# Genetic variation in *TERT* and *TERC* and human leukocyte telomere length and longevity: a cross sectional and longitudinal analysis

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## 1. SUMMARY

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**7. AUTHOR CONTRIBUTIONS**

Mette Soerensen: Generating the study concept and the study design. Acquisition of data. Conduction of data clean up and data analysis. Interpretation of data. Drafting the manuscript.

Mikael Thinggaard: Providing data and data-cleaning. Assisting in establishing and conducting the statistical analysis.

Marianne Nygaard: Contribution to the acquisition of data.

Qihua Tan: Assisting in establishing the statistical methods.

Jacob Hjelmborg: Assisting in establishing the statistical methods.

Karen Andersen-Ranberg: Contribution to the acquisition of data.

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Kaare Christensen: Generating the study concept and the study design. Interpretation of data.

Lene Christiansen: Generating the conception of the study and the study design. Acquisition of data and interpretation of data.

9. SUPPORTING INFORMATION

Supplementary material concerning the 11 *TERT* SNPs investigated with respect to longevity, as well as the 4 *TERT* and 2 *TERC* candidate SNPs investigated with respect to LTL and longevity, is intended for online availability. The material includes a paragraph on the 'selection, genotyping and data analyses of the 11 *TERT* tagging SNPs' as well as Supplementary Table 1–3.

Telomerase is of key importance for telomere maintenance and variants of the genes encoding its major subunits, TERT and TERC, are candidates for inter-individual variation in telomere length. Recently, the two SNPs rs3772190 and rs12696304 in the *TERC* locus were reported to be associated with leukocyte telomere length (LTL) in two genome-wide association studies, while one haplotype of *TERT* (rs2853669, rs2736098, rs33954691, and rs2853691) has been reported to be associated with both LTL and longevity in a candidate gene study.

In this study we investigated the two *TERC* and four *TERT* SNPs in middle-aged, old, and oldest-old Danes (58–100 years) and their association with LTL (n=864) and longevity (n=1069). Furthermore, data on 11 *TERT* tagging SNPs in 1089 oldest-old and 736 middle-aged Danes were investigated with respect to longevity. For all SNPs, the association with longevity was investigated using both a cross-sectional and a longitudinal approach.

Applying an additive model we found association of LTL with the minor *TERC* alleles of rs3772190 (A) and rs12696304 (G), such that a shorter LTL was seen in rs3772190 A carriers (regression coefficient = −0.08, p = 0.011) and in male rs12696304 G carriers (regression coefficient = −0.13, p = 0.014). No *TERT* variations showed association. Moreover, the A allele of rs3772190 (*TERC*) was found to be associated with longevity (HR (AG+AA) = 1.31, p = 0.006). No associations with longevity were observed for the *TERT* SNPs or haplotypes. Our study, thus, indicates that *TERC* is associated with both LTL and longevity in humans.

### Keywords

human longevity; leukocyte telomere length (LTL); telomerase reverse transcriptase (TERT); telomerase RNA component (TERC); association study; cross sectional data and longitudinal data

## 2. INTRODUCTION

Genetic factors explain approximately 25% of the variation in the human life span (Herskind *et al.* (1996)). Such factors exert a minimal effect before age 60 years and the most profound effect after the age of 85 years (Hjelmborg *et al.* (2006)). Candidate longevity genes encode proteins engaged in several biological processes, including the maintenance of genomic stability. These include telomere maintenance genes (Christensen *et al.* (2006)).

The ends of human chromosomes, the telomeres, consist of TTAGGG repeats that undergo shortening with each replication cycle of cells that lack telomerase, the reverse transcriptase which adds telomeres to the ends of chromosomes (reviewed by (Blackburn *et al.* (2006); Campisi and d'Adda de Fagagna F. (2007); Wong and Collins (2003)). Telomere shortening is associated with organismal aging; in humans, leukocytes telomere length (LTL) is inversely related to age (Lindsey *et al.* (1991); Slagboom *et al.* (1994)) and is associated with increased risk of age-related disease and with mortality ((Bakaysa *et al.* (2007); Kimura *et al.* (2008); Njajou *et al.* (2007); Shay and Woodring (2008))). LTL is heritable; its heritability has been estimated to be between 35 and 80 % (Andrew *et al.* (2006); Bischoff *et al.* (2005); Slagboom *et al.* (1994); Vasa-Nicotera *et al.* (2005)). Hence, telomere maintenance genes, particularly those that regulate telomerase activity, might also be longevity genes. The two major telomerase genes are *TERC*, encoding the subunit of the enzyme, which provides the template for the synthesis of the TTAGGG repeats, and *TERT,* encoding its catalytic subunit (Blackburn *et al.* (2006)).

Two recent genome-wide association studies observed association of LTL with two SNPs (rs12696304 and rs3772190) of the *TERC* locus in individuals of European descent (Codd *et al.*(2010); Levy *et al.* (2010)). The association of rs12696304 with LTL was recently confirmed in a Chinese population (Shen *et al.* (2011)). Another study by Atzmon et al.

(Atzmon *et al.* (2010)) reported that Ashkenazi centenarians have a longer LTL and increased frequency of rare *TERT* variants compared with younger controls without a family history of extreme longevity. Moreover, the authors reported that a *TERT* SNP (rs33954691) and three *TERT* haplotypes (rs2853669, rs2736098, rs33954691, and rs2853691) were associated with longevity, while one haplotype was also associated with a longer LTL. Accordingly, we examined the association of LTL and longevity with these *TERC* and *TERT* variants in a large sample of 58–100+ year old Danish individuals.

# 3. RESULTS

The characteristics of the study cohorts are summarized in Table 1 and in the Supplementary material, while lists of all the *TERC* and *TERT* SNPs tested in the study are provided in Table 1 and 3 of the Supplementary material. Genotype distributions of all SNPs were in agreement with Hardy-Weinberg equilibrium (results not shown). For all statistical analyses only genotype and haplotype groups with a frequency above 3% are presented.

## 3.1 LTL association study

First, we analyzed the two *TERC* candidate SNPs for association with LTL using participants from the Unilever Twin Cohort (UT), the Longitudinal Study of Aging Danish Twins (LSADT), the Danish 1905 Birth Cohort Study (1905 cohort) and the Danish Longitudinal Centenarians Study (DLCS) for which LTL data were available (N = 864). The minor alleles for both SNPs were found to be negatively correlated with LTL. Applying an additive model, as was done in the previous studies, the age and gender adjusted regression coefficient for rs3772190 was −0.08 (P = 0.011). Because a test for interaction between rs12696304 and sex indicated a significant effect (p = 0.012), the regression analysis was stratified by sex. When applying an additive model the regression coefficient for rs12696304 was −0.13 (P = 0.014) for males. The two *TERC* SNPs were in high, although not in perfect, linkage disequilibrium (LD) in the study population (r2=0.88). Accordingly the results were correlated, although a slight discrepancy in effect was noticed, most likely as a consequence of the imperfect LD. Next, we investigated the four *TERT* candidate SNPs, however, none of the *TERT* SNPs, nor their haplotypes, showed association with LTL (data not shown). The data is summarized in Table 2. Correction for multiple testing by the Bonferroni Step-down approach left the two estimates borderline significant: p = 0.066 and p = 0.07 for rs3772190 and rs12696304, respectively.

## 3.2 Longevity association study

First, we analyzed the association of the two *TERC* candidate SNPs with longevity using participants from the UT, LSADT, 1905 and DLCS cohorts (N = 1013). Comparison of allele and genotype frequencies between age categories i.e. age < 80 (N = 578) and age ≥ 80 (N = 435), as well as age < 80 (N = 578) compared to octogenarians (N = 177), nonagenarians (N = 131) and centenarians (N = 127) showed a significant increase in minor allele frequency (MAF) of rs3772190 (A) in centenarians compared to the < 80 age group (OR = 1.46, p = 0.016).

In contradiction to this finding, mortality analysis of samples with available longitudinal data (N = 773) indicated a significantly reduced survival in the rs3772190 A allele carriers ≥ 80 years (see Table 3). Compared to the rs3772190 GG group, the AG heterozygotes showed reduced survival (HR = 1.31, p = 0.009), whereas the homozygotes AA showed the same but non-significant tendency (HR = 1.32, p = 0.112). Combining the AG and AA groups strengthened the finding (HR= 1.31 p = 0.006). No significant associations were observed for rs12696304, but the effect estimates were comparable to the effect of

rs3772190 in both size and direction, as would be expected because of the high LD between the two *TERC* SNPs.

Next, we analyzed the association of the 4 *TERT* candidate SNPs with longevity (N = 1069). Allele, genotype and haplotype frequencies were compared between individuals grouped into the same age categories as for the *TERC* SNPs. None of the SNPs or haplotypes showed association with longevity (data not shown). Prospective mortality analysis using samples in which longitudinal data were available (N = 850) did not reveal association of any of the four SNPs with longevity (data not shown). However, individuals heterozygote (AG) for rs33954691 had a decreased mortality risk at an age younger than 85 (HR = 0.50, p = 0.011) compared to the most frequent homozygote (GG) group.

When correcting for multiple testing using the Bonferroni Step-down approach, none of the associations with longevity remained significant (data not shown).

Finally, for an exhaustive evaluation of *TERT* variation and longevity, we also investigated 11 *TERT* tagging SNPs covering the common variation in *TERT* in Caucasians in 1089 members of the 1905 cohort and 736 middle-aged controls (see the Supplementary material, Supplementary Tables 2 and 3). Neither single-marker comparisons and haplotype comparisons ('Sliding window' of 3 consecutive SNPs at a time) nor prospective mortality analysis showed any associations (data not shown). A similar evaluation of *TERC* was not possible since no tagging SNPs were known for Caucasians in the *TERC* encoding region at the time of conducting this study.

## 4. DISCUSSION

In the present study we investigated genetic variations in the *TERT* and *TERC* loci, and the possible association with LTL and longevity. The results point to association of *TERC* variants with both LTL and mortality at advanced ages.

We observed that rs3772190 and rs12696304 of *TERC* were associated with a significantly shorter LTL, in line with the results recently observed by Levy et al. (2010), Codd et al. (2010) and Shen et al. (2011), although in our study the association of rs12696304 with LTL was restricted to males. Correcting for multiple testing by the Bonferroni-Step down approach left the estimates borderline significant (p-corrected: 0.066 for rs3772190 and 0.07 for rs12696304). If instead applying the less conservative Benjamini and Hochberg correction method the effect of rs12696304 remains significant (p=0.042), illustrating the difference in the use of diverse correction methods. We found no significant association of the *TERT* candidate SNPs rs2853669, rs2736098, rs33954691, and rs2853691 or their haplotypes with LTL or longevity, i.e. we did not replicate the findings by Atzmon et al. (2010). Our sample size of 865 was much larger than that of Atzmon et al. (74 centenarians and 49 controls), thus giving our study a higher statistical power. A lack of association of *TERT* variants to LTL is also supported by two recent genome-wide association studies (Codd *et al.* (2010); Levy *et al.* (2010)) and one candidate study (Mirabello *et al.* (2010)). The underlying reasons for the discrepancy in the findings might, however, relate to differences between the populations (Ashkenazi Jews versus Danes) and perhaps different LTL measurement methods. We have employed Southern blot analysis, while the Atzmon et al (2010) used qPCR to measure LTL. We also found that individuals carrying the minor allele A of *TERC* rs3772190 experienced reduced survival during old age, in line with the association of this SNP with shortened LTL, although the finding did not remain significant after Bonferroni step-down correction (p-corrected = 0.108 using a dominant model and p = 0.153 for comparison of GG vs. AG). If instead applying the less conservative Benjamini and Hochberg correction method the p-values were 0.108 for the dominant model and 0.081

for GG vs. AG, respectively. In any case this finding seems relevant, since several reports have provided evidence that LTL is associated with increased risk of age-related disease and mortality in humans (Bakaysa *et al.* (2007); Fitzpatrick *et al.* (2011); Kimura *et al.* (2008)). One puzzling observation was, however, that cross sectional comparison of genotype frequencies in predefined age groups suggested that the minor allele frequency (MAF) of rs3772190 was significantly increased in the centenarians, in an apparent conflict with the mortality analysis using follow-up data. However, this cross sectional estimate was based on the rather small sample size of centenarians (127 out of 1013 study participants), hence it might simply be a chance finding. Moreover, repeating the mortality analysis for rs3772190 with exclusion of the centenarian subgroup did not change the results, while performing the mortality analysis of the centenarians separately eliminated the association. Hence, it appears that the centenarian subgroup did not contribute to the mortality risk estimate of follow-up data.

Finally, despite the very thorough examination of the genetic variation in the *TERT* encoding locus in the present study, we found no evidence for association of *TERT* SNPs or haplotypes with longevity. Applying both a cross sectional and a longitudinal study approach, we investigated the association of mortality to common genetic variation in *TERT* by examining the 4 candidate SNPs investigated by Atzmon et al. (2010) in 1069 individuals in the age range of 58–100+ years, as well as 11 tagging SNPs in 736 middle-aged and 1089 oldest-old individuals. Nonetheless, we cannot rule out that a putative effect of *TERT* variation on longevity might be population specific, i.e. relevant in a population of Ashkenazi Jews, but not in Danes.

In conclusion, we have replicated associations of genetic variation in the *TERC* locus with LTL and, moreover, have found association of variation in the *TERC* locus with longevity. Hence, our study suggests that *TERC* is associated with both LTL and human longevity.

## 5. EXPERIMENTAL PROCEDURES

### 5.1 Subjects

For the investigation of the 2 *TERC* and 4 *TERT* candidate SNPs DNA from participants from the Danish 1905 Birth Cohort Study (1905 cohort), the Danish Longitudinal Centenarians Study (DLCS), the Longitudinal Study of Aging Danish Twins (LSADT), and the Unilever Twin (UT) Cohort Study were used. Briefly, the prospective follow-up studies of the 1905 and DLCS cohorts of oldest old were initiated when the participants were 92 and 100 years of age, respectively (Andersen-Ranberg *et al.* (2001); Nybo *et al.* (2001)). The LSADT study includes all Danish twins age 70+ (Skytthe *et al.* (2002)) and the UT cohort includes 220 female twin pairs, aged 59–81 years (Gunn *et al.* (2009)). Participants were followed with respect to vital status until January 1st 2010 or until death, whichever came first. The information on vital status was retrieved from the Danish Central Population Register (Pedersen *et al.* (2006)). Permissions to collect blood samples and the usage of register based information were granted by the Danish National Committee on Biomedical Research Ethics.

### 5.2 Genotyping

DNA was isolated from whole blood or blood spot cards using the QIAamp DNA Mini and Micro Kits (Qiagen). Genotyping of the four candidate *TERT* SNPs (rs2736098, rs2853669, rs2853691, and rs33954691) and two candidate *TERC* SNPs (rs12696304 and rs37772190) was carried out by allelic discrimination using predesigned TaqMan® SNP genotyping assays (Applied Biosystems). DNA was amplified in a total volume of 5 μl containing 2.5 μl TaqMan Universal Master Mix(Applied Biosystems), 900 nM of each primer, 200 nM of

each probe and approximately 10 ng template DNA. PCR was performed using the StepOne® Real Time PCR instrument (Applied Biosystems) using standard conditions. Due to technical difficulties genotyping of rs2853691 failed for 13.7% of the samples, probably because of poor sample quality. Although these failures were all due to lack of signal, and not because of insufficient discrimination between genotype clusters, this may potentially introduce bias. However, the 86.3% of the samples genotyped was in hardy Weinberg equilibrium, indicating that a differential lack of one of the genotype groups is not the case. Moreover, the mean (age-adjusted and sex-stratified) LTL among the 13.7% non-typed samples was not statistical different from the mean of the 86.3% successfully genotyped samples. The 11 *TERT* tagging SNPs and the genotyping of these are described in the Supplementary material.

### 5.3 Measurement of LTL

LTL measurements were performed by Southern blot analysis of the terminal restriction fragments, which were generated by HinfI and RsaI restriction enzymes, and the mean terminal restriction fragment length, which represents LTL, was calculated as previously described (Kimura *et al.* (2010)).

### 5.4 Statistical analysis

$\chi^2$ test statistics were applied for all cross sectional association studies of genotype, allele and haplotype frequencies using the Plink statistical program (http://pngu.mgh.harvard.edu/purcell/plink (Purcell *et al.* (2007))). Among the subjects genotyped for the *TERT* and *TERC* candidate SNPs were 381 intact twin pairs, which possibly can lead to an increased risk of false positive findings due to the non-independency of twin samples. However, repeating all cross sectional analyses while excluding one arbitrarily chosen twin from each pair completely mirrored the presented findings, thus leading to the same conclusions.

The mortality risk of genotypes in longitudinal data was estimated with STATA 11.1 (Stata Corporation, College Station, TX, USA) using a sex-adjusted, left-truncated Cox proportional hazards model to adjust for late entry into the data set according to age. Because intact twin pairs were included in the study, statistical analyses were performed using the robust estimator of variance assuming independence between the twin pairs. The proportional hazard assumption was evaluated using Schoenfeld residuals and performing an Aalen linear hazard model, and both suggested a change in effect with age for rs2853668, rs33954691, rs3772190 and rs3891064. Hence, an extended Cox model was performed splitting the effect up in age spans which the Aalen model supported.

Sex- and age- adjusted linear regression analysis was performed in STATA 11.1 for inspecting the association of single-SNP genotypes with LTL, using the robust estimator. The assumptions of the linear regression were initially tested, and in case of a sex or age effect, an interaction model was applied, and when indicated gender-specific analysis was performed. Because the individuals investigated belonged to different cohorts an effect of cohort was initially tested in the regression analyses, but since no effect was observed this variable was disregarded.

The Thesias statistical program (Tregouet and Garelle (2007)) was used for investigating associations of all haplotype combinations of the four *TERT* candidate SNPs with LTL. Linear regression analyses were adjusted for sex and age and the most frequent haplotype was used as reference.

Findings were corrected for multiple testing by the Bonferroni Step-down (Holm) correction.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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**Table 1**

Characteristics of the study cohorts investigated for the 2 *TERC* and 4 *TERT* candidate SNPs

| | The Cohorts | | | |
| --- | --- | --- | --- | --- |
| | **UT** | **LSADT** | **1905** | **DLCS** |
| Mean age at intake (years (range)) | 68.2 (58.8–80.9) | 78.8 (73.3–94.2) | 93.2 (92.4–93.7) | 100 (99.1–100.3) |
| Females (%) | 100 | 66.1 | 82.3 | 77.1 |
| N (2 *TERC* candidate SNPs): | 213 | 547 | 125 | 128 |
| MAF of rs12696304 | 0.28 | 0.28 | 0.28 | 0.33 |
| MAF of rs3772190 | 0.26 | 0.26 | 0.25 | 0.33 |
| N (4 *TERT* candidate SNPs) | 213 | 549 | 175 | 132 |
| MAF of rs2853691 | 0.32 | 0.37 | 0.39 | 0.35 |
| MAF of rs33954691 | 0.11 | 0.11 | 0.12 | 0.11 |
| MAF of rs2736098 | 0.32 | 0.24 | 0.25 | 0.25 |
| MAF of rs2853669 | 0.36 | 0.28 | 0.29 | 0.29 |
| N (mTRFL) | 214 | 468 | 119 | 63 |
| Mean age at mTRFL assessment (years) | 68.2 | 79.3 | 99.7 | 102.2 |
| mTRFL ± SD | 6.25±0.042 | 5.71 ±0.023 | 5.20±0.043 | 5.18±0.06 |

Notes: UT: Unilever Twin Cohort Study, LSADT: the Longitudinal Study of Aging Danish Twins, 1905: the Danish 1905 Birth Cohort Study, DLCS: the Danish Longitudinal Centenarian Study, MAF: minor alleleb frequency, mTRFL: mean terminal restriction fragment length (LTL), SD: standard deviation.

**Table 2**

Regression analysis of LTL by *TERC* candidate SNP genotypes

|  | Coefficient | P- value | 95% CI |
|---|---|---|---|
| rs3772190 (N = 864) | −0.08 | **0.011** | −0.15; −0.01 |
| rs12696304 (N = 860) | −0.06 | **0.046** | −0.12; −0.001 |
| rs12696304 males (N = 187) | −0.13 | **0.014** | −0.24; −0.02 |
| rs12696304 females (N = 673) | −0.04 | 0.248 | −0.12; 0.03 |

Notes: 95% CI: 95% confidence interval, P-values below 0.05 are shown in bold. An additive model was applied for the regression analyses.

**Table 3**

Cox regression analysis by *TERC* rs3772190 genotypes

| | Genotype | HR | P- value | 95% CI |
|---|---|---|---|---|
| rs3772190 (age > 80, N = 720) | AG | 1.31 | **0.009** | 1.070; 1.611 |
| | AA | 1.32 | 0.112 | 0.936; 1.862 |
| | AG+AA | 1.31 | **0.006** | 1.081; 1.598 |

Notes: HR: hazard rate, 95% CI: 95% confidence interval.