length and 0.4% for HBG.

Because rLTL measurements do not reflect the actual (absolute) leukocyte telomere length (LTL), we were asked to test alternate commercially available methods. Although whole-genome sequencing data could, in our view, provide the most accurate measure, these were unavailable to us at the time. A reference DNA sample with known absolute telomere length from a commercially available

kit (catalog no. 8918; ScienCell Research Laboratories) was therefore used in order to calculate absolute telomere length in the reference QC sample, and then, by calculating ΔΔCt between the QC sample and the cohort samples, the estimated absolute telomere length was obtained for all other samples.

Statistical Analysis

Data are expressed as the mean ± SD, median (Q1, Q3), or proportion (as a percentage). rLTL with skewness of −0.4 followed a normal distribution, and covariates were natural logarithmic transformed if skewed. Comparisons between groups were performed by using the Student *t* test. When comparing differences in rLTL between sexes, and by baseline or incident CVD, all analyses were adjusted for age, sex, BMI, and diabetes duration by using a general linear model. The χ^2 or Fisher exact test was used as appropriate for categorical variables. Pearson and partial correlations were determined in order to test relationships between rLTL and baseline characteristics. We also applied logistic regression to analyze the association between rLTL and odds of CVD at baseline, and Cox regression to examine associations between rLTL and incident CVD. Because the shape of the association between rLTL (categorized into quartiles) and risk of CVD was linear, we investigated rLTL as a continuous variable in logistic regression and multivariable Cox regression. In addition, competing risk regression models were used in order to estimate the subdistribution hazard ratio (HR) of rLTL, with death not caused by CVD entered as the competing risk. The odds ratio (OR) represented the relative increase in the risk of prevalent CVD with each unit decrease of rLTL, and HR represented the risk of incident CVD. The association between rLTL (by quartiles) and the number of CVD events during follow-up were assessed by applying the Kaplan-Meier method. Mean rLTL values of groups were used as a continuous variable in the model in order to assess trends in risk in relation to CVD. In the sensitivity analysis, we evaluated the HR of rLTL in relation to incident CVD, with rLTL normalized to that of the NTC or QC sample. We computed *z* scores on the basis of the mean value and SD for rLTL and all risk factors, and we repeated Cox regression with all variables defined as per-SD change. We compared the characteristics of subjects with and of those without complete data. A two-tailed *P* value ≤0.05 was considered statistically significant. Data were analyzed with SPSS software for Windows, version 24.0 (SPSS Inc., Chicago, IL) and R version 3.6.1.

RESULTS

Characteristics of Subjects and Telomere Length at Baseline

In this consecutive cohort of 5,506 patients with type 2 diabetes, 157 were excluded because of failed quality control or a missing rLTL measurement. The remaining 5,349 patients had mean follow-up of 13.4 years (SD 5.5 years); 45.2% were male. The cohort had a mean age of 57.5 years (SD 13.3 years) and BMI of 25.3 kg/m^2 (4.0 kg/m^2). At baseline, 808 patients (15.1%) had been diagnosed with CVD; the remaining 4,541 were "CVD-free," of whom 1,140 (25.1%) experienced CVD events during follow-up. There was no significant difference in rLTL between men (4.5 ± 1.2 ΔΔCt) and women (4.6 ± 1.2 ΔΔCt) (P = 0.283). There were statistically significant differences in TC, HDL-C, and TG between subjects with and those without missing variables, but the absolute differences were very small (Supplementary Table 1).

Compared with CVD-free patients at baseline, subjects with CVD were older (65.7 ± 10.8 vs. 56.1 ± 13.2 years, $P <$ 0.001) with a longer diabetes duration (9.3 ± 7.6 vs. 6.7 ± 6.6 years, $P <$ 0.001), a higher proportion of men (49.0% vs. 44.0%, P = 0.015) and smokers, higher systolic blood pressure (SBP) and TG levels, and more extensive renal dysfunction (higher urinary ACR and lower eGFR). Subjects with prevalent CVD also had lower diastolic blood pressure (DBP) and more antihypertensive drug usage. rLTL was shorter in patients with prevalent CVD (4.3 ± 1.2 ΔΔCt) than in those without prevalent CVD (4.6 ± 1.2 ΔΔCt) ($P <$ 0.001), even adjusting for age, sex, BMI, smoking status, and duration of diabetes ($P <$ 0.001) (Supplementary Table 2).

Logistic regression analyses suggested that shorter rLTL was inversely associated with greater odds of CVD at baseline (OR 1.235 [95% CI 1.162–1.313] for each unit decrease, $P <$ 0.001), which remained significant after adjustment for age, sex, duration of diabetes, ever having smoked, BMI, SBP, HbA_{1c}, LDL-C, renal function, ACR, and retinopathy at baseline (OR 1.160 [95% CI 1.082–1.243], $P <$ 0.001) (Table 1).

Relationship Between Telomere Length and Baseline Risk Factors

Pearson and partial correlation coefficients between rLTL and metabolic and cardiovascular risk factors are presented in Supplementary Table 3. In the entire cohort, rLTL was inversely associated with age, diabetes duration, SBP, DBP, HbA_{1c}, FPG, TC, LDL-C, and Ln (ACR), and positively with HDL-C. These relationships were not substantially altered after adjusting for age and sex. The associations with BMI and eGFR were attenuated after adjusting for age and sex. We also analyzed the association between men and women, separately. The associations between rLTL and diabetes duration, blood pressure, glucose control, lipids, and Ln (ACR) were robust and significant in both men and women with diabetes. BMI showed only a modest association with rLTL in men, but not in women (Supplementary Table 3).

Baseline Telomere Length Was an Independent Predictor for Incident CVD

When comparing patients with type 2 diabetes who were free of CVD at baseline and those who remained CVD-free during follow-up, those who developed incident CVD were older (60.7 ± 11.8 vs. 54.5 ± 13.3 years, $P <$ 0.001) and more likely to be male (51.0% vs. 42.4%, $P <$ 0.001). Of those who developed CVD, 37% were current or former smokers and 16.6% were current smokers at baseline. Among those without incident CVD, 26.0% currently or previously smoked and 12.2% smoked at baseline (both $P <$ 0.001). After adjusting for age and sex, patients with incident CVD had worse cardiometabolic risk factors (longer diabetes duration; higher SBP, DBP, HbA_{1c}, FPG, TC, LDL-C, TG, and ACR; and lower eGFR) than those who remained CVD-free during follow-up. They were also more likely to have microvascular complications and to have been prescribed antihypertensive drugs, an ACE inhibitor/angiotensin receptor blocker, and insulin. Patients with incident CVD also had shorter rLTL at baseline (4.3 ± 1.2 ΔΔCt)

Table 1—Association between rLTL and subjects with and those without CVD at baseline

	Model 1		Model 2		Model 3		Model 4		Model 5	
Variables	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
rLTL (each unit decrease)	1.235 (1.162–1.313)	<0.001	1.152 (1.080–1.228)	<0.001	1.140 (1.067–1.217)	<0.001	1.161 (1.083–1.244)	<0.001	1.160 (1.082–1.243)	<0.001
Age (years)			1.064 (1.057–1.072)	<0.001	1.056 (1.048–1.065)	<0.001	1.037 (1.027–1.047)	<0.001	1.038 (1.028–1.047)	<0.001
Male sex			1.250 (1.069–1.461)	0.005	1.124 (0.927–1.362)	0.234	1.132 (0.925–1.384)	0.226	1.117 (0.912–1.367)	0.281
Duration of diabetes (years)					1.023 (1.012–1.034)	<0.001	1.014 (1.002–1.026)	0.024	1.008 (0.996–1.021)	0.180
Ever smoked					1.338 (1.095–1.635)	0.004	1.354 (1.099–1.669)	0.004	1.362 (1.105–1.68)	0.004
BMI (kg/m^2)					1.016 (0.994–1.037)	0.073	1.008 (0.986–1.03)	0.502	1.009 (0.987–1.032)	0.409
SBP (mmHg)					1.006 (1.002–1.010)	0.002	1.005 (1.001–1.01)	0.012	1.005 (1.001–1.009)	0.027
HbA_{1c} (%)							1.044 (0.994–1.095)	0.083	1.033 (0.983–1.085)	0.190
LDL-C (mmol/L)							0.865 (0.792–0.945)	0.001	0.863 (0.79–0.943)	0.001
eGFR (mL/min/ 1.73 m^2)							0.986 (0.981–0.99)	<0.001	0.986 (0.982–0.991)	<0.001
Ln (ACR)							1.021 (0.971–1.072)	0.420	1.006 (0.957–1.058)	0.806
Retinopathy at baseline									1.469 (1.221–1.765)	<0.001

Model 1 included no adjustment. Model 2 was adjusted for age and sex. Model 3 was adjusted for model 2 variables and for duration of diabetes, BMI, SBP, and ever smoked. Model 4 was adjusted for model 3 variables for HbA_{1c}, LDL-C, eGFR, and Ln (ACR). Model 5 was adjusted for model 4 and retinopathy at baseline

than those who remained CVD-free (4.7 $\pm$ 1.2 $\Delta\Delta$Ct) ($P < 0.001$), which remained significant after adjusting for age, sex, smoking status, diabetes duration, and micro- and macroalbuminuria ($P < 0.001$) (Table 2).

The relationship between baseline rLTL and incident CVD among subjects free of CVD at baseline was examined by using multivariate Cox regression including traditional risk factors for CVD, such as age, sex, ever smoked, duration of diabetes, BMI, SBP, HbA_{1c}, LDL-C, ACR, and eGFR (2,22). Baseline rLTL was inversely associated with risk of incident CVD (HR 1.252 [95% CI 1.195–1.311] for each unit decrease, $P < 0.001$), which remained significant after adjusting for the traditional risk factors for CVD (HR 1.141 [95% CI 1.084–1.200], $P < 0.001$) (Table 3). Plate number itself was not a predictor of CVD when entered as a covariate with rLTL. In Cox regression models that included the *z* score of each variable, the fully adjusted models showed that each SD decrease of LTL was independently associated with future risk of CVD (HR 1.180 [95% CI 1.111–1.254], $P < 0.001$), after adjusting for the *z* scores of traditional risk factors (Supplementary Table 4). The competing risk regression models provided similar results to the Cox regression models, which indicated no significant competing risk from non-CVD-related death (Supplementary Table 5).

The Kaplan-Meier event-free survival curves for incident CVD stratified by the quartiles of rLTL are shown in Fig. 1. The median (Q1, Q3) cutoff value of rLTL was 4.640 (3.853, 5.326) in the overall cohort. The survival curve showed a clear separation during follow-up years ($P < 0.001$, log-rank test). Patients from the group with the shortest rLTL had a 2.1-times-higher risk of CVD during follow-up than did those from the group with the longest rLTL (HR 2.144 [95% CI 1.802–2.551], $P < 0.001$). The survival curves for incident CVD in female and male patients are shown in Supplementary Figs. 1 and 2 (both $P < 0.001$).

Patients were categorized according to quartiles of rLTL (<3.853, 3.853–4.639, 4.640–5.326, >5.326), and the HRs of incident CVD increased according to the decreasing quartiles of rLTL. After full adjustment for traditional risk factors, the HRs (95% CIs) for decreasing quartiles of rLTL were 1.00 (longest rLTL as reference), 1.111 (rLTL 0.911–1.354), 1.322 (rLTL 1.094–1.598), and 1.559 (rLTL 1.296–1.875) (*P* for trend <0.001). A similar trend of increasing HRs with decreasing quartiles of rLTL was also observed in female and male groups separately (Supplementary Table 6).

Subgroup analysis for the relationship between rLTL and various composite end points for CVD is presented in Supplementary Table 7. Baseline rLTL was inversely associated with all end points, including MI (HR 1.192 [95% CI 1.091–1.303], $P < 0.001$), CHD (1.256 [1.186–1.331], $P < 0.001$), CVA (1.229 [1.154–1.309], $P < 0.001$), CHF (1.253 [1.174–1.337], $P < 0.001$), and PVD (1.128 [1.006–1.265], $P = 0.039$). After full adjustment of the same covariates for the combined CVD end points, baseline rLTL remained independently associated with CHD, CVA, and CHF. The significance for MI and PVD, however, was attenuated (Supplementary Fig. 3). Additional analyses incorporating

Table 2—Baseline characteristics of subjects by CVD status during follow-up

Baseline variables	Subjects with type 2 diabetes who did not develop CVD (n = 3,401)	Subjects with type 2 diabetes who developed CVD (n = 1,140)	P value	P value adjusted for age and sex
Age (years)	54.5 ± 13.3	60.7 ± 11.8	<0.001	—
Male sex	42.4%	51.0%	<0.001	—
Smoking status				
Current	12.2%	16.6%	<0.001	<0.001
Ever smoked	26.0%	37.0%	<0.001	<0.001
Duration of diabetes (years)	6.0 ± 6.3	8.6 ± 7.1	<0.001	<0.001
BMI (kg/m^2)	25.3 ± 4.2	25.4 ± 3.9	0.461	0.014
SBP (mmHg)	132.0 ± 19.9	139.5 ± 20.3	<0.001	<0.001
DBP (mmHg)	75.3 ± 10.8	77.2 ± 11.3	<0.001	<0.001
HbA_{1c}				
%	7.5 ± 1.8	8.0 ± 1.8	<0.001	<0.001
mmol/mol	58.1 ± 19.3	64.4 ± 20.2	<0.001	<0.001
FPG (mmol/L)	8.4 ± 3.2	9.1 ± 3.6	<0.001	<0.001
TC (mmol/L)	5.1 ± 1.1	5.4 ± 1.2	<0.001	<0.001
HDL-C (mmol/L)	1.3 ± 0.4	1.3 ± 0.4	<0.001	<0.001
LDL-C (mmol/L)	3.0 ± 0.9	3.3 ± 1.0	<0.001	<0.001
TG (mmol/L)	1.3 (0.9–2.0)	1.5 (1.1–2.2)	<0.001*	<0.001*
ACR (mg/mmol)	1.6 (0.7–7.1)	4.6 (1.3–24.7)	<0.001*	<0.001*
eGFR (mL/min/1.73 m^2)	84.6 ± 25.0	74.6 ± 24.1	<0.001	0.002
Diagnosed comorbidity				
Retinopathy	22.6%	35.5%	<0.001	<0.001
Neuropathy	15.6%	26.5%	<0.001	<0.001
Microalbuminuria	23.8%	31.9%	<0.001	<0.001
Macroalbuminuria	12.6%	23.9%	<0.001	<0.001
Use of medications				
Lipid-lowering drugs	15.3%	16.2%	0.449	0.430
Antihypertensive drugs	40.3%	51.6%	<0.001	0.023
Oral antihyperglycemic drugs	66.4%	68.2%	0.250	0.657
Insulin	14.1%	20.0%	<0.001	<0.001
RAS inhibitors (ACE inhibitors or ARBs)	18.3%	24.3%	<0.001	0.058
rLTL (ΔΔCt)	4.7 ± 1.2	4.3 ± 1.2	<0.001	<0.001

All data are expressed as the mean ± SD or median (Q1–Q3), unless otherwise indicated. All comparisons were adjusted for the differences of age and sex by using either a general linear model for continuous data or a logistic regression model for categorical data. *Natural logarithmic transformation was used for TG and ACR.

an interaction term between HbA_{1c} and rLTL was significant, though there was no significant interaction between rLTL and age or sex for incident CVD (data not shown). When further categorized by sex and age, rLTL was still inversely associated with incident CVD in men and women, and in participants aged between 60 and 74 years and those between 45 and 59 years, after fully adjusting for traditional risk factors. In the youngest (<45 years old) and oldest (>74 years old) groups, however, the association became attenuated (Supplementary Table 7).

Receiving operating characteristic analysis was used in order to evaluate the contribution of rLTL as a predictor of CVD. When only rLTL was included in the model, the area under the curve (AUC) was 0.613, suggesting LTL alone is a modest predictor for CVD risk in patients with type 2 diabetes (Supplementary Fig. 5). We compared the AUCs of traditional risk factors both including and not including telomere length (Supplementary Fig. 5). The AUC of traditional risk factors was 0.719 (95% CI 0.702–0.358); it improved to 0.731 (95% CI 0.715–0.748) after including telomere length ($P < 0.001$). Similar results were obtained when we considered risk factors from the UK Prospective Diabetes Study risk engine (23) or the HKDR CHD risk equation (24). When adding rLTL to the model based on the HKDR CHD risk equation, the AUC improved from 0.711 (0.695–0.728) to 0.724 (0.707–0.740).

When the interaction between HbA_{1c} and rLTL was included in the regression model, the interaction term was significant (HR 1.054 [95% CI 1.025–1.084]). More interestingly, when considering the interaction effects, the effect size of rLTL became stronger, increasing from 1.141 (1.084–1.200) to 1.719 (1.373–2.152), whereas that for HbA_{1c} became attenuated (Supplementary Table 8).

Alternative ΔΔCt Calculation

A single repeat sample (QC) was also used in order to calculate a new ΔΔCt to present rLTL. The QC material was from a person in his 40s who was not known to have diabetes and who was younger than most of our patients with diabetes; the rLTL of this control sample was much longer. When using this QC as a reference for calculation, more than half of the ΔΔCt results herein were negative (the mean of the new ΔΔCt

Table 3—Cox regression analysis of the association between baseline telomere length (calculated by using NTC) and incident CVD

	Model 1		Model 2		Model 3		Model 4	
Variables	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
rLTL (each unit decrease)	1.252 (1.195–1.311)	<0.001	1.186 (1.132–1.242)	<0.001	1.164 (1.110–1.221)	<0.001	1.141 (1.084–1.200)	<0.001
Age (years)			1.046 (1.041–1.051)	<0.001	1.036 (1.030–1.041)	<0.001	1.034 (1.027–1.042)	<0.001
Male sex			1.470 (1.309–1.652)	<0.001	1.320 (1.146–1.520)	<0.001	1.330 (1.147–1.542)	<0.001
Duration of diabetes (years)					1.034 (1.025–1.042)	<0.001	1.021 (1.012–1.030)	<0.001
Ever smoked					1.413 (1.221–1.634)	<0.001	1.351 (1.159–1.575)	<0.001
BMI (kg/m^2)					1.023 (1.007–1.038)	0.004	1.020 (1.004–1.036)	0.015
SBP (mmHg)					1.007 (1.004–1.010)	<0.001	1.002 (0.999–1.006)	0.173
HbA_{1c} (%)							1.087 (1.052–1.123)	<0.001
LDL-C (mmol/L)							1.109 (1.040–1.183)	0.002
eGFR (mL/min/1.73 m^2)							0.997 (0.993–1.001)	0.093
Ln (ACR)							1.122 (1.081–1.165)	<0.001

Model 1 received no adjustment. Model 2 was adjusted for age and sex. Model 3 was adjusted for model 2 variables and for duration of diabetes, BMI, SBP, and ever smoked. Model 4 was adjusted for model 3 variables and for $HbA_{1c,}$ LDL-C, eGFR, and Ln (ACR).

was −0.2). Similar to rLTL results with ΔΔCt calculated on the basis of NTC (discussed above), subjects with CVD at baseline had significantly lower values of the new ΔΔCt based on QC materials (−0.5 ± 1.0 vs. −0.2 ± 1.0, $P < 0.001$). Among CVD-free subjects at baseline, subjects with incident CVD had a significantly shorter rLTL (−0.5 ± 1.0) than those without (−0.1 ± 1.0) ($P < 0.001$); after adjusting for age, sex, and other risk factors, the difference was still significant. The Cox regression models showed similar results. Shorter rLTL was associated with an increased risk of incident CVD (HR 1.331 [95% CI 1.264–1.403, $P < 0.001$). In the fully adjusted model, the HR between rLTL and incident CVD (1.166 [95% CI 1.100–1.237], $P < 0.001$) was very similar to that obtained in the main analysis (Supplementary Table 9). The association between estimated absolute telomere length and incident CVD was very similar to that between ΔΔCt (based on QC materials) and CVD risk (Supplementary Table 10). The estimated absolute telomere length results indicated that patients with incident CVD had significantly shorter telomere length (4.4 kb per chromosome [2.8–6.7 kb per chromosome]) than those without (5.9 kb per chromosome [3.7–8.6 kb per chromosome], $P < 0.001$).

CONCLUSIONS

In this large cohort of Chinese patients with type 2 diabetes who had a moderately long follow-up duration, we demonstrated that baseline rLTL was not only associated with CVD risk factors and prevalent CVD at baseline, but also was strongly associated with incident CVD during a mean of 13.4 years' follow-up. Baseline rLTL was an independent predictor for incident CVD events, even after adjusting for age, sex, obesity, and other traditional CVD risk factors.

In this study, rLTL was associated with age, known diabetes duration, blood pressure, glucose control, and lipid levels at baseline. At baseline, shorter rLTL was strongly associated with the presence of CVD after adjusting for traditional CVD risk factors. After a mean follow-up of 13.3 years, shorter rLTL was independently associated with incident CVD events. Indeed, our data showed that, for each unit relative decrease in rLTL, the risk of incident CVD increased by ~25%. rLTL is a well-recognized marker of biological age (25). Biological age, also referred to as physiological age, reflects how old cells appear and behave, and is more clinically relevant and tends to outperform chronological age in predicting healthy aging (26). Linear regression between rLTL and age at baseline indicated that a 1-year increase of age was associated with a −0.010 unit decrease of rLTL in an unadjusted model, and −0.008 in a model adjusted for sex, diabetes duration, and HbA_{1c}. The differences in rLTL between patients with and those without incident CVD were small (0.4 units), but this translates into a significant difference in biological age.

In subgroup analyses, rLTL remained strongly associated with CHD, CVA, and CHF. Associations with MI and PVD, however, were attenuated after full adjustment, which might be due to the low rates of MI (6.1%) and PVD (4.1%) during follow-up. The results of analysis stratified by age and sex showed the same direction, with significance attenuated among patients from the oldest and youngest age groups, although these groups had small sample sizes (Supplementary Fig. 4). Our findings highlight the associations between shorter telomere length and not only baseline CVD status but also CVD end points combined with various end points of incident CVD during a long follow-up period, which were independent of age and sex effects.

Including LTL in the models for CVD added substantial information to the prediction models, despite a relatively modest actual improvement in model performance. This is not uncommon when novel biomarkers are compared against traditional cardiovascular risk factors for predicting cardiovascular risk. rLTL is also associated with age, glucose, lipids, and smoking status, and therefore such factors also contribute to mediating some effects of rLTL on cardiovascular risk. That partly explains the relatively modest increment in AUC when rLTL is added to the models. In the Cox regression analysis using *z* score of rLTL and other risk factors for predicting CVD, the effect size of

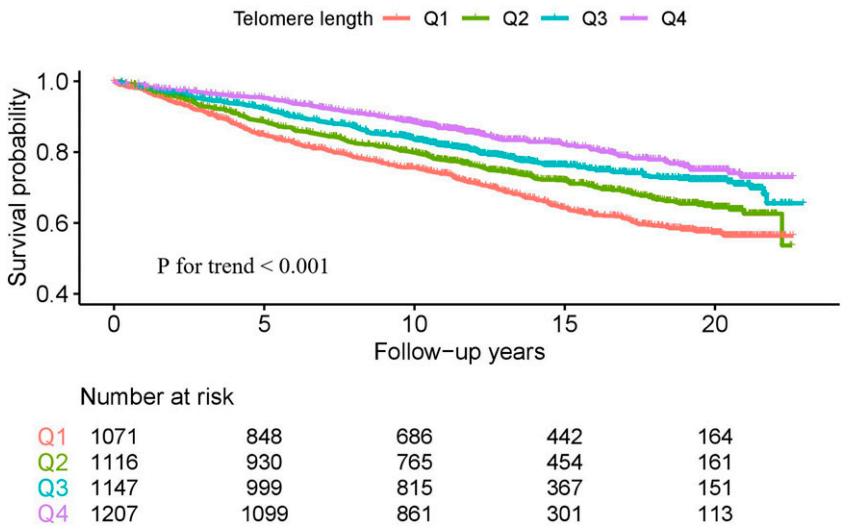

Figure 1—Cumulative survival curve of subjects without CVD, based on quartiles (Q) of rLTL. Subjects were categorized by quartiles of rLTL in the entire cohort: Q1 <3.853, Q2 3.853–4.639, Q3 4.640–5.326, and Q4 >5.326.

rLTL was similar to that of HbA_{1c} per SD change in risk factor.

Other reports have demonstrated an association of rLTL with CVD risk factors—including elevated LDL-C, raised blood pressure, poor glucose control, and carotid intima-media thickness—in patients with diabetes (27,28). Cross-sectional studies of patients with type 2 diabetes showed that patients with previous MI or atherosclerotic plaques had shorter rLTL than did those without either (8,9,12). rLTL was also found to be associated with later risk of CHD in a small cohort of 489 patients with type 2 diabetes, of whom 61 developed CHD (10). In all these previous studies, however, the sample sizes were relatively small, and the findings were suggestive rather than conclusive. In the general population, a relationship between shorter rLTL and CVD has also been reported (6,15,28). In a community-based longitudinal study, 419 participants were followed for 9 years. Among younger participants (defined as <73 years old), shorter telomere length was associated with incidence of MI and stroke, but not PVD (6). These results are consistent with our current findings of the weak association with PVD and among the oldest age group (>74 years old). In a Danish study with 1,397 participants and a 29-year follow-up, after adjusting for age, sex, smoking, BMI, blood pressure, lipids, and other risk factors, shorter rLTL was not associated with future risk of total CVD events and stroke, but it was related to high risk of CHD in the subgroup analysis (15). A meta-analysis of 24 studies including 43,725 individuals reported an association between shortened rLTL and increased risk of CHD, independent of other traditional risk factors (28). Some studies used terminal restriction fragment for rLTL measurement, which requires a large amount of DNA and limits its use in large studies (6). Studies using quantitative PCR reported CVs ranging from 5 to 13% (9,15,29), which may affect the precision of their results.

In a meta-analysis of 17 cohort studies involving 5,575 patients with diabetes and 6,439 controls, the pooled standardized mean difference (−3.41 [95% CI −4.01 to −2.80]) indicated that shortened rLTL was associated with type 2 diabetes (30). Another meta-analysis included three longitudinal studies, with 6,991 participants and 2,011 incident type 2 diabetes events, and the pooled relative risk for type 2 diabetes incidence was 1.31 (95% CI 1.07–1.60) when comparing the shortest with the longest rLTL at baseline (31). These studies support associations between telomere length and type 2 diabetes.

The mechanism underlying the association between telomere shortening and CVD in diabetes remains hypothetical; however, several studies support a role for oxidative stress. For example, animal studies demonstrated that hyperglycemia attenuated endothelial cell nitric oxide production (32), promoted inflammation and oxidative stress (33), and accelerated rLTL shortening and vascular atherosclerotic processes (34). Some clinical studies also confirmed the relationship between rLTL and oxidative stress in patients with diabetes. Salpea et al. (35) reported a positive relationship between rLTL and plasma total antioxidant status in type 2 diabetes. In diabetes, oxidative stress is increased not only in leukocytes but also in pancreatic β-cells, which could result in a shortening of β-cell telomeres and subsequent dysfunction in insulin secretion (36). Oxidative stress may also be increased in adipocytes and muscle cells in patients with diabetes (37). All of these studies suggest that oxidative stress may play a key role in telomere attrition and the development of CVD in diabetes. Chronic inflammation, which is present in subjects with type 2 diabetes, is closely linked to obesity and hyperglycemia, and is implicated in both rLTL shortening and atherogenesis (35).

We observed no statistically significant difference in rLTL between men and women in our study, in which most women would have been postmenopausal. Several studies of Chinese patients with diabetes (38), and of other populations (30), also reported no difference in LTL between males and females. Patients with diabetes have shorter telomeres than those without diabetes (29), which may attenuate the difference in LTL between the sexes. Further, women with diabetes had a longer disease duration (median [interquartile range] 6 years [2–11 years]) than men (4 years [1–11 years]) in our cohort, which, together with other metabolic risk factors, may further induce telomere attrition and attenuate the differences in LTL between males and females. From Supplementary Table 3 we can observe that there was a strong inverse relationship between rLTL and diabetes duration. This relationship remained when the model was adjusted for age and sex. When the relationship was examined separately among male and female patients, however, the inverse association between rLTL and diabetes duration was stronger among female patients than male patients. This association supports our hypothesis that the longer diabetes duration in the women may be a factor contributing to the lack of difference in rLTL between male and

female patients in our cohort. Females with diabetes lose cardioprotection, and this may be reflected by the observed lack of sex differences in LTL.

We found that when we included the interaction term between HbA_{1c} and rLTL, the interaction effects were significant (HR 1.054 [95% CI 1.025–1.084]). More interestingly, when considering the interaction effects, the effect size of rLTL became stronger, increasing from an HR of 1.141 (95% CI 1.084–1.200) to 1.719 (1.373–2.152), which supports our hypothesis that rLTL is independently associated with future risk of CVD in patients with type 2 diabetes. The effect of HbA_{1c}, however, was attenuated after including the interaction term. Patients with poor glycemic control had significantly shorter telomere length (39), and we believe that our result suggests that telomere length could explain some of the effects of glucose on CVD risk. Moreover, whereas HbA_{1c} mainly reflects glucose control over the preceding 3 months, telomere length may in fact reflect the impact of longer-term glucose, lipids, and other metabolic influences.

Study strengths include the analysis of a large number of well-characterized patients with type 2 diabetes and a moderately long follow-up in order to evaluate incident CVD. The cohort size and length of follow-up ensured a large number of CVD end points. The rLTL method we used was a modification of the Cawthon method (20,21). We used 96-well PCR plates instead of 384-well plates, which improved both inter- and intraplate CVs (decreasing from the 5–18% calculated by other groups to 1–3%) (9,15,29); identical study samples assessed in Hong Kong and Sydney, Australia, yielded highly reproducible results. The HBG used as the single-copy gene provided a better negative background. In addition to total CVD events, we explored the relationships between rLTL and individual CVD end points, obtaining consistent findings.

We recognize several limitations. Findings of a study of Hong Kong Chinese patients with type 2 diabetes may not extend to other populations. Nevertheless, the consistent association between shortened rLTL and CVD risk factors in studies from different populations suggests that the biological significance of shortened rLTL is similar between populations. Second, we did not examine rLTL during follow-up to explore the impact of attrition rate of rLTL on the risk of CVD. We found that rLTL was strongly associated with smoking status, but the study lacks a more detailed assessment of smoking, such as pack years. We did not evaluate HbA_{1c} variability in relation to LTL. We also recognize that CVD in type 2 diabetes may be subclinical. Last, we used an rLTL assay, which is suitable for large studies such as this and is economical, but we used a recently available commercial absolute LTL kit to measure LTL in our QC sample, and a mathematical formula to convert rLTL to absolute LTL.

In summary, our results highlight the relationship between rLTL and CVD. In Chinese patients with type 2 diabetes, shorter rLTL was associated with prevalent and incident CVD, and various CVD end points. Further studies of other populations may be needed in order to validate the use of rLTL as a possible biomarker for predicting diabetes-related complications.

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Author Contributions. F.C. measured telomere length, performed statistical analysis, and wrote the manuscript. A.O.L., C.H.T.T., B.F., H.W., A.Y., E.S.H.L., and E.C. analyzed the data and interpreted results. A.C.W.N., C.K.P.L., and H.M.L. contributed to study logistics and prepared samples. M.V.J. and A.A.H. developed the modified rLTL measurement method and trained F.C. A.C.K., M.V.J., A.J.J., and A.A.H. analyzed data. J.C.N.C. is the principal investigator of the HKDR and designed the research, contributed to study logistics, obtained funding, and interpreted results. A.O.L., A.P.K., W.Y.S., J.C.N.C., and R.C.W.M. recruited subjects and contributed to study logistics. R.C.W.M. designed the research, obtained funding to support the study, supervised the research work, performed statistical analysis, and wrote the manuscript. All authors approved the final version. R.C.W.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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