RESEARCH ARTICLE

Evolution of TP53 abnormalities during CLL disease course is associated with telomere length changes

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Abstract

Background: Telomeres are protective structures at chromosome ends which shorten gradually with increasing age. In chronic lymphocytic leukemia (CLL), short telomeres have been associated with unfavorable disease outcome, but the link between clonal evolution and telomere shortening remains unresolved.

Methods: We investigated relative telomere length (RTL) in a well-characterized cohort of 198 CLL patients by qPCR and focused in detail on a subgroup 26 patients who underwent clonal evolution of TP53 mutations (evolTP53). In the evolTP53 subgroup we explored factors influencing clonal evolution and corresponding changes in telomere length through measurements of telomerase expression, lymphocyte doubling time, and BCR signaling activity.

Results: At baseline, RTL of the evolTP53 patients was scattered across the entire RTL spectrum observed in our CLL cohort. RTL changed in the follow-up samples of 16/26 (62%) evolTP53 cases, inclining to reach intermediate RTL values, i.e., longer telomeres shortened compared to baseline while shorter ones prolonged. For the first time we show that TP53 clonal shifts are linked to RTL change, including unexpected RTL prolongation. We further investigated parameters associated with RTL changes. Unstable telomeres were significantly more frequent among younger patients ($P=0.032$). Shorter telomeres were associated with decreased activity of the B-cell receptor signaling components p-ERK1/2, p-ZAP-70/SYK, and p-NFκB ($P=0.04$, $P=0.01$, and $P=0.02$, respectively).

Conclusions: Our study revealed that changes of telomere length reflect evolution in leukemic subclone proportion, and are associated with specific clinico-biological features of the explored cohort.

Keywords: Chronic Lymphocytic Leukemia, Telomere, TP53, Clonal evolution, BCR signaling

Background

Chronic lymphocytic leukemia (CLL) is a biologically heterogeneous entity with variable clinical outcomes [1–3]. CLL tumor cell mass can be generally composed of multiple genomically distinct subclones endowed with different capacities to proliferate and survive, and their relative composition evolves during the disease course, especially under therapy pressure [4, 5]. This process, generally known as clonal evolution, crucially drives CLL disease progression. Nowadays, many established prognostic biomarkers are used to stratify CLL patients into clinically distinct subgroups. Among these, the aberrations affecting the TP53 gene have a particularly adverse effect on patient prognosis and chemotherapy response [6, 7]. TP53 defects frequently
bound by multiple interacting proteins, which pro-

tected. More specifically, it was revealed for the first time an interplay between telo-
group) over the disease course, TP53

mutations (evol-

tion/mutated

and 17p deletion/mutated

TP53 gene [23–30], with short time to first treatment

[31] and short overall survival (OS) [17, 27, 32, 33]. Tel-
omere length is considered stable in most CLL cases; however, its changes have been observed in a subset of follow-up samples, especially in those undergoing clonal evolution [27, 34]. Furthermore, the level of telomerase activity in CLL cells is similar to its level in normal B lymphocytes, although a subgroup of IGHV-mutated CLL cells had a significantly lower telomerase activity than healthy B cells [23]. In some studies, high telomerase activity was observed and correlated with poor prognosis [31, 35].

This present study follows up our previous efforts focused on investigating clonal evolution of TP53 aberrations [8] and on the associations of ATM aberrations with short telomere length [29] in CLL. Here we explored a well-characterized cohort of 198 CLL patients to assess associations between CLL genomic features and telomere length. Sequential samples from 26 patients, who underwent clonal evolution of TP53 mutations (evolTP53 group) over the disease course, revealed for the first time an interplay between telomere length of subclones, BCR signaling activity, and CLL clinico-biological features.

Methods

Primary CLL samples

Two hundred and thirty-five CLL samples from 198 CLL patients were included in the present study; clinical and laboratory data were available for all tested cases (Tables 1 and 2). CLL cells were separated from peripheral blood with high purity (> 98%) as we described previously [36] using Ficoll-Paque PLUS (GE Healthcare, USA) combined with RosetteSep Human B cell Enrichment Kit and CD3 + Depletion Kit (StemCell Technologies, Canada) to deplete non-B cells. The study was approved by the Ethical Committee of the University Hospital Brno and all patients provided informed consent to the research use of biological material and clinical data in agreement with the Helsinki declaration.

Molecular characterization of the cohort

IGHV gene somatic hypermutation status was analyzed as described previously [37, 38].

Exonic regions and splice sites of the TP53 and ATM genes were amplified using in-house designed primers [8, 29]. Sequencing libraries were prepared using Illumina Nextera XT library preparation kit (Illumina, USA) and sequenced using Illumina MiSeq or NextSeq machines in paired-end mode (2 × 150 bp). The results were analyzed with an in-house bioinformatic pipeline [8, 29]. The cut-off variant allelic frequency (VAF) of 10% recommended in routine clinical diagnostics [39] was used to identify TP53 and ATM mutated cases. However, for the characterization of the evolTP53 group, ultra-deep sequencing with a cut-off 0.1% VAF was used to confirm TP53 clonal evolution [8].

Genomic aberrations were assessed by FISH for del(11q), del(13q), del(17p), and trisomy 12 (XL CLL Probe Kit, MetaSystems). Genome-wide copy number and heterozygosity profiles were obtained using CytoS
can HD arrays (Thermo Fisher Scientific). Metaphase cytogenetics was performed on peripheral blood samples ($n=169$) stimulated with IL 2 and DSP30 CpG Oligonucleotide. Complex karyotype and highly complex karyotype were defined as $\geq 3$ or $\geq 5$ abnormalities, respectively.

Relative telomere length (RTL) measurement

Telomere length was measured by the quantitative polymerase chain reaction (qPCR) method described previously [29, 40] with certain modifications (see Supplementary Material) using primers specific for telomeric repetition [41]. HBG3 and HBG4 primers [42] were
used for the amplification of β-globin (HBB) gene, serving as a single-copy gene control.

Each sample was measured in triplicates for both RTL and HBB, the reactions were assembled by Freedom EVO automated system (Tecan, Switzerland) in 384-well plates, and qPCR was performed by QuantStudio 12 k Flex (Applied Biosystems, USA). Each reaction contained 15 ng DNA, 100 nM F and R primers, 5 mM DTT, 10 mM Tris–HCl (pH 8), 130 mM DMSO, 1.5 µM BSA, 50 nM ROX in the final volume 10 µl. Quantitative PCR was performed on QuantStudio 12 k Flex (Applied Biosystems) with the cycling conditions as follows: 10 min 95 °C, 40x (20 s 95 °C, 1 min 56 °C). The RTL was calculated as -ΔCT for CLL samples divided by -ΔCT for control human DNA sample (G3041, Promega, USA) isolated from leukocytes of healthy donors. The -ΔCT values represent the difference between average Ct values of telomere length and HBB gene. The cut-off value for RTL change in consecutive CLL cases was 5% difference of RTL between baseline and follow-up, which corresponded to the complete loss of telomeres on two chromosomes (calculated approximately as 46 chromosomes with 2 telomeric regions each = 92 telomeric regions, 5% loss corresponds to loss of ~ 4 telomeric regions).

Human telomerase reverse transcriptase (hTERT) expression
Reverse transcription was carried out with SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). One ng of reverse-transcribed cDNA was used for real-time PCR containing 2×ABsolute QPCR Mix (low ROX, Thermo Fisher Scientific) and either hTERT or GAPDH TaqMan assay (Hs00972650_m1 for hTERT, Hs03929097_g1 for GAPDH, Thermo Fisher Scientific) following the manufacturer protocol. PCR was performed on QuantStudio 12 k Flex (Applied Biosystems) with the cycling conditions: 15 min 95 °C, 40x (15 s 95 °C, 1 min 60 °C).

BCR signaling assessment
Fresh frozen CLL cells were thawed, fixed, and permeabilized with 1.6% paraformaldehyde and 100% methanol. Supplementary Table 1 shows that the samples were incubated with antibodies against phosphorylated AKT, BTK/ITK, ERK1/2, IKKα/β, NF-κB, p38, PLCγ2, and ZAP-70/SYK at 20 °C for 60 min, and measured using FACSVerse flow cytometer (BD Biosciences). Median fluorescent intensity (MFI) value, representing the amount of basal phosphorylation of BCR signaling pathway components, was used for further analysis.

Statistical analysis
All statistical analyses were performed using software JASP (Version 0.11.1) [43]. Specific statistical tests used for different study variables are described in the figure legends. T-tests were Student’s two-tailed, RTLs of paired samples were evaluated by Wilcoxon signed rank test, correlations were counted as Pearson’s r. P values < 0.05 were considered statistically significant.

Data sharing statement
For the original data and detailed protocols please contact the corresponding author.

Results
Telomere length associates with clinical features of the disease
In order to comprehensively assess the association between genomic features and telomere length in CLL, we studied a set of 198 clinically and genetically well-characterized CLL cases (Table 1). The majority of samples (n = 153; 77.3%) was collected prior to first treatment, and for patients with multiple available samples, only the first sample was included here. Forty samples (20.2%) carried a TP53 mutation above 10% VAF (with concomitant del(17p) in 23/40 cases).

The median RTL of the entire CLL cohort was 0.81 (range 0.46 – 1.25; Supplementary Figure 1), meaning that telomere length in CLL cells was generally shorter than in the control mixed human DNA sample. Additionally, we observed significantly longer RTLS in patients at Rai and Binet early disease stages at diagnosis (Supplementary Figure 2). To assess the impact of RTL on OS, we split the cohort into two groups with RTLS above and below median RTL (median RTL = 0.84) and detected statistically significant difference between the groups.

Table 1 Composition of the basic CLL cohort (n = 198)

| Parameter                          | n (%)          |
|-----------------------------------|---------------|
| Sex (female/male)                 | 77/121 (38.9% / 61.1%) |
| Age at diagnosis (median)         | 62.4 years    |
| Untreated at the time of analysis | 153 (77.3%)   |
| Time to first treatment (median; n = 182) | 30 months    |
| IGHV status (mutated/unmutated)   | 79/119 (39.9% / 60.1%) |
| Hierarchical cytogenetics (FISH)  |               |
| del(17p)                          | 28 (14.1%)    |
| del(11q)                          | 45 (22.7%)    |
| trisomy 12                        | 18 (9.1%)     |
| normal                            | 37 (18.7%)    |
| del(13q)                          | 70 (35.4%)    |
| Complex karyotype (≥ 3 changes; n = 169) | 47 (27.8%)    |
| Highly complex karyotype (≥ 5 changes; n = 169) | 23 (13.6%)    |
| ATM mutation > 10% VAF (n = 191)  | 37 (18.9%)    |
| TP53 mutation > 10% VAF           | 40 (20.2%)    |
Clonal evolution of TP53 defects affects telomere length

We focused in detail on the associations between telomere length and TP53 mutational status in our CLL cohort. First, we evaluated the impact of RTL on patient OS in the subgroup with TP53 mutations \( (n = 40) \). We observed that OS of this subgroup divided by the RTL value above and below median was statistically different favoring cases with longer RTL (median RTL = 0.78; median OS 86 and 55 months, respectively; \( P = 0.046 \); Supplementary Figure 3B).

Next, available retrospective ultra-deep NGS data \( [39] \) were used to divide the patients from the basic cohort into three groups: (I) patients with wild-type TP53 \( (wtTP53; \ n = 135) \), (II) patients with mutated TP53 above 10% VAF \( (mutTP53; \ n = 40; \ median VAF = 57.5\%) \), and (III) patients with TP53 mutation expanding above 10% VAF later in a disease course \( (n = 23) \). All group III patients except one expanded a TP53 mutation after therapy. Median TP53 VAF of group III was 0.2% \((0\% – 5.9\%\)) at baseline and 43.4% \((12.1\%–100\%)\) at follow-up, corresponding to median fold change of 211. Figure 1 summarizes the RTL dynamics in the context of TP53 mutation clonal evolution. When we compared RTLS between the patients with \( wtTP53 \) (group I) and \( mutTP53 \) (group II), we found that the \( mutTP53 \) samples had significantly shorter telomeres (median RTL 0.87 and 0.78, respectively; \( P < 0.001 \); Fig. 1A). Interestingly, RTLS of group III (with TP53 mutation expanding above 10% VAF later) were intermediate (median RTL 0.83; Fig. 1A) and they did not differ significantly either from group I \( (P = 0.25) \) or group II \( (P = 0.10) \).

Apart from the above-mentioned 23 cases from group III manifesting TP53 mutation expansion, we identified another 3 cases from group II \( (mutTP53) \) who experienced replacement of a certain TP53 mutation by another one after therapy. Thus, 26 cases out of the entire cohort experienced TP53 mutation evolution \( (evolTP53 \) group) during the disease course (Table 2, Supplementary Table 3). In order to discover whether \( mutTP53 \) evolution showed any association with telomere length, we studied pre- and post-evolution samples. All \( evolTP53 \) cases were analyzed in at least 2 time points. The median follow-up between the pre- and post-evolution samplings was 38.5 months (range 18 – 122 months). The median RTL was 0.82 (range 0.52 – 1.25) before and 0.78 (range 0.65 – 0.98) after the \( mutTP53 \) evolution (Fig. 1B). When focusing on individual patients, the RTL change course was inconsistent. In detail, we observed that telomeres became shorter, remained stable, or became longer in 10/26 (38.5%), 10/26 (38.5%), and 6/26 (23.1%) patients, respectively (Fig. 1C). Interestingly, short telomeres became longer over time, while in contrast, long telomeres became shorter (Pearson \( r = -0.75, P < 0.001 \); Fig. 1C). Additionally, for 6 and 2 of the 26 patients, 3 and 4 time points were analyzed, respectively, to follow further RTL development. Evolutions of genomic aberrations related to changes of RTLS in two CLL cases with the shortest basal telomere length are illustrated in Figs. 2A and 2B. Importantly, the apparent correlation between RTL and genomic abnormalities indicates that telomere length was likely a feature of CLL clones, not a hallmark of the entire CLL population changing in time.

Association of RTL dynamics with clinico-biological features in cases evolving TP53 mutations

hTERT expression

Since the changes of telomere length could potentially be caused by deregulated telomerase activity \( [31, 45] \), we tested hTERT expression in the 26 \( evolTP53 \) patients. However, only weak or null hTERT expression was detected. Supplementary Figure 4 and Supplementary Table 3 show that we did not find any correlation with telomere length or its change.

Lymphocyte doubling time (LDT)

Intensive cell division, which could possibly cause RTL shortening, can be estimated from LDT \( [24] \). Supplementary Figure 5 and Supplementary Table 3 show the correlation of LDT of the \( evolTP53 \) cases with the change of RTL between the baseline and follow-up. Short doubling time was observed in available baseline samples (median 6.5 months, range 0.5 – 39.6; \( n = 21 \)) as well as in follow-up samples (median 2.6 months; range 0.4 – 81.5; \( n = 25 \)). Nevertheless, no significant association of LDT and change of RTL was found.
To study the relation between RTL dynamics and the activity of BCR signaling, we applied flow cytometry to detect basal phosphorylation of selected intracellular markers in CLL cells obtained prior and after mut\(TP53\) expansion (\(n = 11\) and 10, respectively, of these 7 samples were paired; Supplementary Table 3). Figure 3 illustrates the correlation of RTL and BCR signaling activity. Significantly longer telomeres were detected prior as well as after mut\(TP53\) expansion in cases with higher phosphorylation of ERK1/2 (prior mut\(TP53\) expansion: Pearson \(r = 0.60; P = 0.05\); Fig. 3B; after mut\(TP53\) expansion: Pearson \(r = 0.79; P = 0.01\); Fig. 3D; Supplementary Table 3). Additionally, significantly longer telomeres after mut\(TP53\) expansion were detected in cases with higher phosphorylation of NF-κB (Pearson \(r = 0.70; P = 0.02\); Fig. 3E; Supplementary Table 3). Moreover, the phosphorylation of ZAP-70/SYK and ERK1/2 closely correlated mutually (Pearson \(r = 0.82; P = 0.002\) in baseline samples; Pearson \(r = 0.92; P < 0.001\) in follow-up samples; Supplementary Figure 6A, B).

**Fig. 1** Relative telomere length dynamics in the context of \(TP53\) mutation clonal evolution. (A) The analysis of the RTL and \(TP53\) mutation status (the cut-off VAF 10%) within the basic CLL cohort (\(n = 198\)), showing a difference between the subgroups of patients characterized by wt\(TP53\) (blue; \(n = 135\)), patients expanding mut\(TP53\) above 10% VAF later in a disease course (yellow; \(n = 23\)), and mut\(TP53\) (red; \(n = 40\)). (B) RTLs in baseline and follow-up samples in the evol\(TP53\) cohort (\(n = 26\); Wilcoxon signed rank \(P = 0.291\)). (C) RTL change in individual patients in the evol\(TP53\) cohort. Telomeres became shorter in 10/26 (38.5%), remained stable in 10/26 (38.5%), or became longer in 6/26 (23.1%) patients. Higher RTL shrank and lower RTL extended with an inverse relationship during the disease course (Pearson \(r = -0.75, P < 0.001\)). RTL change marks: ▼ shortening, ○ stable, ▲ prolonging. The cut-off value for RTL change was set to 5% difference of RTL between baseline and follow-up samples.
Clinical and biological features

All patients with changing RTLs (both shortening and extending) could be considered as one group of patients with impaired telomere maintenance resulting in unstable RTLs during the disease course. Figure 4 shows that when we examined all clinical and biological features (as listed in Supplementary Table 3) of the patients with unstable RTLs (n = 16), we observed that they were statistically significantly younger at diagnosis compared to the patients with stable telomeres (median 55.5 vs. 63.7; \( P = 0.032 \)).

Discussion

Dysregulation of telomeres is common in many cancer types and represents a means for overcoming senescence and achieving uncontrolled cell growth [46]. In CLL, short telomere length has been linked to poor prognosis [23, 31–33, 35]. Here, we used a previously described qPCR method [40, 41] to study relative telomere length (RTL) in a large cohort of 198 CLL patients. The use of pure samples containing > 98% of CLL cells enabled us to follow the RTL very accurately. We confirmed that RTL in CLL patients was generally shorter compared to the control DNA pooled from healthy donors. Short telomeres were associated with mutations in ATM gene as we reported previously [29] and with other markers of inferior prognosis such as unmutated IGHV, complex karyotype, del(17p) and del(11q), and mutations in TP53 gene in concordance with published results [23–28, 30, 31].

The shortest detected telomeres were in patients with mutated ATM gene, while the longest in patients with 13q deletion as a sole cytogenetic aberration. Additionally, our study provided an insight into the relationship between clonal evolution of CLL cells with TP53 gene aberrations and their telomere length. Within our cohort of 26 evolTP53 cases evolving TP53 mutations, we measured RTL at several time points. We demonstrated that the RTL can evolve diversely with the clonal evolution of genomic abnormalities in CLL.

Clonal evolution is an adverse event during the course of CLL and other cancers, which gives rise to subclones of cells with novel aberrations and features supporting increased fitness. In CLL, TP53-aberrant subclones tend to expand in disease relapse frequently and have a crucial impact on CLL patients’ prognosis and survival as we reported previously [8, 47]. Jebaraj et al. [27] hypothesized that telomere shortening preceded the appearance of TP53 and ATM defects, and could provide selective pressure on CLL subclones favoring those with abilities of uncontrolled cell growth [27]. In our study, patients with TP53 mutation expanding above 10% VAF later in a disease course did show a tendency towards shorter telomeres prior to TP53 clonal expansion when compared to telomeres of the wtTP53 cases. Thus, it was not possible to anticipate a forthcoming TP53 mutation expansion in individual CLL cases based on the RTL.

In our evolTP53 cohort, we performed measurements of paired samples and showed RTL changes between baseline and follow-up samples. Mansouri et al. [34] have found a high concordance between RTLs at diagnostic

### Table 2 Composition of the CLL cohort evolving TP53 mutation during the disease course, which underwent the telomere length measurements in multiple time points (n = 26)

| Parameter | n (%) |
|-----------|-------|
| Sex (female / male) | 12 / 14 (46.2% / 53.8%) |
| Median age at diagnosis | 57.7 years |
| Median time to first treatment | 20.0 months |
| IGHV status (mutated /unmutated) | 2 / 24 (7.7% / 92.3%) |
| Previously treated for CLL | 5 (19.2%) / 26 (100%) |
| Hierarchical cytogenetics (FISH) | follow-up sample |
| del(17p) | 3 (11.5%) / 10 (38.5%) |
| del(11q) | 10 (38.5%) / 9 (34.6%) |
| trisomy 12 | 3 (11.5%) / 2 (7.7%) |
| normal | 6 (23.1%) / 4 (15.4%) |
| del(13q) | 4 (15.4%) / 1 (3.8%) |
| Copy-neutral loss of heterozygosity 17p (n = 21) | 0 (0%) / 5 (23.8%) |
| Complex karyotype (≥ 3 changes; n = 24) | 7 (29.2%) / 9 (37.5%) |
| Highly complex karyotype (≥ 5 changes; n = 24) | 4 (16.7%) / 6 (25.0%) |
| ATM mutation > 10% VAF (n = 22) | 4 (15.4%) / 3 (12.5%) |
| TP53 mutation > 10% VAF | 3 (11.5%) / 26 (100%) |
and follow-up CLL samples [34] suggesting that the telomere length does not change during the disease course. Recent study of Jeebaraj et al. [27] focused on telomere length in 41 CLL patients without clonal evolution and 24 CLL patients with clonal evolution between samplings (9/24 had clonal evolution of TP53 mutation detected by whole-exome sequencing). They identified significant telomere shortening between samplings only within patients with clonal evolution. Concurrently with these changes of clonal proportions, higher RTL was detected. In the patient pt1 studied in three time points with three distinct clones carrying different TP53 mutations, telomeres initially lengthened and subsequently shortened, closely following an exchange of a dominating TP53 mutation (the first follow-up sample was collected 27 months after the baseline sample and the second follow-up sample was collected 10 months later, arrows indicate each sampling).

**Fig. 2** Examples of RTL change associated with shifts in a proportion of clones bearing different aberrations. **A)** In the patient pt1, the RTL increased in disease relapse (the follow-up sample collected 81 months after the baseline sample, arrows indicate each sampling). The proportion of a new clone bearing del(11q), del(8p), del(15p), and a TP53 mutation increased to approximately 40% of the CLL population in the follow-up sample. At the same time, the proportion of the clone with sole del(11q) shrunk to approximately 35% of the CLL population and the clone with del(13q) disappeared. Concurrently with these changes of clonal proportions, higher RTL was detected. **B)** In the patient pt2 studied in three time points with three distinct clones carrying different TP53 mutations, telomeres initially lengthened and subsequently shortened, closely following an exchange of a dominating TP53 mutation (the first follow-up sample was collected 27 months after the baseline sample and the second follow-up sample was collected 10 months later, arrows indicate each sampling).
Increased telomere length was frequently found in the corresponding follow-up samples, suggesting selective pressure on subclones with longer telomeres with presumably better fitness. In this context, the prognosis of CLL cases with RTL prolongation remains to be evaluated. It seems plausible that these cases, while having longer telomeres suggesting a better prognosis, probably overcame a critically short telomere crisis and gained a wide range of new aberrations, which is typical of inferior prognosis. Nonetheless, longer telomeres in baseline samples were not necessarily maintained at follow-up. Our findings, thus, highlight an unexpected consequence.

**Fig. 3** Relative telomere length dynamics and BCR signaling activity. Prior clonal evolution, RTL was **A** lower in CLL samples with less phosphorylated ERK1/2 (Pearson $r = 0.63; P = 0.04$) and **B** less phosphorylated ZAP-70/SYK (Pearson $r = 0.60; P = 0.05$). In later stages, RTL was **C** lower in CLL cases with less phosphorylated ERK1/2 (Pearson $r = 0.66; P = 0.04$), **D** less phosphorylated ZAP-70/SYK (Pearson $r = 0.79; P = 0.01$), and **E** less phosphorylated NFkB (Pearson $r = 0.70; P = 0.02$).
of clonal evolution leading to both shorter or longer telomeres, associated with changing genomic abnormalities. A larger study, specifically addressing the fate of individual clones carrying certain telomere lengths and distinct cytogenetic abnormalities, is needed to elucidate the causality between the telomere length, genomic features, and a clone fitness.

To uncover the molecular mechanisms behind the RTL changes in our cohort, we studied two possible causal processes – hTERT expression and cellular proliferation. We did not confirm any clear role of hTERT in CLL telomere maintenance in concordance with others [23, 48]. Although the telomere shortening rate was previously attributed to a rapid proliferation rate [23, 31], neither the cellular proliferation, represented by lymphocyte doubling time, could explain the changing RTLs in our samples. Telomerase activity is highly restricted in normal somatic cells and was detected in normal B-lymphocytes in germinal centers [21]. CLL cells are well known to circulate between blood and lymph nodes [49]. Possibly telomerase activity in CLL could be restricted to the time when cells are staying in the supportive microenvironment of lymph nodes. Since our CLL samples are isolated from peripheral blood, we cannot anticipate the telomerase activity of CLL cells located in lymph nodes.

Interestingly, we found that patients with unstable telomeres, both shortening or prolonging, with concurrent evolution of TP53 mutations, have a significantly lower age at diagnosis. We have not found any association of unstable telomeres with clinical or biological features. Some more complex factors of CLL disease nature in younger patients could be involved.

Finally, for the first time, we studied the interplay between tonic BCR signaling and telomere length maintenance in CLL. CLL cells depend on BCR signaling, supporting their survival, proliferation [50], therapy resistance, and gene expression [50–52]. BCR pathway is successfully targeted by novel inhibitor agents introduced in clinical practice in the last decade [12–14]. Using a targeted selection of phosphorylated kinases, we aimed to analyze different branches of BCR signaling to uncover possible associations with RTL changes. Patients with shorter telomeres had lower basal phosphorylation of ZAP-70/SYK and ERK1/2 in the baseline samples and lower basal phosphorylation of ZAP-70/SYK, ERK1/2, and p-NF-κB in the follow-up samples, indicating weakened BCR signaling at both time points. Other phosphorylated signaling components, especially BTK/ITK and PLC-γ2, showed strong, although not significant, trends towards lower basal phosphorylation in patients with shorter telomeres. This could anticipate that CLL cells with shorter telomeres are less dependent on survival and proliferative signals delivered to the cells by tonic BCR signaling.

**Conclusions**

To summarize, we have performed an assessment of the telomere length in a well-defined cohort of CLL cases. Apart from confirming known associations between genomic features and telomere shortening, we focused on the relationship between telomere length and TP53 mutation evolution. We discovered that long telomeres can shorten, while short telomeres can, in some cases, even extend their length during the clonal evolution process. Thus, our study shows that shifts in subclone equilibriums can be reflected in telomere length changes, while telomere length per se is likely not the only factor affecting the survival capacity of a clone expanding in the evolution process.

**Abbreviations**

CLL: Chronic lymphocytic leukemia; evolTP53: Clonal evolution of TP53 mutations; HBB: β-Globin; hTERT: Human telomerase reverse transcriptase; LDT: Lymphocyte doubling time; MFI: Median fluorescent intensity; qPCR: Quantitative polymerase chain reaction; RTL: Relative telomere length; VAF: Variant allelic frequency.
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-09221-z.

Additional file 1: Supplementary Table 1. Antibodies used for BCR signaling activity assessment. Supplementary Table 2. Results of relative telomere length (RTL) quantification in the basic CLL cohort (n = 198).

Supplementary Table 3. TP53 mutation evolution cohort overview - results of clinico-biological features and results in baseline and follow-up samples, PART A Abbreviations: ALZ – alermtuzumab; BR - bendamustine and rituximab; CLB – chlorambucil; CR - cyclophosphamide and rituximab; del – deletion; F – female; FCR - fludarabine, cyclophosphamide, and rituximab; FISH - fluorescence in situ hybridization; hyper CVAD - hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone; HGIV – immunoglobulin heavy chain variable region; M – male; M – mutated; mo – months; U – unmutated; yrs – years. Supplementary Table 3. TP53 mutation evolution cohort overview - results of clinico-biological features and results in baseline and follow-up samples, PART B Abbreviations: LTD – lymphocyte doubling time; mo – months; mut – mutated; p – phosphorylated; RTL – relative telomere length; VAF – variant allele frequency; wt – wildtype. Supplementary Figure 1. RTL of the entire CLL cohort was compared to the telomere length of a control DNA sample pooled from healthy individuals, arbitrarily set to RTL = 1 (green line). Median RTL of CLL cases (black line) was 0.81, range 0.46 – 1.23. Supplementary Figure 2. A) Rai (n = 130) and B) Binet (n = 133) stages at diagnosis in untreated CLL samples and their associations with RTL. The significant associations were p = 0.0018; P (n = 130) = 0.0108, P A V C. Supplementary Figure 3. Overall survival (OS) of A) the entire CLL cohort (n = 198) divided by RTL above (‘RTL long’) and below (‘RTL short’) median RTL value (median RTL = 0.84, OS long RTL = 119; OS short RTL = 91; OS HP = 0004), and B) of CLL patients with mutated TP53 status (n = 40) divided by RTL above (‘RTL long’) and below (‘RTL short’) median RTL value in this subgroup (median RTL = 0.78; OS long RTL = 86; OS short RTL = 55; OS HP = 0.046). Supplementary Figure 4. hnRT expression in A) the baseline samples (P shortening vs. stable = 0.066, P stable vs. prolonging = 0.088; P shortening vs. prolonging = 0.045) and in B) the follow-up samples (P shortening vs. stable = 0.77, P stable vs. prolonging = 0.52, P shortening vs. prolonging = 0.82). (RTL shortening n = 10, stable n = 10, prolonging n = 6). CI Comparison of hTERT values in serial samples. hnRT expression did not associate with RTL prolongation. Supplementary Figure 5. Lymphocyte doubling time did not correlate with a change of RTL time during the disease course (Pearson r = -0.175; P = 0.45). Supplementary Figure 6. p-ZAP70/SYK significantly correlated with p-ERK1/2 in A) baseline samples (Pearson R = 0.82; P = 0.0002) and B) follow-up samples (Pearson R = 0.92, P < 0.001).

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Authors’ contributions

K.P. designed the study. H.O., K.P.1, J.M., J.K., S.P.1, K.S., M.S., V.N., B.D., and M.J. performed experiments. J.K., Y.B., A.P. and M.D. provided samples and clinical data. H.O., N.T., and K.P.2 evaluated results. K.P.1 and S.P.2 supervised the study. H.O., K.P.1, J.K., S.P.1, K.S., M.T., and S.P.2 wrote and edited the manuscript. All authors provided final approval of the manuscript.

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Availability of data and materials

The data used during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The whole study and written informed consent obtained from all patients were approved by the Ethical Committee of University Hospital Brno in concordance with the Declaration of Helsinki.

Consent for publication

Written consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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References

1. Puiggros A, Blanco G, Espinet B. Genetic abnormalities in chronic lymphocytic leukemia: Where we are and where we go. Biomed Res. Int: Hindawi Publishing Corporation, 2014.
2. Guèze R, Wu CJ. Genomic and epigenomic heterogeneity in chronic lymphocytic leukemia. Blood Am Soc of Hematol. 2015;126(4):445–53.
3. Balakas P, Jéromin S, Ikas M, Puiggros A, Pievova K, Nguyen-Khac F, et al. Cytogenetic complexity in chronic lymphocytic leukemia: Definitions, associations, and clinical impact. Blood Am Soc of Hematol. 2019;133:1205–16.
4. Hernández-Sánchez M, Kotaskova J, Rodríguez AE, Radova L, Tamborero D, Abáigar M, et al. CLL cells cumulate genetic aberrations prior to the first therapy even in outwardly inactive disease phase. Leukemia Nature Publishing Group. 2019;33:518–58.
5. Schuh A, Becq J, Humphray S, Alexia A, Burns C, Clifford R, et al. Monitoring chronic lymphocytic leukemia progression by whole genome sequencing reveals heterogeneous clonal evolution patterns. Blood. 2012;120:4191–6.
6. Zenz T, Eichhorst B, Busch R, Denzel T, Häbe S, Winkler D, et al. TP53 mutation and survival in chronic lymphocytic leukemia. J Clin Oncol. 2010;28:4473–9.
7. Rossi D, Cerri M, Deambrogi C, Sozzi E, Cretsa S, Rasi S, et al. The prognostic value of TP53 mutations in chronic lymphocytic leukemia is independent of Del17p13: Implications for overall survival and chemorefractoriness. Clin Cancer Res Am Association for Cancer Reseach. 2009;15:995–1004.
8. Malcikova J, Stano-Kozubik K, Tichy B, Kantorova B, Pavlova S, Tom N, et al. Detailed analysis of therapy-driven clonal evolution of TP53 mutations in chronic lymphocytic leukemia. Leukemia. 2015;29:887–85.
9. Landau DA, Tausch E, Taylor-Weiner AN, Stewart C, Reiter JG, Bahlo J, et al. Mutations driving CLL and their evolution in progression and relapse. Nature. 2015;526:518–52.
10. Hallek M, Cheson BD, Catovsky D, Jakobovits A, Maloney D, Gratschew M, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: A report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood Am Soc of Hematol. 2008;111(12):5446–56.
11. Stilgenbauer S, Schnaier A, Paschka P, Zenz T, Rossi M, Döhner K, et al. Gene mutations and treatment outcome in chronic lymphocytic leukemia: Results from the CLL8 trial. Blood Am Soc of Hematol. 2014;123:3247–54.
12. Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum KA, et al. Targeting BTK with ibrutinib in Relapsed Chronic Lymphocytic Leukemia. N Engl J Med. 2013;369:32–42.
13. Furman RR, Shamaran JP, Coutre SE, Cheson BD, Pagel JM, Hillman P, et al. Idelalisib and Rituximab in Relapsed Chronic Lymphocytic Leukemia. N Engl J Med Massachusetts Sci. 2014;370:997–1007.
14. Burger JA, Tedeschi A, Barr PM, Robak T, Owen C, Ghia P, et al. Ibrutinib in Initial Therapy for Patients with Chronic Lymphocytic Leukemia. N Engl J Med Massachusetts Sci. 2015;373:2425–37.
15. Roberts AW, Davids MS, Pagel JM, Kahl BS, Puvvada SD, Gerecitano JF, et al. Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. N Engl J Med Massachusetts Sci. 2016;374:311–22.
16. Okuda K, Bardegeuz A, Gardner JP, Rodriguez P, Ganesh V, Kimura M, et al. Telomere length in the newborn. Pediatr Res Lippincott Williams and Wilkins. 2002;52:377–81.
17. Lin TT, Norris K, Heppel NH, Pratt G, Allan JM, Allsup DJ, et al. Telomere dysfunction accurately predicts clinical outcome in chronic lymphocytic leukaemia, even in patients with early stage disease. Br J Haematol Blackwell Publishing Ltd. 2014;167:214–23.
18. Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, et al. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci U S A. 1992;89:1014–8.
19. Collins K, Mitchell JR. Telomerase in the human organism. Oncogene. 2002;21:564–79.
20. Wright WE, Shay JW. Telomere biology in aging and cancer. J Am Geriatr Soc. 2005;53:5292–4.
21. Norntack HF, Dahlenborg K, Carlsson R, Roos G. Telomerase activation in normal B lymphocytes and non-Hodgkin’s lymphomas. Blood. 1996;88:222–9.
22. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, et al. ATM mutations in major stereotyped subsets of chronic lymphocytic leukemia. Blood. 2004;103:375–82.
23. Damle RN, Batliwalla FM, Ghiotto F, Valetto A, Albesiano E, Sison C, et al. Ibrutinib as initial therapy in mantle cell lymphoma. Blood. 2015;126:3978–86.
24. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, et al. ATM mutations in major stereotyped subsets of chronic lymphocytic leukemia. Blood. 2004;103:375–82.
25. Wright WE, Shay JW. Telomere biology in aging and cancer. J Am Geriatr Soc. 2005;53:5292–4.
26. Norntack HF, Dahlenborg K, Carlsson R, Roos G. Telomerase activation in normal B lymphocytes and non-Hodgkin’s lymphomas. Blood. 1996;88:222–9.
27. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, et al. ATM mutations in major stereotyped subsets of chronic lymphocytic leukemia. Blood. 2004;103:375–82.
28. Wright WE, Shay JW. Telomere biology in aging and cancer. J Am Geriatr Soc. 2005;53:5292–4.
29. Norntack HF, Dahlenborg K, Carlsson R, Roos G. Telomerase activation in normal B lymphocytes and non-Hodgkin’s lymphomas. Blood. 1996;88:222–9.
30. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, et al. ATM mutations in major stereotyped subsets of chronic lymphocytic leukemia. Blood. 2004;103:375–82.
31. Wright WE, Shay JW. Telomere biology in aging and cancer. J Am Geriatr Soc. 2005;53:5292–4.