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Short telomeres are associated with inferior outcome, genomic complexity, and clonal evolution in chronic lymphocytic leukemia

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Abstract

Telomere length in chronic lymphocytic leukemia (CLL) has been shown to be of prognostic importance, but the analyses have largely been executed on heterogeneous patient cohorts outside of clinical trials. In the present study, we performed a comprehensive analysis of telomere length associations in the well characterized CLL8 trial ($n = 620$) of the German CLL study group, with validation in a representative cohort of the CLL4 trial ($n = 293$). Absolute telomere length was

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Compliance with ethical standards

Conflict of interest M.K.W. and G.F.-R.: employment at F. Hoffmann-La Roche; C.W.: consultancy, membership in board of directors or advisory committees, research funding from Hoffmann-La Roche; K.F.: travel grants from Roche; M.K., U.J., M.H., and S.S.: consultancy, honoraria, and research funding from Roche. The remaining authors declare no conflict of interests.

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analyzed using quantitative-PCR. Apart from identifying associations of short telomere length with adverse prognostic factors and survival, the study identified cases with 17p- and 11q- associated with *TP53* and *ATM* loss, respectively, to have the shortest telomeres, even when these aberrations were present in small subclones. Thus, telomere shortening may precede acquisition of the high-risk aberrations, contributing to disease evolution. In line with this, telomere shortening was associated with an increase in genomic complexity as well as clonal evolution, highlighting its importance as a biomarker especially in monitoring disease progression in non-high-risk CLL.

Introduction

Chronic lymphocytic leukemia (CLL) has a variable clinical course ranging from indolent to high-risk disease with short survival despite intensive treatment. Prognostication, therefore, is of key importance, and various factors such as clinical characteristics, immunoglobulin heavy chain gene (IGHV) mutation status, genomic aberrations, and gene mutations have been proposed [1,2]. Also, telomere length in CLL has been associated with biological and clinical disease characteristics as well as outcome [3–9].

Telomeres are nucleoprotein complexes at the ends of each chromosome whose main function is capping of chromosomal ends to prevent them from being recognized as DNA damage. In somatic cells, telomeres shorten with cell division owing to the end-replication problem. Cells with short telomere length undergo senescence and upon abrogation of the senescence checkpoints, the cells can undergo proliferation with further telomere shortening until the crisis stage leading to cell death. In stem cells and germ cells, telomere length is maintained by the enzyme complex telomerase [10–12].

Telomere dysfunction is described to be associated with tumorigenesis across various cancers [13–19] and short telomeres may favor genomic instability, checkpoint deregulation, and activation of telomerase, leading to malignant transformation [12]. Different treatment strategies exploit the crucial role of telomere dysfunction, and targeting telomerase dependence of cancers shows promising impact in clinical trials [20]. In CLL, cases with short telomeres are associated with high telomerase expression and activity, presumably to maintain the critical telomere length for the survival of the cell. Telomerase expression and activity along with telomere length were shown to be of prognostic value in CLL [3, 4, 8]. However in CLL, the impact of drugs targeting telomerase is more difficult to assay, as the cells show limited proliferation in vitro.

The prognostic value of telomere length in CLL has mainly been tested on small and heterogeneous patient cohorts. Here, we performed a comprehensive analysis of telomere length associations with disease characteristics and outcome using the large, well-characterized CLL8 trial of the German CLL study group (GCLLSG) with mature follow-up [21]. The findings were validated using the CLL4 trial of the GCLLSG [22].

The associations of short telomere length with poor prognostic factors in CLL are largely presumed to be a consequence of the underlying genetic characteristics. Here, we verified if telomere shortening, in addition to being a marker of cell proliferation, could have a role in acquisition of high-risk aberrations, favoring genomic complexity and clonal evolution.

Materials and methods

Patient cohort

The CLL8 trial was an international prospective, multi-center, randomized phase III trial for first-line treatment comparing fludarabine and cyclophosphamide (FC) with FC and rituximab (FCR) as previously reported [23]. In total, 817 patients in need for treatment were enrolled. Among these, 620 (76%) cases with available DNA were included in the analysis of telomere length. In terms of validation, the CLL4 trial of the GCLLSG was used, which included 362 patients that were randomized to first-line treatment with fludarabine alone (F) vs. FC [22]. In all, 293 (81%) cases with available DNA were included for telomere-length analysis. The analyzed cohorts were representative of the whole trial populations (Suppl. Table 1). CLL4 and CLL8 were open-label studies without blinding (investigators and study personnel were not blinded to treatment assessment). B cells from age-matched healthy probands ($n = 20$) were isolated from peripheral blood samples by CD19 sorting and used as control. The study was conducted in accordance with the Declaration of Helsinki, approved by our institutional review board, and informed consent was obtained from all patients before inclusion in the study.

Telomere length measurement using Q-PCR

Telomere length measurement was carried out using a quantitative-PCR (Q-PCR)-based technique, favouring measurement of large numbers of samples with relatively low quantities of DNA [24]. The primers used to amplify telomere and single-copy genes (SCG) were tel1b, tel2b, and HBG3, HBG4, respectively [24]. The absolute telomere length was obtained by using synthetic oligonucleotide standards for telomere (84 bp) and SCG (81 bp) PCR. In brief, a serial dilution of the telomere and SCG standard was prepared and the amount of DNA molecules in each standard was calculated as previously described [25]. In total, 12 ng of DNA was used per reaction (total volume of 10 μ l) in triplicates for the telomere and SCG PCRs and amplified using Qiagen quantitect SYBR green (Qiagen, Hilden, Germany) in 384-well plates and analyzed using 7900HT fast real-time PCR system (Applied Biosystems, Darmstadt, Germany). The Q-PCR technique was validated by terminal restriction fragment length (TRF) analysis. Six telomere length controls with known telomere length, analyzed using TRF analysis were included in every plate to detect variation. The TRF value of telomere length for each sample was calculated from the linear regression of Q-PCR versus TRF.

Validation of Q-PCR technique with TRF analysis

The Q-PCR technique was validated by TRF analysis and Southern hybridization. A total of 6 μ g of non-degraded DNA was digested overnight using *HinfI* and *RsaI* (New England Biolabs, Frankfurt, Germany) and resolved on a 0.8% agarose gel. In gel hybridization was carried out by drying the gel and hybridizing with a telomere-specific probe, end labeled with 32 P. The mean telomere length was analyzed from the autoradiograph as previously described [26]. A correlation of $R^2 = 0.8516$ was obtained upon comparison of telomere length measured using Q-PCR and TRF in a control sample set ($n = 18$) (Suppl. Fig. 1a).

Genetic analyses

Genomic aberrations were analyzed using fluorescence in situ hybridization (FISH) [27] and IGHV status [28] by Sanger sequencing, respectively. Mutations in *TP53* were identified using Amplichip (Roche Molecular Systems, Mannheim, Germany) and confirmed by Sanger sequencing (Big Dye Terminator Kit and ABI 3100 sequencer; Applied Biosystems, Darmstadt, Germany). *NOTCH1* mutations were identified using Sanger sequencing and *SF3B1* mutations were identified using dHPLC on a WAVE 3500HT System (Transgenomic Inc., Crewe, UK) and confirmed by Sanger sequencing [29]. Detection of copy number alteration and of loss of heterozygosity was performed by single-nucleotide polymorphism (SNP) array analysis (Affymetrix 6.0., Santa Clara, CA, USA) [30]. Whole-exome sequencing (WES) was performed on HiSeq 2000 (Illumina, San Diego, CA, USA), as previously described [31].

Statistical analyses

Associations between patient characteristics and telomere length were evaluated both as grouped variable (with median as cutoff) and as a continuous variable using chi-square test and non-parametric rank-sum test as appropriate. Correlations of telomere length with genomic complexity, CLL drivers and clone size of aberrations were analyzed using Spearman rank correlation (r_s) or linear regression (R^2), as appropriate. Time-to-event parameters included progression free survival (PFS) and overall survival (OS). Kaplan-Meier estimates and two-sided non-stratified log-rank tests were used for analyzing survival times. Hazard ratios (HR) and confidence intervals (CI) were calculated using Cox proportional-hazard regression analyses. Multivariable analyses were performed using Cox regression with stepwise selection including only those variables that were significantly associated with PFS or OS in univariate analyses. All statistical tests were two-sided and a P value of <0.05 was considered as significant. No adjustments for multiple testing were done. The analyses were performed using SPSS 23.0 (SPSS, Chicago, IL) and Graphpad prism (versions 5.0 and 8.0.2).

Results

Telomere length variation in CLL is largely restricted to tumor cells

To investigate whether telomere shortening in CLL is restricted to the tumor cell fraction, 48 cases from CLL8 with paired tumor (CD19 +), non-tumor (CD19-), and unsorted samples were analyzed. Also, CD19 + samples were included from 20 age-matched healthy donors (median: 56 years), out of which eight cases had a paired CD19- sample. The median telomere length of CLL cells (CD19 +) was significantly shorter compared with the non B cells (CD19-) of CLL patients (median: 3.37 kb vs. 5.42 kb; $P=0.0004$), whereas, in healthy donors, non-malignant B cells had significantly longer telomeres than their CD19- counterparts in each case (7.54 kb vs. 5.40 kb, respectively; $P<0.001$) (Fig. 1a). Telomere length of tumor B cells significantly differed from that of normal B cells (median: 3.37 kb vs. 7.54kb, $P<0.0001$), whereas the CD19- cells from CLL patients and healthy donors had similar telomere length distribution (5.42 kb vs. 5.40 kb). Further, the influence of the disease features on telomere length was apparent in the CD19 + tumor cell fraction where a correlation with patient's age was absent ($R^2=0.002$, $P=0.919$) (Suppl. Fig. 1b). In the

non-tumor fraction, an association of telomere length with age was retained ($R^2 = 0.191$, $P = 0.002$) (Suppl. Fig. 1c).

CD19 enriched samples were available only for a subset of cases hence in the analysis of the full CLL8 and CLL4 cohorts, DNA from either the CD19 sorted or the unsorted cells were used for telomere length measurement. Of note, telomere length was similar between the CD19 + sorted and unsorted samples from same patients (median: 3.38 kb vs. 3.48 kb; $P = 0.197$) (Fig. 1a). Absence of adverse influence of non-tumor fraction on telomere length associations in samples with low WBC count ($< 50 \times 10^9$ cells/L) without CD19 enrichment ($N = 128$) was verified by analyzing correlation of telomere length with WBC count as well as survival (Suppl. Fig. 2).

Telomere length in CLL8 and its association with clinical characteristics

The telomere length of the CLL8 samples ($n = 620$) was found to be highly variable, ranging from 1.94 kb to 33.56 kb (median 4.56 kb). Analysis of the frequency distribution of the telomere lengths revealed a skewing toward shorter telomeres, with 46.9% of the cases represented in the telomere length range of > 3 kb and < 5 kb. In total, 11.7% of the cases analyzed had very short telomeres of < 3 kb. A subset of CLL cases (1.6%) displayed extremely long telomeres of > 15 kb (15.57–33.56 kb) (Suppl. Fig. 3a) and the telomere lengths obtained by q-PCR were validated using TRF for cases with available DNA with a strong correlation ($R^2 = 0.981$, $P < 0.001$) (Suppl. Fig. 3b, c). Although an overestimation of telomere length by q-PCR was observed in increasing telomere lengths, these cases had significantly longer telomeres compared with that of normal B cells as measured by TRF ($P < 0.001$).

Associations of telomere length and clinical characteristics in the CLL8 cohort were analyzed by comparing groups defined by short and long telomeres (cutoff at the median of 4.56 kb). Telomere length showed no significant association with age ($P = 0.629$), sex ($P = 0.168$), presence of B-symptoms ($P = 0.297$), Eastern Cooperative Oncology Group (ECOG) status ($P = 0.224$), and high serum beta-2 microglobulin ($s\text{-}\beta_2\text{MG} > 3.5$; $P = 0.653$). In contrast, short telomeres were significantly associated with other adverse risk characteristics such as high WBC (> 50 Giga/L; $P < 0.001$), high serum thymidine kinase ($s\text{-TK} > 10.0$ U/L; $P < 0.001$) and Binet stage B ($P = 0.002$) (Table 1), whereas longer telomeres were associated with Binet C stage ($P = 0.007$).

Association of telomere length with genetic characteristics

Among the genetic characteristics, unmutated IGHV, mutated *TP53*, and mutated *SF3B1* were significantly associated ($P < 0.001$) with short telomeres, whereas *NOTCH1* ($P = 0.696$) mutations were not (Suppl. Table 2). The hierarchically categorized genomic aberration subgroups 17p- and 11q- had significantly shorter telomeres (both $P < 0.001$), whereas cases with 13q- as the sole abnormality ($P < 0.001$) and no abnormality ($P = 0.020$) had longer telomeres. The presence of + 12q ($P = 0.071$) showed no association with telomere length (Suppl. Table 2). Figure 1b, c show the distribution of telomere length (as continuous variable) in the different gene mutation and genomic aberration subgroups. ZAP70 + ($> 20\%$) cases were found to be significantly associated with short telomeres ($P =$

0.010). All cases with very long telomeres belonged to the favorable prognostic subgroup, mutated IGHV, predominantly with 13q- as sole abnormality (70%).

Impact of telomere length on clinical response, MRD, and survival

Though significantly associated with adverse clinical and genetic characteristics, telomere length in CLL8 had no association with clinical response (CR: 37.7% vs. 41.2% and PR: 53.2% vs. 52.4%; $P = 0.400$) (Suppl. Table 3). Also, telomere length did not impact MRD response in peripheral blood ($P = 0.751$) and bone marrow ($P = 0.213$) (Suppl. Table 4). At a median observation time of 69.7 months, there were 422 (FC 228 vs. FCR 194) events for PFS and 208 (FC 117 vs. FCR 91) for OS. The short telomere subgroups (median) had significantly shorter PFS in both the FC and FCR treatment arms (FC: 27.1 vs. 44.0 months, HR 1.934, 95% CI 1.486–2.517, $P < 0.001$; FCR: 44.8 vs. 69.9 months, HR 1.863, 95% CI 1.395–2.489, $P < 0.001$) (Fig. 2a, b). Similarly, short telomeres were significantly associated with shorter OS in both FC and FCR treatments (FC: 70.9 vs. 89.5 months, HR 2.166, 95% CI 1.489–3.151, $P < 0.001$; FCR: 90.2 months vs. median not reached, HR 1.680, 95% CI 1.098–2.570, $P = 0.017$) (Fig. 2c, d).

Multivariable analysis for PFS was performed including variables that were significant in univariate analyses (treatment arm, gender, genomic aberrations by FISH, mutational status of IGHV, *TP53*, and *SF3B1*, s-TK, s- β_2 MG and telomere length categorized based on median). Although FCR treatment ($P < 0.001$), 17p- ($P < 0.001$), 11q- ($P < 0.001$), unmutated IGHV ($P < 0.001$), mutated *TP53* ($P = 0.006$), *SF3B1* mutation ($P = 0.018$), and s-TK > 10 ($P = 0.016$) were significant, telomere length was not identified as an independent prognostic factor for PFS (Suppl. Table 5). For OS, treatment arm, age, ECOG status, CIRS score, genomic aberrations by FISH, mutational status of IGHV and *TP53*, s-TK, s- β_2 MG, and telomere length categorized based on median were identified as potential candidates in univariate analyses and therefore included as variables for multivariable analysis. Whereas telomere length was not significant, FCR ($P = 0.012$), ECOG ≥ 1 ($P = 0.023$), CIRS score ($P = 0.001$), 17p- ($P = 0.001$), unmutated IGHV ($P = 0.001$), *TP53* mutation ($P < 0.001$), s-TK > 10 ($P = 0.020$), and s- β_2 MG ($P = 0.014$) were identified as independent prognostic factors (Suppl. Table 6).

Analysis of associations with telomere length as a continuous variable in CLL8

Associations with different clinical and genetic characteristics were additionally studied considering telomere length as a continuous variable. The results obtained confirmed the observations from categorical (median) analysis (Suppl. Table 7, Fig. 1b, c). Telomere length associations with genomic aberrations within the IGHV subgroups were similar to that of the whole cohort, with 17p- and 11q- associated with the shortest telomere length (Suppl. Fig. 4). When analyzed as a continuous variable, telomere length showed a significant association with PFS (FC: HR 0.921, 95% CI 0.864–0.980 $P = 0.010$; FCR: HR 0.838, 95% CI 0.778–0.902, $P < 0.001$) and OS (FC: HR 0.806, 95% CI 0.723–0.898, $P < 0.001$; FCR: HR 0.801, 95% CI 0.709–0.905, $P < 0.001$) in both FC and FCR treatment groups (Suppl. Table 8). Of note, telomere length included as continuous variable was an independent prognostic factor for OS (HR 0.890, 95% CI 0.805–0.983, $P = 0.022$) along with FCR ($P = 0.009$), ECOG ≥ 1 ($P = 0.020$), CIRS score ($P = 0.001$), 17p- ($P = 0.001$), unmutated IGHV

($P=0.043$), *TP53* mutation ($P<0.001$), s-TK > 10 ($P=0.032$), and s- β_2 MG ($P=0.008$) (Table 2).

Telomere length associations in the validation cohort CLL4

The telomere length associations identified in the CLL8 were validated using the CLL4 trial cohort. Telomere length in CLL4 was found to be highly variable similar to CLL8, varying from 2.48 kb to 32.41 kb. CLL4 had a higher representation of cases with low and intermediate risk genomic aberration (+12q, normal karyotype and 13q-) compared with CLL8 (73.0% vs. 68.4%, Suppl. Table 1). The longer median telomere length of CLL4 (5.98 kb) may also be owing to differences in DNA extraction method (Qiagen vs Trizol) and DNA quality. Hence, a unifying telomere length cut-point was not used between the two trials for dichotomization and analyses. Among the clinical and genetic characteristics, association of short telomeres with high WBC count ($P<0.001$), high s-TK (<0.001) (Table 1), unmutated IGHV ($P<0.001$), *SF3B1* mutations ($P=0.022$), and presence of 11q- ($P<0.001$) (Suppl. Table 2) were confirmed in CLL4. Suppl. Fig. 5a, b represent the distribution of telomere length (as continuous variable) in the different gene mutation and genomic aberration subgroups. A full comparison of telomere length associations in CLL4 as grouped and continuous variable is shown in Table 1, Suppl. Table 2 and 7. As in CLL8, all cases with very long telomeres belonged to the favorable prognostic subgroup harboring mutated IGHV and predominantly 13q- as sole abnormality (72%).

Similar to CLL8, short telomeres in CLL4 were significantly associated with shorter PFS in both the treatment arms (F: 23.4 vs. 26.4 months, HR 1.459, 95% CI 1.032–2.062, $P=0.033$; FC: 41.0 vs. 51.0 months, HR 1.476, 95% CI 1.012–2.154, $P=0.043$; Suppl. Fig. 6a, b). Short telomeres in CLL4 were significantly associated with shorter OS in F but the difference was not statistically significant in FC treatment (F: 77.1 vs. 122.6 months, HR 1.906, 95% CI 1.218–2.983, $P=0.005$; FC: 87.6 vs. 110.1 months, HR 1.591, 95% CI 0.998–2.536, $P=0.051$; Suppl. Fig. 6c, d). When analyzed as a continuous variable, telomere length showed a significant association with PFS in FC (HR 0.898, 95% CI 0.834–0.968, $P=0.005$) and OS in both F (HR 0.819, 95% CI 0.731–0.917, $P=0.001$) and FC (HR 0.870, 95% CI 0.786–0.964, $P=0.007$) treatment groups (Suppl. Table 8). Multivariable analysis (Suppl. Table 9) including variables that are significant for PFS in univariate analysis (treatment arm, genomic aberrations, mutational status of IGHV and *TP53*, s-TK and telomere length) showed the FC treatment arm ($P=0.033$), 17p- ($P<0.001$), 11q- ($P<0.001$), *TP53* mutation ($P=0.004$), and high s-TK ($P=0.003$) to be independent prognostic factors for PFS. For OS, when telomere length, age, sex, genomic aberrations by FISH, mutations of IGHV and *TP53*, s-TK and s- β_2 MG were included as variables, 17p- ($P<0.001$), 11q- ($P=0.003$), *TP53* mutation ($P<0.001$), high s-TK ($P=0.002$), unmutated IGHV ($P<0.001$), male sex ($P=0.012$), and age >60 ($P=0.041$) were significant in multivariable analysis. Telomere length analyzed either as grouped or continuous variable was not an independent prognostic factor for PFS and OS in CLL4.

Correlation of telomere length with genomic complexity and CLL driver mutations

As telomeres play a key role in maintaining genomic stability, the association of telomere length with genomic complexity was analyzed. The number of copy number alterations

(CNA) and loss of heterozygosity (LOH) were identified as a measure of genomic complexity in 353 cases analyzed using SNP array [30]. The sum of CNA and LOH varied from 0 (no detectable alteration) to 9 alterations per case. Genomic complexity was associated with progressive telomere shortening ($P < 0.001$; $r_s = -0.411$). Also, an increase in genomic complexity was associated with increase in percentage of cases with high-risk genomic aberrations such as 17p-, 11q-, and *TP53* mutations ($P = 0.002$; $R^2 = 0.723$, Fig. 3a). Similar associations were also identified within the IGHV subgroups (Suppl. Fig. 7a, b). In addition, associations of telomere length and driver mutations were analyzed from WES of 279 patient samples [31]. Similar to the association with genomic complexity, telomere shortening was significantly associated with increase in the number of clonal and subclonal driver events, including ($P < 0.001$; $r_s = -0.454$, Fig. 3b) and excluding ($P < 0.001$; $r_s = -0.365$, Fig. 3c) genomic aberrations. Within the IGHV subgroups, the unmutated IGHV cases showed no association between number of driver mutations and telomere length (Suppl. Fig. 7c, d)

Association of short telomeres with acquisition of poor-risk genetic aberrations and clonal evolution

Among the different genomic aberration subgroups, cases with 17p- and 11q-, associated with *TP53* and *ATM* loss, respectively, had the shortest telomere length (median: 3.4 kb). Therefore, to verify if this presence of short telomeres is a mere consequence of higher proliferation rate of these poor-risk tumors, we compared telomere length with the clone size of these aberrations. Of note, telomere length did not correlate with the clone size of 17p- ($R^2 < 0.001$) and 11q- ($R^2 = 0.027$) as analyzed by FISH. Similarly, telomere length did not correlate with the variant allele frequency of *SF3B1* ($R^2 = 0.004$) and *TP53* mutations (excluding 17p-, $R^2 = 0.072$) analyzed using WES (Fig. 4a–e). Thus, even cases with very low variant allele frequency of these sub-clonal drivers had very short telomere lengths.

To study if telomere shortening may precede and therefore contribute to clonal evolution in CLL, paired baseline and follow-up samples from 65 cases were analyzed for telomere length dynamics. The median follow-up time for included cases was 44 months (range: 5–83 months). Clonal evolution was present in 24 cases (36.9%) as detected by SNP array analysis. Although there was no significant difference in baseline telomere lengths of cases with and without clonal evolution ($P = 0.770$), telomere length at follow-up was significantly shortened in cases with clonal evolution ($P = 0.003$), whereas this was not the case in the group without clonal evolution ($P = 0.204$; Fig. 4f). The most frequent recurrent genetic alteration acquired upon clonal evolution was 17p- (33.3%) and 41.7% of cases had an acquired 11q- or a 17p- at follow-up. Also, among cases which underwent clonal evolution, 54% had a 17p- or 11q- at baseline. In addition, WES analysis of samples at baseline and follow-up identified nine cases that acquired *TP53* mutation. Five of nine cases had a pre-existing 17p- or 11q- aberration that is known to be associated with short telomeres. Three of four non-high-risk cases with 13q-, 12q+, and normal karyotype had short telomeres (3.28, 3.57, and 4.25 kb, respectively) even before the acquisition of *TP53* mutation. The results indicate that in a subset of cases, critical telomere shortening may precede acquisition of high-risk genomic abnormalities.

Discussion

The relationship between telomere length and cancer biology is highly complex: telomere length of tumors may reflect the telomere of the initially transformed cell, as well as the history of cell divisions since transformation through telomere attrition. Furthermore, telomere shortening may induce cellular adaptation to overcome proliferation checkpoints. Telomere shortening may accelerate cancer evolution by facilitating acquisition of genomic copy number variation, yielding novel cancer genotypes favoring increased proliferation [11, 12]. Disentangling these complex relationships may be particularly challenging in the context of human cancer, as largely correlative data are available for analysis. Nevertheless, the large size of the studied CLL8 ($n = 620$) and CLL4 ($n = 293$) cohorts enables examining several such hypotheses directly in patient data.

First, we considered that telomere length of the cancer cell may correspond to that of the initiating transformed cell. This is of particular importance in CLL, as it tends to affect older individuals [1], and telomere shortening is known to be a part of normal aging [32]. Our data do not support a significant age-related contribution, to the telomere length of the tumor (Table 1). Furthermore, our analysis of age-matched normal B cells, and the CD19⁻ subsets show that telomere shortening in CLL does not stem from a defect in the hematopoietic progenitors, but rather seems restricted to the CLL cell fraction (Fig. 1a). Thus, the evolutionary age of the malignant population, reflecting hundreds of divisions after transformation, seems to be more important than pre-transformation aging related shortening. Roth et al. reported significant telomere shortening in T-cells from CLL however the difference in absolute telomere lengths in the T cells from CLL vs. healthy was much smaller in the study. Such a difference could be masked by the limited number of CD19⁻ samples from healthy donors included in the present study. The presence of an association of CD19⁻ telomere length with patient's age indicate that the non-tumor telomere lengths are only minimally affected by the disease [33].

As each cell division results in further telomere attrition, the length of the telomeres may reflect the number of divisions in the cell's past. Indeed, serially sampled cases (median time interval 44 months) demonstrated ongoing telomere shortening ($P < 0.001$, Fig. 4f) over time. Consistent with this hypothesis, we observed that large tumor burden in the form of higher circulating cell numbers or a more advanced stage is associated with shorter telomeres (Table 1), especially within the more aggressive IGHV unmutated subtype (data not shown). Somatic driver variations associated with adverse outcome (e.g., *SF3B1* mutations) were also linked with shortening, providing further support to the fact that more aggressive disease progression leads to greater telomere attrition (Fig. 1b, c, Suppl. Fig. 5a, b).

On the other hand, when considering the impact of the transformed cell of origin, we found a strong association between longer telomeres and mutated IGHV (Fig. 1b, Suppl. Fig. 5a). This association is well documented [4–9], and can be attributed to the fact that CLL with mutated IGHV are considered to develop from CD5(+), CD27(+), post-germinal center (GC) B-cell subsets [34], which activate telomerase during the GC reaction [35]. Short telomeres in unmutated IGHV may therefore represent an early event in pathogenesis, favoring

selection of high-risk clones in these cases [36], to overcome telomere dysfunction induced genomic instability.

Telomere shortening may also contribute to further diversification through a higher rate of copy number variation, allowing the tumors to more efficiently explore the evolutionary fitness landscape. In support of this notion, we found that shorter telomeres were closely associated with greater number of copy number variations and greater clonal diversity (Fig. 3a–c). In addition, telomere shortening may exert a strong selection pressure for variants such as ATM or TP53 deficiency that will be permissive to ongoing growth despite critical shortening [37]. We have found a close association between these aberrations and shortened telomeres (Fig. 3a). Importantly, this was confirmed even when these mutations were found in small subclones (with relatively small impact on the mean telomere length of the tumor bulk) (Fig. 4a–e), supporting the hypothesis that telomere shortening preceded the appearance of TP53 and ATM defects, and serves as a selection force to favor the emergence of clones bearing these variants. Further studies with multiple follow-up samples from single cases to track emergence of high-risk genomic aberration along with analysis of telomere length dynamics over a longer period of time may be important to confirm the temporal relationship between telomere length and high-risk aberrations.

Collectively, these data sets suggest that telomere erosion in CLL reflects a more-aggressive CLL phenotype, resulting from greater proliferative history, as well as the propensity to increase clonal diversification and specifically select genotypes that will enable further growth despite telomere attrition. Given these factors, it is not surprising that short telomeres in CLL are associated with adverse outcome in both cohorts (Fig. 2, Suppl. Fig. 6), as it may constitute a summation of multiple risk factors such as IGHV unmutated status, advanced stage, chromosomal instability, high-risk genetic alterations, and greater clonal heterogeneity. As telomere shortening seems to be an early event, preceding acquisition of high-risk aberrations, profiling telomere length at diagnosis and through the disease course in non-high-risk patients could provide valuable information on disease progression and evolution, thus contributing to the decision of therapy. With the advent of novel non-genotoxic agents such as inhibitors of BTK, PI3K, and BCL2, initial response to therapy is similar across the favorable and unfavorable genetic subgroups [38]. However, identification of complex karyotype as an independent prognostic factor for PFS and OS in CLL treated with ibrutinib [39] may indicate a possible contribution of underlying telomere dysfunction, thus further studies on telomere length associations in the context of novel, non-genotoxic treatments are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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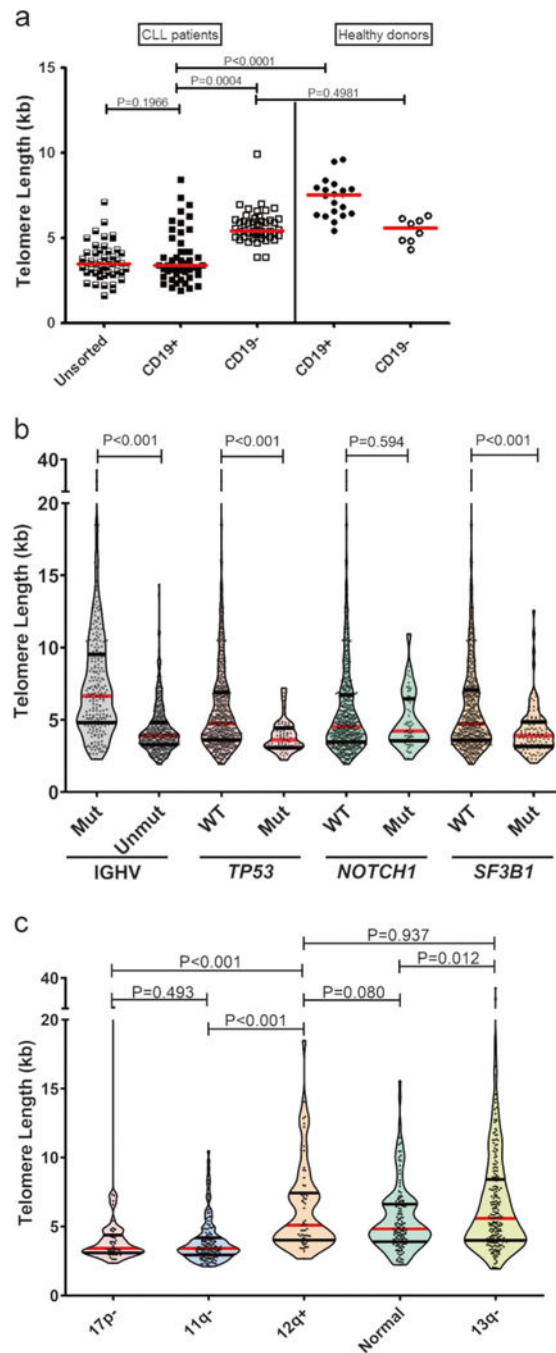


Fig. 1. **a** Comparison of telomere length between CD19 +, CD19–, and unsorted CLL samples ($n = 48$) and with age-matched healthy donors $n = 20$ (CD19 +) and $n = 8$ (CD19–). **b** telomere length in cases with mutated and unmutated IGHV; mutated and wildtype *TP53*, *NOTCH1*, *SF3B1*. **c** Telomere length in the hierarchically classified genomic aberration subgroups namely, 17p–, 11q–, + 12q, normal karyotype and 13q– in CLL8 (red bars indicate the median telomere length). All P values from Mann–Whitney test

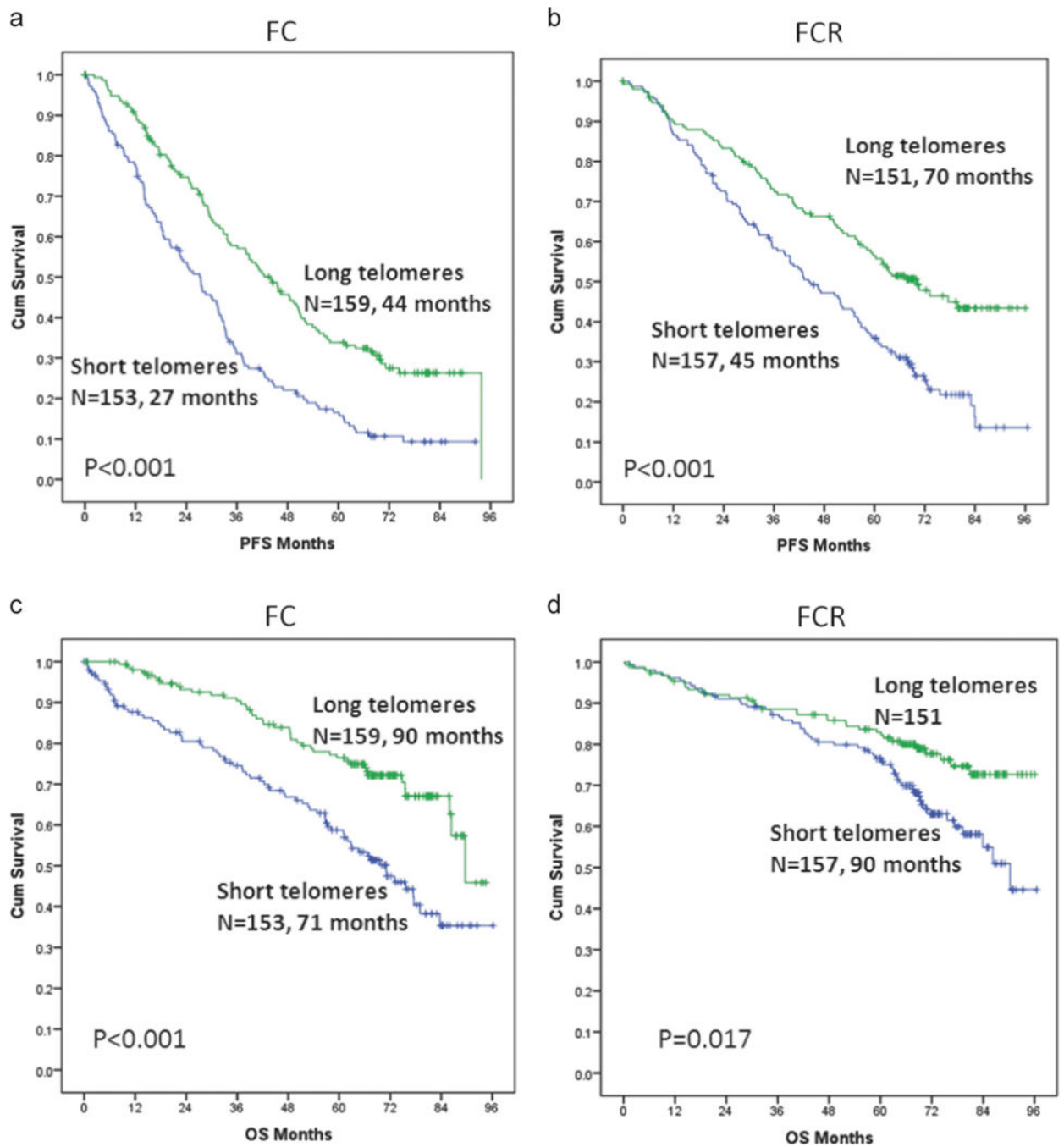
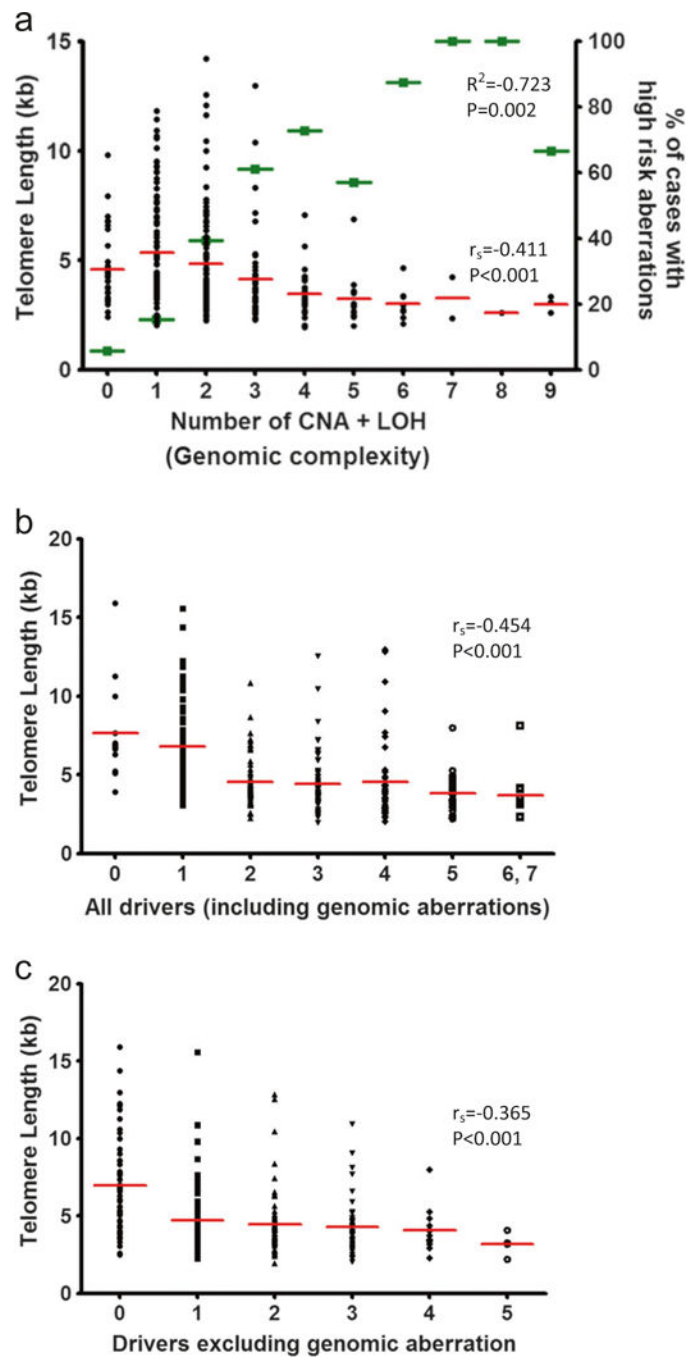


Fig. 2. Comparison of **a, b** PFS (FC: median 27.1 vs. 44.0 months, $P < 0.001$; FCR: median 44.8 vs. 69.9 months, $P < 0.001$) and **c, d** OS (FC: median 70.9 vs. 89.5 months, $P < 0.001$; FCR: median 90.2 months vs. median not reached, $P = 0.017$) between cases with short (median) and long ($>$ median) telomere length, treated with FC ($n = 153$ vs. 159) and FCR ($n = 157$ vs. 151) in CLL8

**Fig. 3.**

a Telomere length association with genomic complexity (copy number alterations (CNA) and loss of heterozygosity (LOH)) and high-risk aberrations (17p-, 11q-, and mutated *TP53*). The red lines indicate the median telomere length (left Y axis), the green symbols indicate the percentage of high-risk aberrations (right Y axis) and the black dots show the distribution of telomere length corresponding to the number of genomic alterations **b**. Telomere length distribution in relation to the number of clonal and subclonal driver events

including and **c** excluding genomic aberrations. r_s in Figs. **a–c** is derived from spearman rank correlation and R^2 in **a** from linear regression

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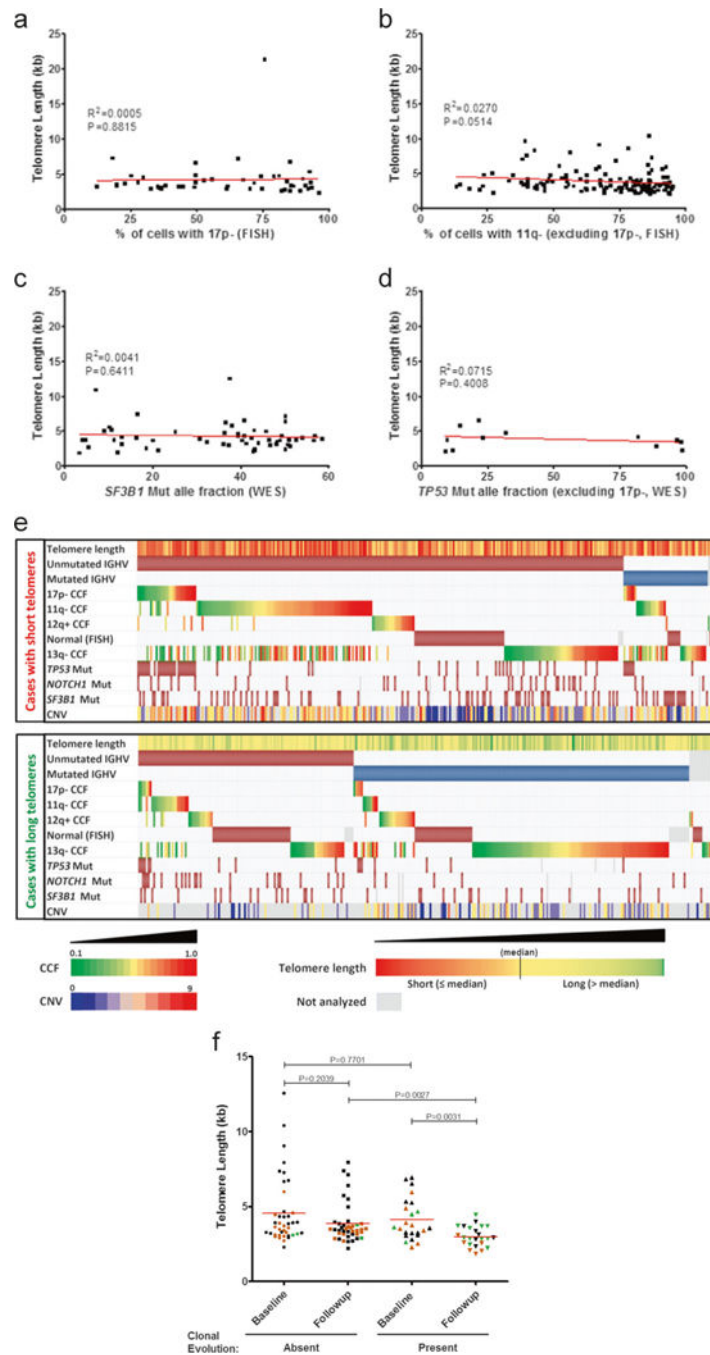


Fig. 4. Telomere length association with percentage of cells with **a** 17p- and **b** 11q- as analyzed using FISH and with mutant allele frequency of **c** *SF3B1* and **d** *TP53* mutations as analyzed using whole-exome sequencing (WES). **e** Distribution of telomere length in IGHV, genomic aberration (with their respective cancer cell fractions—CCF, as analyzed by FISH) and gene mutation subgroups as well as association with copy number variation (analyzed by SNP array). **f** Telomere length at baseline and follow-up in cases with ($n = 24$) and without clonal evolution ($n = 41$). Cases with hierarchical 17p- and 11q- are represented as green and

brown symbols, respectively. P values and R^2 in **a–d** from linear regression and P values in **f** from Mann–Whitney test

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Table 1
Correlation of telomere length (TL) with clinical characteristics (*P*-value from chi-square test)

Patient characteristics	CLL8		CLL4		<i>P</i>
	Short TL N (%)	Long TL N (%)	Short TL N (%)	Long TL N (%)	
Analysis cohort, <i>N</i>	310	310	146	147	
Age at entry (years)	310	310	146	147	
>60	169 (54.5)	163 (52.6)	56 (38.4)	44 (29.9)	0.128
Sex	310	310	146	147	
Male	238 (76.8)	223 (71.9)	113 (77.4)	107 (72.8)	0.362
Binet stage	310	310	145	147	
A	14 (4.5)	20 (6.5)	11 (7.6)	15 (10.2)	0.073
B	215 (69.4)	178 (57.4)	71 (49.0)	87 (59.2)	
C	81 (26.1)	112 (36.1)	63 (43.4)	45 (30.6)	
<i>B</i> -symptoms	310	308	140	144	
Yes	134 (43.2)	146 (47.4)	57 (40.7)	62 (43.1)	0.689
ECOG status	300	293	141	137	
1	146 (48.7)	128 (43.7)	65 (46.1)	61 (44.5)	0.792
WBC count ($10^9/L$)	304	307	143	147	
50	220 (72.4)	178 (58.0)	105 (73.4)	71 (48.3)	<0.001
<i>s</i> -TK (U/L)	282	263	121	131	
>10.0	232 (82.3)	177 (67.3)	110 (90.9)	81 (61.8)	<0.001
<i>s</i> - β_2 MG (mg/L)	282	263	121	134	
>3.5	93 (33.0)	82 (31.2)	62 (51.2)	46 (34.3)	0.006

Multivariable analysis for identification of independent prognostic factors in CLL8 including telomere length as continuous variable

Table 2

COX regression	Progression-free survival (PFS)			Overall survival (OS)					
	Hazard ratio (HR)	95% Confidence interval	P value	Hazard ratio (HR)	95% Confidence interval	P value			
		Lower			Upper				
		Upper							
FCR	0.510	0.409	0.635	<0.001	FCR	0.661	0.485	0.901	0.009
TK > 10 (U/L)	1.386	1.062	1.808	0.016	ECOG 1	1.465	1.061	2.023	0.020
Unmut IGHV	1.724	1.329	2.237	<0.001	CIRS	1.166	1.062	1.280	0.001
17p-	3.441	2.018	5.868	<0.001	Unmut IGHV	1.545	1.014	2.356	0.043
11q-	1.581	1.226	2.038	<0.001	17p-	2.577	1.476	4.501	0.001
TP53 mut	1.881	1.201	2.946	0.006	TP53 mut	2.640	1.602	4.351	<0.001
SF3B/ mut	1.374	1.056	1.787	0.018	s-TK > 10 (U/L)	1.626	1.042	2.537	0.032
					s-β ₂ MG > 3.5 (mg/L)	1.559	1.121	2.168	0.008
					Telomere length *	0.890	0.805	0.983	0.022

* Continuous variable