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Telomere length predicts survival independent of genetic influences

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Summary

Telomeres prevent the loss of coding genetic material during chromosomal replication. Previous research suggests that shorter telomere length may be associated with lower survival. Because genetic factors are important for individual differences in both telomere length and mortality, this association could reflect genetic or environmental pleiotropy rather than a direct biological effect of telomeres. We demonstrate through within-pair analyses of Swedish twins that telomere length at advanced age is a biomarker that predicts survival beyond the impact of early familial environment and genetic factors in common with telomere length and mortality. Twins with the shortest telomeres had a three times greater risk of death during the follow-up period than their co-twins with the longest telomere measurements [hazard ratio (RR) = 2.8, 95% confidence interval 1.1–7.3, *P* = 0.03].

Key words: biomarker; human; longevity; replicative lifespan; telomere; twins.

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Introduction

Telomeres are DNA protein complexes of repetitive noncodon hexanucleotide sequences at the end of eukaryotic chromosomes that provide protection from recombination and degradation during cellular division (de Lange, 2002). Telomeric DNA structures are inadequately replicated, and as a consequence, telomere length declines in mitotic tissue with each cell division (Olovnikov, 1973). The enzyme telomerase, which prevents telomere shortening, is highly expressed in germline tissue, whereas expression is low or absent in most somatic tissue (Blackburn *et al*., 1997; Blackburn, 2001). Progressive shortening of telomeres during cellular replication *in vitro* eventually leads to loss of chromosomal stability, changes in gene expression within the subtelomeric region, senescence and apoptosis. Shortening beyond a critical length leads to loss of telomere protection and induces cellular senescence (Blasco, 2005). Aberrant activation of telomerase maintains telomere length above the critical threshold, resulting in excessive cellular proliferation and prolongation of cellular lifespan (Blackburn, 2001).

Telomere length in DNA from human blood lymphocytes is inversely related to age (Jeanclos *et al*., 2000) and is influenced by genetic factors (Slagboom *et al*., 1994; Bischoff *et al*., 2005a,b). Individual differences in telomere length can also be explained by differences in expression of telomerase during embryonic development (Okuda *et al*., 2002), in white blood cell turnover rate, and in accumulated oxidative damage (von Zglinicki, 2002). Reduction in telomere length has been implicated in the pathology of several diseases, although it is not clear whether increased telomere loss is a marker of poor health status or whether the loss has a direct biological effect on the causal network that underlies human health. Premature aging syndromes, such as congenital dyskeratosis, are associated with accelerated telomere shortening (Aviv *et al*., 2005; Hofer *et al*., 2005). In contrast, 80% of human cancers show increased telomerase activity (Kim *et al*., 1994), resulting in retained telomere length in spite of replication of the tumor cells. There have been previous population-based studies of telomere length that both support and contradict the suggestion that telomere length influences survival in human populations (Cawthon *et al*., 2003; Martin-Ruiz *et al*., 2005; Bischoff *et al*., 2006). Whether causal or not, evidence is growing that telomere length may reflect the biological aging rate of the organism.

Because genetic factors are important for individual differences in both telomere length and mortality, this association could reflect genetic pleiotropy rather than a direct biological effect of telomeres on survival. In the present investigation, we examine the relationship between telomere length and all-cause mortality within a population-based cohort of 175 like-sexed

Table 1 Descriptive data of 350 twins within the Swedish Twin Registry by survival status at the end of follow-up

	Total sample (*n* = 350)	Survivors (*n* = 174)	Dead* (*n* = 176)
Age at blood draw (years), mean (SD)	78.8 (7.8)	74.7 (7.1)	82.8 (6.2)
Follow-up time (years), mean (SD)	6.9 (3.1)	9.3 (1.1)	4.5 (2.6)
Sex, *n* (%)			
Men	108 (30.9)	52 (29.9)	56 (31.8)
Women	242 (69.1)	122 (70.1)	120 (68.2)
Zygosity, *n* (%)			
MZ twins	186 (53.1)	101 (58.1)	85 (48.3)
DZ twins	158 (45.1)	73 (42.0)	85 (48.3)
Unknown zygosity	6 (1.7)	0 (0)	6 (3.4)
Smoking status, *n* (%)			
Current smoker	77 (22.0)	34 (19.5)	43 (24.4)
Former smoker	42 (12.0)	20 (11.5)	22 (12.5)
Never smoked	231 (66.0)	120 (69.0)	111 (63.1)

*Mean (SD) age at death is 87.4 (6.2) years overall, 84.0 (6.2) years for men, and 89.0 (5.4) years for women.

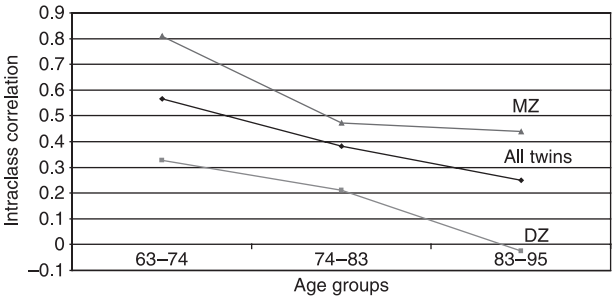

Fig. 1 The within-pair correlation of telomere length decreases with increasing age groups.

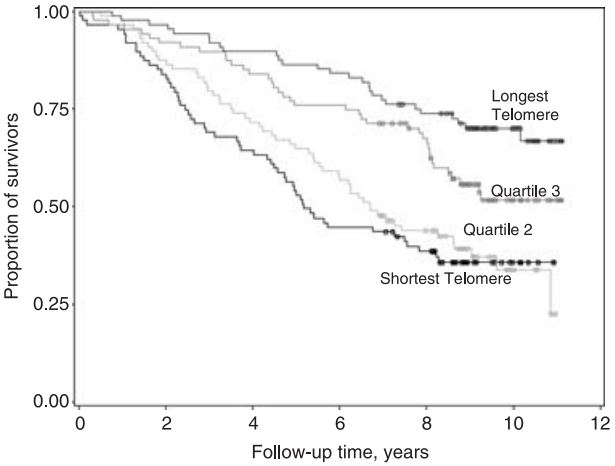

Fig. 2 Kaplan–Meier survival curve for the effect of telomere length on overall survival among Swedish twins. Individuals were divided into quartiles based on their telomere lengths. Longest telomere with mean length, in kilo base pairs, is 7.6 (range: 7.1–9.3), Quartile 3: 6.9 (6.6–7.1), Quartile 2: 6.3 (6.0–6.6) and shortest telomere is 5.6 (4.5–6.0). Mantel–Haenszel log-rank $P = 0.025$. Telomere lengths are corrected for inter-batch measurement variation.

Swedish twin pairs (McClearn *et al.*, 1997; Finkel *et al.*, 1998), using a co-twin analysis. The study design takes advantage of the fact that monozygotic (MZ) and dizygotic (DZ) twins share 100% and 50% of their (segregating) genes, respectively, and that twins reared together share the same environment during their formative years. Thus, co-twin analyses eliminate potential pleiotropic genetic and familial influences on telomere length and mortality as the explanations of associations between telomere length and survival.

Results

Twins in our cohort were born between 1900 and 1928 with ages ranging from 63 to 95 years at blood draw. The mean age at study entry was 79 years (Table 1) and the mean follow-up was 6.9 years. As expected, participants who were older or who were current smokers were more likely to die during follow-up.

Mean (SD) telomere length in the cohort was 6.6 (0.8) kb. Adjusting for batch differences, older individuals had shorter telomere lengths compared to younger individuals, with a 0.83-unit decrease in length for each additional 5 years in age ($P = 0.019$). In the cohort, the women were older than the men at blood draw (80.0 vs. 76.0 years). Accounting for these differences, women had on average shorter telomeres than their male counterparts (–0.21 units, $P = 0.009$). Neither smoking ($P = 0.69$) nor drinking ($P = 0.81$) were associated with telomere length in this cohort.

We observed significant familial similarity for telomere length. Within-pair similarity for standardized telomere length was greater in MZ twin pairs ($r = 0.57$, $P < 0.0001$) than DZ twin pairs ($r = 0.20$, $P = 0.01$), resulting in a heritability estimate of 56% [95% confidence interval (CI) 0.42–0.67]. To look further at the correlation of telomere length within twin pairs as a function of age, we calculated intraclass correlations separately for three equal-sized age groups. There was an inverse relationship between age group and within-pair correlation, with the greatest associations in the youngest age group. This was true in both zygosity groups (Fig. 1).

The Kaplan–Meier survival curve for the effect of telomere length on survival for the overall cohort is depicted in Fig. 2. Among individuals with the longest telomeres, the cumulative mortality was 30.0% during the 11 years of follow-up, compared to 64.2% among those with the shortest telomeres (Fig. 2: log-rank $P = 0.025$). A similar trend in the Kaplan–Meier curve indicating reduced survival for those in the-shortest-telomere-length group was also observed for twins with deaths attributable to cardiovascular disease (CVD) and to cancer.

Accounting for batch differences and comparing the standardized telomere length of the twin that died first, the telomere length was an average 0.3 units shorter ($P = 0.003$) than the twin who died later or survived to the end of the follow-up

Table 2 Hazard ratios per unit decrease in telomere length for mortality, measured as a survival function within twin pairs, by twin zygosity in the Swedish Twin Registry

	Hazard ratio (95% CI)	*P* value
All twin pairs	1.7 (1.2–2.6)	0.004
MZ twin pairs	2.1 (1.1–4.0)	0.03
DZ twin pairs	1.6 (1.0–2.8)	0.03

period. Twins in the shortest 25th percentile of telomere length had the greatest risk of dying (RR = 2.8, 95% CI 1.1–7.3, $P = 0.03$) when compared to their co-twins in the longest 25th percentile of telomere length. The risk estimate did not differ by zygosity, indicating that the association does not reflect pleiotropic genetic influences on telomere length and mortality. Furthermore, for every unit decrease in telomere length, there was a twofold increased risk of death (95% CI 1.1–4.0) among MZ pairs (Table 2), and the association was similar for DZ pairs.

Because there may be potential gender differences in conditions such as myocardial infarction, stroke, and various cancers, we looked at men and women separately in terms of both mortality and telomere length. There was a significant difference in the mean age at death between men (84 years) and women (89 years) ($P < 0.0001$), while at the same time the mean standardized telomere length was 0.27 units longer for women than men ($P = 0.02$). The age-adjusted mortality incidence was 45.5 deaths per 100 women and 61.1 deaths per 100 men for our study period. We stratified and conducted a co-twin (within-pair) analysis on men and women separately to examine if the contribution of telomere length to survival was stronger in either gender. For every unit decrease in telomere length, adjusted for smoking, the RR for death increased by 1.7 (95% CI 0.9–3.4, $P = 0.1$) among men and by 1.7 (95% CI 1.1–2.7, $P = 0.02$) among women.

There is a known gel-to-gel variation associated with the Southern blot method of telomere length analysis (Slagboom *et al*., 1994; Bischoff *et al*., 2005a). We observed the same significant positive relationship between telomere length and survival time for twin pairs measured on the same gel, using both our standardized and unstandardized telomere measurements, as we did for the overall cohort. Men/women and MZ/DZ pairs were not disproportionately measured in the same batch. The age distribution of twins was not equal across the 18 telomere batches. To address this, we performed two separate analyses of the older twins and the younger twins and found that neither cohort was singularly responsible for our results.

Discussion

In the present investigation, we examine the relationship between telomere length and all-cause mortality within a population-based cohort of 175 like-sexed Swedish twin pairs (McClearn *et al*., 1997; Finkel & Pedersen, 2004), using a co-twin analysis in which risk for survival as a function of telomere length was examined within twin pairs. Co-twin analyses minimize potential pleiotropic genetic and familial influences on telomere length and mortality and as explanations of remaining associations between telomere length and survival. We found that shorter telomere length is related to a higher risk of mortality, independent of genetic factors. The pattern of results was also similar for CVD and cancer deaths.

Our matched analysis adds strength to the inverse association between telomere length and mortality in unrelated populations demonstrated by Cawthon *et al*. (2003). In contrast, two later studies failed to demonstrate that telomere length was predictive of mortality (Martin-Ruiz *et al*., 2005; Bischoff *et al*., 2006). The Danish study was composed of both twin pairs and additional members of the population selected for their advanced age (Bischoff *et al*., 2006), and found that when the hazard analyses included age, an association between mortality and telomere length disappeared. Within-pair analyses were restricted to a computation of the percentage of pairs at which the twin with the shortest telomere length died first (55%, CI 48–63%). Although not significant, the Danish within-pair findings are compatible with ours. The mean age of individuals in the Leiden 85-plus study (Martin-Ruiz *et al*., 2005) was 10 years older than the mean age in our cohort and the telomere length variation was measured by a polymerase chain reaction (PCR)-based method instead of by Southern blotting, probably making a direct comparison between populations unfeasible. The authors state that their null findings may have been due to the high degree of telomere instability in populations of advanced age (Martin-Ruiz *et al*., 2005). The findings of both these negative studies were unexpected in light of the abundance of *in vitro* studies (Harley *et al*., 1990) that support the role of telomere length in cellular lifespan. To the best of our knowledge, we present the first prospective within-pair twin study to demonstrate the relationship between telomere length and mortality.

Previous studies, notably those by the authors that did not find telomere length to be associated with survival (Bischoff *et al*., 2005a, 2006), have mentioned the difficulties in controlling for interbatch variation of telomere measurements. It is a strength of the current study that approximately 80% of the twin pairs do not have their telomeres measured in the same batch as their co-twins. This randomization decreases the probability of false-positive effects. Researchers in the Danish study found that restricting their analyses to only those twin pairs whose telomere measurements were from the same batch produced the same null finding as seen in their entire cohort (Bischoff *et al*., 2006). We too restricted our analyses to twin pairs whose telomere measurements were from the same batch and found the same relationship between telomere length and survival as seen in the overall sample. We conclude, as Bischoff *et al*. did, that the gel-to-gel variation was not responsible for the noted effect.

We also demonstrate that the heritability of telomere length is greater in younger adults than in older adults, which is in agreement with previous Dutch (Slagboom *et al*., 1994) and Danish (Bischoff *et al*., 2005a) findings. At older ages variation

in telomere length appears to reflect individual-specific and stochastic aging processes. Moreover, earlier population-based twin studies show that genetic variation influences both the aging process and the risk of adverse health outcomes, both of which affect survival (Iachine *et al.*, 1998; Zdravkovic *et al.*, 2002, 2004). Despite the role of the telomerase gene on variation in telomere length and the importance of genetic influences on mortality, our study clarifies that the association is not exclusively a function of these genetic influences.

The findings of our study have important implications for evaluating potential biological mechanisms underlying the association between telomere length and aging and disease, in that the association appears not to be a proxy for genetic variation for telomere length. Indeed, the within-pair differences in relative risk are thus shown to be under substantial environmental influence, making plausible the search for pertinent 'environmental' mechanisms, some of which may also be 'pleiotropic' such that they contribute to both shortening of telomeres and accelerated aging. A number of biological mechanisms may be involved. First, at the cellular level, a critically short telomere length arrests the cell into senescence (Counter *et al.*, 1992), and thus telomere length may affect aging by impairing cell proliferation and viability. Second, shorter telomeres might mark exhausted proliferative capacity of T and B lymphocytes (immunosenescence) or stem cell depletion. Nevertheless, it is unclear which mitotic tissues accumulate senescent cells throughout the lifespan to such an extent that this would serve as the basis for organismal aging. Short telomeres in one tissue might cause systemic effects or might simply indicate a history of high stress and damage in the individual. For example, there is accumulating evidence that telomere shortening may contribute to the development of atherosclerosis, through aging of the vascular endothelium (Edo & Andres, 2005). Telomere shortening may also be an indicator of an imbalance in the homeostasis of the immune system, leading to greater risk for mortality. The rate of shortening may be a proxy of the pace of biological aging (Blasco, 2005).

Although the pathophysiological mechanism remains unclear, these data provide strong evidence that telomere length is a biomarker of mortality.

Experimental procedures

The present study on telomere length and survival is nested within the Swedish Twin Registry (Lichtenstein *et al.*, 2002), currently the largest population-based twin registry in the world registering more than 85 000 twin pairs born since 1886. The subset of twins for the current analyses participated in studies of aging (McClearn *et al.*, 1997; Finkel & Pedersen, 2004).

Zygosity for these liked-sex twin pairs had been previously determined by asking pairs if they were 'as similar as peas in a pod' or 'no more alike than siblings in general'. Zygosity was confirmed for all pairs by either restriction fragment length polymorphism (RFLP) or serologic testing and microsatellite markers.

Whole blood for telomere analysis was available for 175 complete twin pairs. Telomere length was assessed by terminal restriction fragment (TRF) analysis, which relies on restriction enzyme digestion and Southern blot hybridization of a minimum of 10^5 cells to measure the average length of telomeres. This was one of the first and most widely used techniques and produces reliable results, although it biases the results against the detection of short telomeres. Telomere length for study participants was measured in a series of 18 batches. In order to account for potential batch-specific differences in telomere measurements, telomere lengths from each respective batch were standardized separately to fit a normal distribution and then the standardized telomere lengths from each batch were pooled for the analysis of a continuous telomere length variable. When telomere length was analyzed as a categorical variable, each batch was divided independently into quartiles based on length, and then each quartile was pooled across the batches. Both the standardization and the quartile methods were measures that control for interbatch measurement variation. To verify our method of controlling for between-batch variations, we restricted our analyses with standardized telomere lengths to the 33 twin pairs where co-twins were measured in the same batch.

Death dates were available through the Registry of the Total Population until the end of 2003 and causes of death were available through linkage with the Swedish Cause of Death Registry using each twin's personal registration number (PRN). The Cause of Death Registry, established in 1961, is 99% complete for all those of the Swedish population who died after 1961. Causes of death were updated until the end of 2001. Deaths from specific causes are obtained from death certificates and were coded according to the International Classification of Diseases (ICD) standards. In addition to looking at overall mortality, we evaluated primary causes of death due to cancer (ICD9 140 to 239, ICD10 C00 to D48) and CVD (ICD9 401 to 459 or ICD10 I10 to I99).

Information on age and sex were derived from the Swedish Twin Registry. Information on potential confounders was assessed via self-administered questionnaires. Observation time for each twin was calculated from date of entry into the cohort, as defined by the date of blood draw (1992–1996), until the occurrence of death or censoring (survival) at the end of the observation period (March 31, 2003).

Statistical analysis

The difference in survival time was compared across two groups, the 25% with the longest telomere lengths and the remaining 75% with shorter telomere lengths, batch corrected, using Kaplan–Meier survival estimates (Proc Lifetest; SAS Institute, 2002–2003). Survival differences were measured via the Mantel–Haenszel log-rank statistic for all-cause mortality and separately for deaths due to CVD and cancer. A paired *t*-test was used to compare the standardized baseline telomere measurements between the twin that died first and their respective partner and a *t*-test was used to compare the difference in the

means of standardized baseline telomere lengths and mean ages of men and women. Significance for both tests was measured with a two-tailed $\alpha = 0.05$. Mortality incidences were calculated separately for men and women.

In the analyses at the twin pair level, we looked both at the extent to which variation in telomere length could be attributed to genetic factors (heritability) and at the extent to which within-pair differences in telomere length were predictive of survival. MZ twins by definition share 100% of their genes while DZ twins share on average 50% of their segregating genes, which is similar to siblings. Intraclass correlations, that is, indicators of intrapair similarity, by zygosity were computed using Proc Corr (SAS Institute, 2002–2003). Heritability was estimated using variance-covariance matrices in the Mx program (Neale *et al*., 2003). We estimated hazard ratios, stratifying on twin pair, as an approximation of the relative risk and 95% CI of overall survival by telomere length at baseline using stratified proportional hazard models (Proc Phreg) (SAS Institute, 2002–2003). By stratifying on twin pair in the co-twin approach, we inherently control for confounding by age, sex, as well as genetic factors for the exposure and outcome. We also analyzed MZ twins separately. The extent to which we observed a difference in hazard ratios between the entire population and MZ pairs provides evidence of residual confounding by genetic and familial environmental effects.

Confounding factors

Information on smoking (categories: current smoker, former smoker, and nonsmoker, as well as a continuous-smoking variable) and alcohol use was derived from self-administered questionnaires and evaluated as additional confounders. The smoking data from each source (cigarettes, cigars, or pipes) was converted to cigarette equivalents per week and pooled across each source for the continuous tobacco measurement. We calculated total alcohol-intake by combining usual intake of wine, beer, and liquor. We classified twins as nondrinkers, light drinkers, or heavy drinkers based on total alcohol gram equivalents per month. Those who drank less than 150 g of alcohol per month, equivalent to 15 drinks, were classified as a light drinker. Only the continuous-smoking variable changed the main effect for telomere length on survival by more than 10% and was retained in the final model.

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