

Research Article

Leukocyte Telomere Length in Relation to Pancreatic Cancer Risk: A Prospective Study

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Abstract

Background: Several studies have examined leukocyte telomere length (LTL) as a possible predictor for cancer at various organ sites. The hypothesis originally motivating many of these studies was that shorter telomeres would be associated with an increase in cancer risk; the results of epidemiologic studies have been inconsistent, however, and suggested positive, negative, or null associations. Two studies have addressed the association of LTL in relation to pancreatic cancer risk and the results are contrasting.

Methods: We measured LTL in a prospective study of 331 pancreatic cancer cases and 331 controls in the context of the European Prospective Investigation into Cancer and Nutrition (EPIC).

Results: We observed that the mean LTL was higher in cases (0.59 ± 0.20) than in controls (0.57 ± 0.17), although this difference was not statistically significant ($P = 0.07$), and a basic logistic regression model showed no association of LTL with pancreas cancer risk. When adjusting for levels of HbA1c and C-peptide, however, there was a weakly positive association between longer LTL and pancreatic cancer risk [OR, 1.13; 95% confidence interval (CI), 1.01–1.27]. Additional analyses by cubic spline regression suggested a possible nonlinear relationship between LTL and pancreatic cancer risk ($P = 0.022$), with a statistically nonsignificant increase in risk at very low LTL, as well as a significant increase at high LTL.

Conclusion: Taken together, the results from our study do not support LTL as a uniform and strong predictor of pancreatic cancer.

Impact: The results of this article can provide insights into telomere dynamics and highlight the complex relationship between LTL and pancreatic cancer risk. *Cancer Epidemiol Biomarkers Prev*; 23(11); 2447–54. ©2014 AACR.

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Introduction

Pancreatic cancer is one of the leading causes of cancer-related deaths in the European Union and in the United States, with a 5-year relative survival of less than 5% (1, 2). Established risk factors for pancreas cancer are cigarette smoking, heavy alcohol consumption, preexisting diabetes mellitus (diagnosed at least 3 years before diagnosis of pancreatic cancer), obesity, chronic pancreatitis, and family history of pancreatic cancer (3, 4). In addition, elevated fasting blood glucose levels (also within the nondiabetic range) and a small number of genetic polymorphic variants have been related to an increased risk (3, 5–11). Overall, however, these known risk factors can account for only a modest proportion of pancreas cancer occurrences, and taken together, they can provide only a very weak prediction of an individual's pancreas cancer risk. The identification of further and stronger risk predictors, for example, in the form of integrative biomarkers of biologic response to various exposures, could help identify higher-risk individuals who would benefit from targeted prevention measures.

Several recent studies have examined leukocyte telomere length (LTL) as a possible predictor for cancer at various organ sites (12), including the pancreas (13, 14). The hypothesis originally motivating many of these studies was that shorter telomeres would be associated with an increase in cancer risk, because LTL has been found to be inversely related to a number of cancer risk factors, including age, smoking, diabetes, hyperinsulinemia, low-grade chronic inflammation, and exposure to environmental air pollution (15–17). The results of epidemiologic studies have been inconsistent, however, and suggested positive, negative, or null associations (12).

Interestingly, considerable evidence on molecular cancer biology indicates that tumor development may be generally related to telomere dysfunction and activation of telomerase, the enzyme that lengthens telomeres (18–20). Genetic studies have shown that the telomerase reverse transcriptase (*TERT*) gene, an enzyme that is fundamental for the accurate *de novo* synthesis of telomeric ends, is one of the few identified risk *loci* for pancreatic cancer (8) as well as other tumor types (21). There is evidence pointing to a strong correlation in telomere length across somatic tissues and suggesting that the variability across cell and tissue types is remarkably lower

than the interindividual variability of LTL (22–24), and natural rates of telomere shortening with age appear to follow a pattern of remarkable synchrony across different somatic tissue types (25). These observations identify LTL as a good proxy for overall telomere length and therefore a possibly useful marker for cancer risk.

So far, only two studies have addressed the association of LTL in relation to pancreatic cancer risk and the results are contrasting (13, 14). To further clarify the association between LTL and pancreatic cancer development, we performed a case–control study of 331 cases and 331 matched control subjects nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.

Materials and Methods

Study population

The EPIC is a large prospective cohort study including more than 500,000 men and women, recruited between 1992 and 2000 in 23 centers in 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom). The study was approved by the local ethical review boards and all participants provided informed consent. Detailed descriptions of study design, population, and methods used were described previously (26). Incident cancer cases were identified by population cancer registries (Denmark, Italy, the Netherlands, Spain, Sweden, and the United Kingdom) or by a combination of health insurance and/or active follow-up with systematic verification of clinical and pathology records for cases identified through insurance data or self-reports (France, Germany, and Greece).

Cases subjects were selected according to ICD-10 (www.who.int/classifications/icd/en/) and included all invasive exocrine pancreatic cancers (ICD-10 codes 25.0–25.3, 25.7–25.9). Exclusion criteria were the occurrence of other malignant tumors preceding the diagnosis of pancreatic cancer, except for nonmelanoma skin cancer. By the end of December 2006, 638 incident cases of pancreatic cancer were identified, of which 578 were primary exocrine pancreatic tumors. For 435 of the 578 case subjects with primary exocrine pancreas cancer, a DNA sample was available, and, for each of these 435 cases, one control participant, alive and free of cancer at time of diagnosis of the index case, was selected using incidence density

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

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sampling. Matching characteristics were study center, sex, age at enrollment (± 6 months), date at entry in the cohort, time between blood sampling, and time of last consumption of food and drink (<3 , $3-6$, and ≥ 6 hours). A total of 104 case-control pairs were discarded from the statistical analysis because of the poor quality of the LTL measurements, according to control criteria for our quantitative real-time PCR assay. Thus, a total of 331 matched case-control pairs were left for statistical analyses. Among these 331 case-control sets, 245 cases of pancreas cancer (74%) were microscopically confirmed, and for the remaining 26% diagnosis had been confirmed by clinical symptoms, imaging results, and/or physical examination.

Sample preparation

Blood samples were collected at baseline recruitment and fractionated into serum, plasma, erythrocytes, and buffy coat from which DNA was extracted. In seven of the EPIC countries (France, Germany, Greece, Italy, the Netherlands, Spain, and the United Kingdom) aliquots were stored in liquid nitrogen (-196°C) in a central biorepository. In the other three countries, aliquots were stored only locally in liquid nitrogen vapor. For this study, DNA was extracted from buffy coat on an Autopure robotic work station (Qiagen) with Puregene chemistry (Qiagen). The DNA of cases and control subjects were extracted using the same techniques, at the same time and by the same personnel.

Telomere length measurement

The experimental procedures were carried out at the Harvard School of Public Health laboratories. The average relative telomere length as represented by the telomere repeat copy number to single gene copy number (T/S) ratio was determined by quantitative real-time PCR using a modification of Cawthon's (27) using an Applied Biosystems 7900HT Thermocycler in a 384-well format. All samples for both the telomere and single-copy gene (*36B4*) reactions were performed in triplicate on different plates and the threshold value for both reactions was set to 0.5. In addition to the samples, each 384-well plate contained a 10-point standard curve from 1.25 to 50 ng using pooled buffy coat-derived genomic DNA. Blinded quality control samples were interspersed throughout the dataset to assess interplate and intraplate variability of threshold cycle (C_t) values. The T/S ratio ($-\Delta C_t$) for each sample was calculated by subtracting the average *36B4* C_t value from the average telomere C_t value. The relative T/S ratio ($-\Delta\Delta C_t$) was determined by subtracting the T/S ratio value of the 5 ng standard curve point from the T/S ratio of each unknown sample. The order of DNAs from cases and controls was randomized on PCR plates to ensure that an equal number of cases and controls could be analyzed simultaneously.

Measurement of other biomarkers

Measures of C-peptide and glycated hemoglobin (HbA1c) on cases and controls of this study have been

performed in a previously published study (28). Briefly, serum or plasma samples from cases and individually matched controls from one center were analyzed within the same analytic batch. HbA1c was measured with the Bio-Rad Variant Haemoglobin Analyzer, while C-peptide was analyzed by a double-antibody radioimmunoassay (Diagnostic Systems Laboratories).

Statistical analysis

Case-control comparisons of the baseline distributions of selected variables that are putatively associated with pancreatic cancer risk, such as type II diabetes (T2D) status, alcohol consumption, and smoking status were made using the *t* test for continuous variables and the χ^2 test for categorical variables. LTL measurements were log-transformed to obtain a variable with an approximately normal distribution, and we compared the geometric mean LTL values between cases and controls and at different levels of baseline variables to identify any possible association between LTL and the variables used in the study. We calculated Pearson correlation coefficients to examine the associations of (log) LTL with blood levels of C-peptide and glycated hemoglobin (HbA1c) that are two pancreatic risk factors.

Conditional logistic regression (matching variables age, gender, and country of origin) was used to calculate ORs and the corresponding 95% confidence intervals (CI) for the associations between LTL and pancreatic cancer risk. LTL was analyzed both as a continuous (log-transformed) variable and as a categorical variable, using quartiles according to its distribution in control subjects. Tests for statistical trend of pancreas cancer risk with increasing LTL were performed with LTL as a continuous variable as well as for quartile categories.

In addition to linear models, we assessed the risk association with restricted cubic spline regression with four knots using the LGTPHCURV9 macro (29).

Smoking status, diabetes alcohol consumption, and, additionally, levels of C-peptide and HbA1c that were suggested as possible pancreatic risk factors by a previous publication (28) were examined as possible confounding factors. Because only the latter two showed a statistically significant association with LTL, they were included as additional, continuous variables in the logistic regression model.

Stratified analyses by follow-up time since the date of blood donation (\leq or >5 years) were conducted to assess the potential for reverse causation biases, and to assess whether the association of LTL with risk changed with increasing duration of prospective follow-up. The 5-year cutoff point for these stratified analyses was motivated by a previously published study on LTL measures and pancreatic cancer risk (13). As an exploratory analysis, we examined the effects of removing all diabetic individuals from our analyses, because T2D is associated with both pancreatic cancer and LTL.

All analyses were performed using SAS software (Version 9.2, SAS Institute Inc.).

Table 1. Baseline characteristics of pancreatic cancer cases and controls

Variable	Cases (n = 331)	Controls (n = 331)	P
Men, women (n)	150, 181	150, 181	Matched
Age at recruitment (y), mean (range)	57 (30–75)	57 (30–75)	Matched
Age at diagnosis (y), mean (range)	63 (37–82)		
Follow-up (y), mean (range)	5.3 (0–13)		
Telomere length, mean + SD	0.59 ± 0.20	0.57 ± 0.17	0.07
Men	0.57 ± 0.18	0.56 ± 0.17	0.48
Women	0.60 ± 0.22	0.57 ± 0.18	0.08
Smoking status, n (%)			0.01
Never	125 (38)	152 (46)	
Former	99 (30)	109 (33)	
Current	103 (31)	66 (20)	
Unknown	4 (1)	4 (1)	
Alcohol intake at recruitment (g/d), median range			
Men	10.56 (8.37–13.31)	8.94 (6.93–11.52)	0.339
Women	4.77 (3.73–6.10)	4.14 (3.28–5.22)	0.401
BMI (kg/m ²), mean ± SD	26.4 ± 4.5	25.7 ± 4.1	
Men	26.5 ± 3.3	26.4 ± 3.6	0.742
Women	26.4 ± 5.2	25.2 ± 4.3	0.019
Diabetes status			
Self-reported diabetes at recruitment (%)	26 (8)	14 (4)	0.143
HbA1c ≥6.5% (48 mmol/mol; %)	40 (12)	21 (6)	0.011
Self-reported diabetes or HbA1c ≥6.5% (%)	44 (14)	25 (8)	0.017

Results

We measured the relative telomere length in 331 pancreatic cancer cases and in 331 matched control subjects recruited in the context of the prospective EPIC study. The distribution of relevant baseline characteristics of cases and controls is shown in Table 1. For none of the selected variables, except smoking, did baseline distributions show significant differences between cases and control subjects. Current smoking, however, was reported more frequently among the cancer cases. Furthermore, a higher proportion of cases than of controls had diabetes mellitus, as indicated by self-report or by elevated HbA1c levels.

Regarding the distribution of LTL measures across different strata of risk covariates (Table 2), age was inversely related to LTL, among the cases as well as among cases and controls combined. Among the cases only, smoking and higher levels of alcohol consumption both also showed suggestive inverse relationships with LTL, although these were not statistically significant ($P \approx 0.07$ – 0.08). There was no association of LTL with gender. HbA1c and C-peptide levels both showed weak but statistically significant inverse associations with LTL [coefficient of correlation = -0.07 ($P = 0.049$) and -0.13 ($P < 0.001$), respectively; Table 3] and therefore were used as adjusting variables in the logistic regression.

We observed that the mean LTL was higher in cases (0.59 ± 0.20) than in controls (0.57 ± 0.17 ; Table 1), although in conditional logistic regression models adjust-

ing for gender, age, and country of origin (matching variables), this difference was not statistically significant ($P = 0.07$). Analyzing LTL as a categorical variable, conditional logistic regression models showed no significant association of pancreatic cancer with LTL (OR, 1.18; 95% CI, 0.71–1.97; for highest versus lowest quartile; Table 4). When adjusting for levels of HbA1c and C-peptide, however, there was a weakly positive association between longer LTL and pancreatic cancer risk [for LTL as a continuous variable, OR, 1.13; 95% CI, 1.01–1.27; $P = 0.028$; for top versus bottom quartile, OR, 1.38; 95% CI, 0.78–2.41; $P = 0.08$; Table 4]. These latter results remained practically unchanged when individuals with T2D were excluded from the statistical analyses (for LTL as continuous variable, OR, 1.15; 95% CI, 1.01–1.30; $P = 0.026$; for top versus bottom quartiles, OR, 1.49; 95% CI, 0.80–2.76; $P = 0.10$). Analysis adjusting for smoking showed results that did not materially differ from those found without this adjustment (Supplementary Table S1), and the same was true for analyses restricting to histologically confirmed pancreatic cancer cases (Supplementary Table S2). We additionally performed a cubic spline regression that suggested a possible nonlinear relationship between LTL and pancreatic cancer risk ($P = 0.022$; Supplementary Fig. S1), with a statistically nonsignificant increase in risk at low LTL levels, and statistically significant increase at high levels of LTL. Considering cases that had either developed pancreatic cancer up to 5 years after blood donation ($N = 171$) or after a time interval of more

Table 2. Comparison of geometric mean LTL values at different levels of baseline variables

Variable	All (n = 662)			Cases (n = 331)			Controls (n = 331)		
	LTL	P ^b	n	LTL	P ^b	n	LTL	P ^b	n
Gender									
Men	0.54	0.160	300	0.54	0.198	150	0.54	0.503	150
Women	0.56		362	0.57		181	0.55		181
Age, y									
20–49	0.59	0.002	123	0.60	0.018	63	0.58	0.206	60
50–54	0.55		108	0.56		53	0.55		55
55–59	0.55		154	0.57		78	0.53		76
60–64	0.56		163	0.56		80	0.55		83
65–69	0.50		75	0.49		37	0.51		38
70+	0.49		39	0.49		20	0.49		19
Alcohol ^a									
<5 g/d	0.56	0.393	312	0.59	0.068	152	0.53	0.411	160
5–14 g/d	0.53		161	0.54		80	0.53		81
15–29 g/d	0.56		104	0.54		56	0.57		48
30+ g/d	0.53		77	0.52		40	0.55		37
Diabetes ^a									
No	0.55	0.290	588	0.56	0.625	289	0.54	0.187	299
Yes	0.52		40	0.54		26	0.49		14
Smoking status ^a									
Life-long nonsmoker	0.56	0.494	277	0.59	0.080	125	0.54	0.389	152
Current smoker	0.55		141	0.54		87	0.56		54

^aTotal may not add up due to missing values.

^bP = P value for trend test.

than 5 years, we observed no statistically significant heterogeneity of the association of LTL with risk (data not shown).

Discussion

Telomeres are highly specialized structures that have a key role in various cellular processes such as control of chromosomal stability, regulation of cell growth (30–32), and the proper segregation of chromosomes to daughter cells (33). Associations of LTL with multiple cancer-related risk factors, plus the correlation of telomere length across different tissue types, including blood cells, in humans and animals suggest that LTL levels may reflect telomere length in tissues where tumors may later develop and serve as a proxy risk marker for cancer.

Cross-sectional analyses of our data did not show any major association between LTL and smoking status or alcohol consumption overall, contrary to previous reports

(34–36). Among the cancer cases, however, alcohol consumption and smoking did appear to be associated with borderline significant reductions in LTL, in line with associations previously reported both among patients with cancer and healthy individuals (34–37). Likewise, our data also confirmed an inverse relationship between LTL and age (Tables 1–3).

In recent years, many studies have addressed the possible association between telomere length measured in the blood and cancer risk, but with inconsistent results. Some studies suggested a significant association between shorter LTL and increased risk while others reported the opposite, that is, no association at all or even an association with the two extremes (very short and very long LTL) with increased risk of several cancer types (reviewed in ref. 12). One key factor that seems to make a difference is whether the studies were retrospective (i.e., the blood sample was taken after diagnosis, in a case–control study)

Table 3. Pearson correlation between HbA1c and C-peptide and LTL

Variable	Cases and controls (N)	Correlation coefficient	P	Cases (N)	Correlation coefficient	P	Controls (N)	Correlation coefficient	P
HbA1c	653	–0.077	0.049	326	–0.080	0.152	327	–0.101	0.067
C-peptide	617	–0.131	0.001	309	–0.116	0.042	308	–0.150	0.008

Table 4. Associations between LTL and pancreatic cancer risk

Relative LTL	Quartile boundaries	Conditional analysis ^a		Conditional analysis adj. by C-peptide and HbA1c ^b	
		OR (95% CI)	P	OR (95% CI)	P
Quartile 1	(0.18–0.45)	Reference	—	Reference	—
Quartile 2	(0.46–0.54)	0.86 (0.54–1.37)	0.32	0.86 (0.51–1.76)	0.28
Quartile 3	(0.55–0.67)	0.94 (0.59–1.51)	0.71	0.92 (0.55–1.56)	0.49
Quartile 4	(0.68–1.55)	1.18 (0.71–1.97)	0.25	1.38 (0.80–2.41)	0.08
Continuous		1.09 (0.99–1.21)	0.07	1.13 (1.01–1.27)	0.028

NOTE: $P_{\text{trend}} = 0.3081$.^aConditional analysis with matching variables (age, gender, country of origin, and fasting status).^bConditional analysis with matching variables (age, gender, and country of origin) and adjustment by C-peptide and HbA1c levels.

or prospective (the blood was taken before diagnosis, in a cohort study), with the retrospective studies generally showing stronger associations than the prospective studies (12). It is worth emphasizing that in several recent publications, which include prospective studies, longer telomeres were found to be associated with an increase in cancer risk (12, 13, 38–45).

For pancreatic cancer, only two studies on the relationship with LTL have been performed so far: one retrospective case-control study, which showed an inverse association between shorter LTL and pancreas cancer risk (14), and one prospective study that showed an association between longer LTL and increased pancreatic cancer risk (13). Although our basic statistical analyses showed a null result, analyses adjusted for blood levels of C-peptide and HbA1c, two metabolic factors that are thought to be associated with pancreas cancer risk (28), suggested a modest positive association between longer LTL and increased pancreatic cancer risk. Using nonlinear methods, we observed a suggestive U-shape risk pattern between LTL and pancreatic cancer as already observed by Skinner and colleagues (14). Although several important risk factors for cancer, such as age and smoking, have been reported to be associated with shorter LTL, findings of a positive association between longer LTL and cancer risk are also plausible, in view of reports that short telomeres may induce cellular senescence, whereas longer telomeres generally mark actively reproducing cells that are at an increased risk of acquiring tumor-causing mutations (46). In agreement with the hypothesis that longer telomere increase cancer risk, a recent report by Robles-Espinoza and colleagues (47) describes a mutation in the protection of telomeres (*POT1*) gene that predisposes to familial melanoma and that is strongly associated with longer telomeres. Taken together, our results and those from Skinner and colleagues suggest a possibly more complex, nonlinear association of pancreas cancer risk with telomeres length, with increased risks both at long and very short telomeres. The association between short LTL and increased risk might represent the lifetime exposure to pancreatic risk factors such as obesity and smoking

that tend to reduce LTL, while the association between longer LTL and increased pancreatic cancer might indicate an increased capacity of cells to divide, and therefore a constitutional increased risk for the disease.

Clear strengths of our study are its prospective design, reducing by a certain extent the possibility of reverse causation that is certainly one of the critical points when looking for the relationship between LTL and cancer risk, as pointed out by various authors (12, 45, 48, 49) and the possibility to avoid possible confounders by using a matched analysis and adjusting for variables that are pancreatic cancer risk factors and/or possible LTL determinants, such as smoking and alcohol consumption, C-peptide and HbA1c levels. A possible limitation is the relatively small sample size, and therefore we cannot exclude the possibility that we could have missed a true but weak association, although the previously reported studies on LTL and pancreas cancer risk were performed on populations of comparable size (13, 14). Moreover, due to DNA availability and quality, we only included 331 of the 578 exocrine pancreatic cancer that were identified in the EPIC cohort till 2006 and, therefore, we cannot completely exclude that the cases selected are not representative of the whole case set. In addition, the strength and the magnitude of the association are moderate, and we also conducted multiple tests. Considering all these limitations, our results must be taken with caution.

In conclusion, although it was originally hypothesized that shorter LTL should be predictive of increased risk for cancer, and despite support for this hypothesis from some epidemiologic studies (reviewed in ref. 12), recent and repeated observations by different epidemiologic studies have indicated longer LTL among individuals developing cancer (12, 13, 38–44). Our present data seem to be in line with this latter hypothesis; however, they do not support LTL as a uniform and strong predictor of pancreas cancer risk.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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