Telomeric damage in early stage of chronic lymphocytic leukemia correlates with shelterin dysregulation

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Introduction

Telomere erosion in proliferating cells leads to senescence and is thereby a potent barrier for tumor progression. In normal proliferating cells, such as germ and stem cells and in cancer cells, this erosion is compensated mainly by a specialized reverse transcriptase named telomerase. This enzyme is crucial in cancer cells for tumor progression. However, telomere shortening because of telomerase invalidation can also cooperate with p53 deficiency to facilitate carcinogenesis in aged mice. In chronic lymphocytic leukemia (CLL), despite a low proliferative index, telomeres are abnormally shortened and we previously reported a low telomerase expression level. In this context, both the consequence and the origins of telomere shortening are still a matter of debate. This point is of great importance as telomere shortening has been shown to be an even better prognosis marker than the reference one, namely the mutational status of IGHV gene.

In this way, recent publications reported the presence of deprotected telomeres in radio-resistant patient cells and fused telomeres bearing extremely short telomeres in patients mainly at advanced stages of the disease. Both of these studies proposed that telomere dysfunction or crisis can be at the origin of genomic instability and karyotype alteration. A priori, the telomere injuries observed in CLL could stem from an excessive telomere shortening or alteration in the protective chromatin structure that cap telomeres or both. Indeed, if maintenance of telomere length depends on telomerase, telomere protection involves various telomere-associated proteins, such as the shelterin complex composed of 6 proteins (TRF1, TRF2, RAP1, TIN2, TPP1, POT1) (Figure 1). This complex appears to act as a hub that protects telomeres from being recognized as a double-strand break, thereby avoiding inappropriate DNA damage response (DDR) activation and aberrant repair (Figure 1). This complex also controls telomere length, by regulating both the telomerase activity and the alternative lengthening of telomere (ALT) mechanism. These proteins are also involved in oncogenesis. For instance, TRF2 is up-regulated in various types of human tumors and has been found to display oncogenic properties in mouse keratinocytes and human cancer cell lines. Human telomeres are also composed of nucleosomes that exhibit repressive chromatin marks, such as H3K9 and H4K20 trimethylation and low histone acetylation level associated to a hypermethylation of CpG in the subtelomeric regions. In agreement with a role of these chromatin modifications in telomere length control, the invalidation of enzymes responsible for their formation, that is, histone methyltransferase such as Suv39h1, Suv39h2, Suv4-20h, or DNA methyl transferase as DMNT1, 3a and 3b, leads to changes in telomere size.

We have previously shown a global deregulation of the expression of shelterin proteins in an heterogeneous cohort of CLL patients. Here, we address the question of whether telomere dysfunction occurs at early stages of CLL and, if yes, whether it can
be correlated with telomere shortening and/or changes in the telomere chromatin cap. As an early sign of telomere dysfunction, we monitored the recruitment at telomeres of DDR proteins. Thus, we restricted our analysis to B cells from 23 patients of first stage of CLL leukemogenesis (Binet stage A, ≤ 2 years of disease history, previously untreated). We used B cells from 12 healthy age-matched donors as control.

We found a significant increase in telomeric damage in CLL patients in comparison with healthy donors. Strikingly, the presence of dysfunctional telomere at this early stage of the disease did not correlate with short telomeres but rather with a down-regulation of 2 shelterin genes: ACD and TINF2. We thus propose that early telomere deprotection in CLL results from shelterin dysregulation rather than from telomere shortening.

Methods

Patients and healthy donors

After informed consent and according to institutional guidelines, total blood samples were collected from 23 CLL patients at the Hospices Civils de Lyon and Grenoble University and from 12 age-matched healthy donors at the Etablissement Français du Sang. The protocol has been approved by the institutional review board. The diagnosis was based on morphologic criteria and immunophenotyping (Matutes score > 3/5). Patients were newly diagnosed (< 2 years of disease history) stage A patients, not previously treated by chemotherapy. Disease stage, FISH data, and IGH mutational

Table 1. Sample characteristics

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NA indicates nonapplicable; ND, not done; M, mutated; UM, unmutated; A, abnormal; N, normal; del, deletion; m, monoallelic; b, biallelic; del, deletion; +12, trisomy 12; and t, translocation.
status are provided for each patient in Table 1. Briefly, del13q, del11q, del17p, and t12 were, respectively, found in 56%, 8.7%, 4%, and 4% of patients and 22% of patients are unmutated. These percentages for some are inferior to what is found in general CLL patients,\(^29,30\) are in accordance with those observed in stage A patients.

**Isolation of human B cells**

B lymphocytes were purified from peripheral blood by using the human CD19 Microbeads kit (Miltenyi Biotec). Briefly, Ficoll prepared PBMCs were incubated with a cocktail of anti-CD19 antibody-conjugated magnetic microbeads. The magnetically labeled CD19-positive cells are collected by passing over a MACS column in a magnetic MACS separator. Cells were washed 3 times, eluted from the column and tested for CD19 enrichment. Percentage of CD19-positive cells was determined by flow cytometry (LSRII; BD Biosciences Pharmingen) using an anti-CD19–PE antibody (Amersham Pharmacia Biotech). Over 93% of CD19-positive labeling was obtained for normal B cells and CLL cells. For CHIP experiments only, patient blood samples showing > 80% B-CLL cells were not further purified after Ficoll gradient separation.

**Immunofluorescence**

Cells were fixed in cold methanol (−20°C), washed twice in PBS, incubated 40 minutes in 1× Target Retrieval Solution (DAKO) at 95°C, cooled 20 minutes at room temperature, and rinsed 3 times in distilled water. Slides were then incubated 1 hour in blocking solution (50 mM NaCl, 0.5% Triton X-100, 3% dry skimmed milk in PBS) and immunostained overnight at 37°C by 1:300 rabbit anti-53BP1 (NB 100-305; Novus Biologicals) and mouse anti-TRF1 (ab10579; Abcam) in blocking solution. After 3 rinses in 0.8%, PBS, 1.5% dry skimmed milk, 50% NaCl, slides were incubated 1 hour in blocking solution containing Alexa Fluor 555–conjugated anti–mouse (A-31 570; Molecular Probes/Interchim) and Alexa Fluor 488 anti–rabbit (A-21 206; Molecular Probes) antibodies at 37°C. Slides were mounted in VectaShield antifade reagent (Vector Laboratories/CliniSciences) and observed thanks to a confocal microscope (Axioplan2 LSM510, Carl Zeiss) with plan achromatic 63×/1.4 oil objective and the Zeiss LSM510 v3.2 acquisition software. Optical sections were recorded at room temperature with a z-step < 200 nm to determine the occurrence of 53BP1/TRF1 colocalizations, at least 30 nuclei per condition were analyzed.

**Chromatin immunoprecipitation**

Formaldehyde-fixed cells (0.75%) were washed and lysed. After nuclei membrane lysis, chromatin was recovered and sonicated on BioRuptor Sonicator (Diagenode). Chromatin (about 800-bp fragments) was separated in different fractions (corresponding to 2.5 million cells for the IgG and 1 million cells for the input and each histone directed antibody used), and precleared (premixed protein A and G sepharose 4 Fast-Flow 50%/50%; Amersham). Fractions were incubated overnight at 4°C with 5 μg of anti-H3K9 trimethylated (07-442; Millipore), H3K9 acetylated (07-352; Millipore), total H3 (ab1791; Abcam), or 10 μg of irrelevant mouse immunoglobulin (IgG, Sigma-Aldrich). After 1 hour of incubation precleared beads were washed 4 times in buffer of increase stringency. Eluates were, subjected to RNase A treatment (1 hour, 37°C, 1 mg of RNAse A; Roche), reverse cross-linked (65°C, overnight in 20 mM NaCl). Protein digestion was done at 45°C for 1 hour with 20 μg of Proteinase K (Roche). DNA was purified (classic phenol/chloroform method) before being spotted thanks to Bio-Dot SF system (Bio-Rad). Ten percent of DNA precipitated was spotted on a Hybon N+ membrane (GE Healthcare). DIG-labeled (DIG-High Prime kit; Roche Applied Bioscience) telomeric (400 bp of repeated 5′-T2AG3-3′ motif) or ALU (173 bp of genomic DNA amplified using primers 5′-TGGAAACCCGTCCTCCTACTAAAAA-3′ and 5′-GTTCTGCTGCTGTCGCCCA-3′). (Plasmids are available on request to E.G. or B.H.) Probes were used for membrane hybridization. Chemiluminescent signals were formed double immunofluorescence analyses: interphasic cells (arbitrary units) in function of the percentage of input.

**Southern blot determination of telomere size (Teloblot)**

Telomere size was determined by terminal repeat fragment (TRF) experiments, considering the maximal intensity peak by using Image J software. As expected,31,32 the CLL patients exhibited an increased BCL2 expression and decreased Ki67 expression, known to be responsible for a higher antiapoptotic activity in a low proliferating rate context (Figure 2A). Importantly, this cohort of patients harbors the previously published features of telomere changes, that is, a general decrease in telomere length (P = .0025) in CLL lymphocytes compared with normal B cells and an even shorter length in IGVH unmutated versus mutated patients (P = .0015; Figure 2B). Therefore, we are studying a representative panel of mutated and unmutated patients as far as classic CLL markers and telomere length are concerned.

**Results**

**Study design**

We performed our experiments on B lymphocytes purified from the peripheral blood of 12 healthy donors and 23 newly diagnosed and previously untreated stage A CLL patients coming from 2 different hospitals. The patients fulfilled the classic CLL phenotypic and molecular criteria (Table 1 and see “Patients and healthy donors”). As expected,31,32 the CLL patients exhibited an increased BCL2 expression and decreased Ki67 expression, known to be responsible for a higher antiapoptotic activity in a low proliferating rate context (Figure 2A). Importantly, this cohort of patients harbors the previously published features of telomere changes, that is, a general decrease in telomere length (P = .0025) in CLL lymphocytes compared with normal B cells and an even shorter length in IGVH unmutated versus mutated patients (P = .0015; Figure 2B). Therefore, we are studying a representative panel of mutated and unmutated patients as far as classic CLL markers and telomere length are concerned.

**Telomere dysfunction in early stages of CLL oncogenesis**

Recent studies8,10,33-35 have reported telomeric damage11,12 and imbalances of shelterin protein expression in CLL,8 but no study has as yet addressed the question of the origin of telomere damage in these patients. To determine whether CLL telomerases are deprotected and thereby recognized as DNA damages, we performed double immunofluorescence analyses: interphasic cells were immunostained with antibodies against TRF1 (a marker of telomeres) and 53BP1 (a DDR factor). The data were analyzed by...
confocal microscopy to score the number of 53BP1 foci colocalizing with telomeres forming the so-called TIFs (telomere dysfunction–induced foci). In our experimental conditions, we only rarely detected the presence of 53BP1 foci in B cells from healthy donors (median of 0.03 focus per nucleus; Figure 3B-C). However, in CLL we found a significant 6-fold increase in 53BP1-positive foci (median of 0.18 focus per nucleus, \( P = 0.0485 \)). As a comparison, we found a 17-fold increase in 53BP1 foci after 5 Gy \( \gamma \) irradiation in one of the patients (Figure 3A). We observed an even more statistically relevant increase in TIF in CLL versus healthy donors (median of 0.08 vs 0 TIF per nucleus, \( P = 0.02352 \); Figure 3B-C). Thus, TIF represent roughly 44% of 53BP1 foci in B-CLL cells, thereby indicating that a significant proportion of DNA damage is linked to telomere dysfunction in CLL. In agreement with these median values, we were unable to detect TIF in 63% (7/11) of healthy and 22% (4/18) of CLL cells, allowing us to classify healthy and CLL donors in subgroups named TIF+ (presence of TIF) and TIF− (absence of TIF). These data confirm a previous study showing the presence of TIF in CLL cells and further show that TIF occur during early stage of CLL. It is worth noting that the increase of TIF in CLL might have been underestimated because very short telomeres might not lead to detectable TRF1-positive foci.

**Telomere deprotection in CLL patients does not correlate with mean telomere length**

Because CLL cells have shorter telomeres than normal B cells, we asked whether the increased frequency of TIF in CLL patients is because of an excessive telomere shortening. Strikingly, whereas

![Image of Figure 2](image-url)

**Figure 2. CLL classic molecular and telomeric features.** (A) BCL2, MKI67, and CDKN2A (respectively coding for Bcl2, Ki67, and p16) normalized gene expression level, quantified in purified B cells from 6 healthy donors and 20 stage A CLL patients. Wilcoxon test \( P \) values are indicated in each corresponding box plot. (B) Telomeric size was analyzed by Teloblotting on B lymphocytes purified from 12 healthy donors and 20 CLL patients. Box plot showing telomere size, with corresponding Wilcoxon test calculated \( P \) values in healthy (Co) versus CLL (CLL) B cells (left) and in mutated (M) versus unmutated (UM) CLL B cells (right). A representative picture of telomeric restriction fragment analysis of one normal B cells (Co), 5 CLL mutated patients (CLL M), and 5 CLL unmutated patients (CLL UM) is shown (right). Telomere size (in kb) was calculated thanks to specific ladder, size is indicated on each side of the Teloblot.

![Image of Figure 3](image-url)

**Figure 3. Telomeric damage induced foci in B cells from healthy donors and CLL patients.** Confocal immunofluorescence analyses of 53BP1 foci and TRF1/53BP1 colocalization (TIF). (A) CLL B cells isolated from one CLL patient were subjected (IR) or no (Co) to 5 Gy \( \gamma \)-IR and fixed for immunostaining 1 hour latter. Median number of 53BP1 focus per nucleus is shown. (B-C) B cells from 11 healthy donors (Co) and 18 CLL patients (CLL) were immunostained for 53BP1 and TRF1; 53BP1 foci and TIF were scored after confocal analysis. (B) Box plot showing number of 53BP1 foci (left) and TIF (right) per nucleus; Wilcoxon-calculated \( P \) values are indicated in each box plot. (C) Immunofluorescence representing pictures of 53BP1 foci (left), TRF1 labeling (middle), and TIF (right) in B cells from healthy donor (top) and CLL patient (bottom). Arrows indicate TIF; TOTO staining is outlined in the equatorial plane of the nucleus. Detailed of \( \times 60 \) magnification are shown after z-stack reconstruction (Image J software).
telomeres tend to be shorter in the TIF\(^+\) healthy subgroup, this is not the case in the TIF\(^+\) CLL subgroup (Figure 4). We also used as a cutoff the maximal number of TIF observed in healthy donors, this segregated CLL patients into 2 equal groups (TIF low and TIF high). Even using this method we failed to observed a diminution in telomere size in the TIF-high subgroup (supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Thus, if a shorter telomere length could be the principal cause of telomere dysfunction in normal B cells, it does not seem to be the case in CLL cells of stage A patients. This suggests that the appearance of TIF in early stages of the disease does not mainly result from replicative erosion but rather from altered capping functions. This does not exclude the possibility that some TIFs result from a complete or nearly complete deletion of telomeres as previously observed in more advanced stage.\(^{12}\) In agreement with this view, whereas no clear correlation is found between telomere shortening and TIF increase when normal individuals are compared with stage A patients (\(P = .1526\)), the correlation is significant (\(P = .0047\)) comparing normal individuals to 4 cases of stage B and C patients (supplemental Figure 1).

**Telomeric nucleosome status in CLL**

Next, we wonder whether telomeric chromatin alterations could explain TIF formation in CLL cells. Thus, we performed ChIP experiments against histone modifications known to characterize “repressive” (H3 lysine 9 trimethylation: H3K9trim), or “active” chromatin (H3 lysine 9 acetylation: H3K9ac) and against total histone H3. We used an irrelevant IgG as a control and estimated telomeric versus Alu sequences enrichment in the immunoprecipitated fractions. We did not observe any significant alterations of the tested histone marks in CLL compared with normal samples, although we noted a trend toward an increased H3K9ac in CLL samples (\(P = .0539\); Figure 5A-B). Interestingly, we also observed a propensity of the TIF\(^+\) subgroup toward an increased presence of the H3K9ac mark at telomeres while this trend is totally lost in the TIF\(^+\) subgroups of CLL patients (Figure 5C). Although we failed to establish any statistically significant difference in the telomeric chromatin status of healthy versus CLL cells, our results are compatible with a common modification of chromatin state in CLL with a trend toward a more open conformation.

**A decreased expression of TINF2 and ACD correlates with TIF formation in CLL**

Because loss or imbalance of shelterin components can induce TIF formation and because we previously reported a deregulation in shelterin gene expression in CLL, we asked whether TIF formation correlates with deregulation of shelterin expression in CLL cells. Therefore, we compared the mRNA expression levels of TERF1, TERF2, TINF2, TERF2IP (coding for RAP1), POT1, and ACD (coding for TPP1) between the TIF\(^-\) and TIF\(^+\) subgroup of CLL patients. There is a global tendency toward a down-regulation of shelterin component mRNA level in the TIF\(^+\) CLL subgroup.
The presence of telomere DNA damage-induced foci (TIF) in CLL patients correlates with a reduced expression of the ACD and TINF2 shelterin genes but not with short telomeres.

It was previously reported that telomere shortening because of cell proliferation (in the absence of telomerase) results in TIF accumulation associated with an increase in telomeric H3K9 acetylation. In agreement with this scheme, we found that the presence of TIF in normal B cells tends to correlate with shorter telomeres and an increased presence of H3K9ac at telomere. However, there is no tendency toward telomere shortening in patients harboring a detectable level of TIF. Thus, the TIF increase in our cohort of stage A patients cannot be merely explained by telomere shortening because of cell overproliferation. In agreement with previous works showing an increased rate of proliferation and telomere crisis in advanced stage of CLL, we observed a significant correlation between the presence of TIF and short telomeres in patients at stages B and C. We therefore conclude that early stages of CLL is associated with telomere deprotection, which cannot be merely explained by telomere shortening, while advanced stages, probably as a consequence of an increased number of cell divisions, lead to critically short telomeres triggering DNA damage response and chromosome end fusion.

We noted a global decrease in the expression of shelterin component encoding genes in the stage A CLL patients harboring telomere damage and in particular a significant decrease in the expression of both ACD and TINF2 genes. Considering the shelterin complex conformation, TRF1 and TRF2 bind the duplex telomeric DNA and POT1 the single-strand 3’ overhang. POT1 function as an heterodimer with TPP1 (encoded by ACD) which enhances its binding to telomeres. TIN2 (encoded by TINF2) plays a major role in the complex stabilization, by bridging TPP1, TRF1, and TRF2. Thus, both ACD and TINF2 encode proteins playing a central role in the formation of the shelterin protein bridge linking the duplex part to the 3’ overhang of telomeric DNA. Their loss is thus expected to dissociate an essential capping structure. Therefore, we propose that increased TIF occurrence in stage A CLL patients results, at least in part, from a partial disassembly of the shelterin complex.

What could be the role of damaged telomeres in CLL pathogenesis? Because the presence of TIF is a hallmark of senescent cells and of senescent T lymphocytes in particular, we propose that early stage CLL is associated to accelerated B-lymphocyte senescence. This might result from the accumulation of various telomere dysfunctions in the B-cell lineage, including telomerase and shelterin down-regulation (and this work). In agreement with this view, CLL B cells are known to be initially G0 arrested and we observed an increase in p16 expression in our cohort of CLL patients (Figure 2A). Escape from senescence induce telomere-excessive shortening, fusion, and cell-crisis entry and promote genome instability. Increase in cell proliferation, fused-telomere and crisis-like phenotype have very recently been reported in more advanced stage of CLL. It could thus be possible that progression toward more advanced stage or transformation in the aggressive Richter syndrome are associated with senescence bypass.

To our knowledge, this work provides the first evidence of telomere deprotection in a human malignancy in link with an alteration of the shelterin complex. Interestingly, these damages occur at an early stage of the disease, suggesting that they contribute to the early step of malignant transformation. This enlightens the concept, well documented in mouse models but still hypothetical in human cancer, that telomere dysfunction facilitates cancer initiation.

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Authorship

and E.G. and D.P. designed the research, analyzed the results, made the figures, and wrote the paper.
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References

Telomeric damage in early stage of chronic lymphocytic leukemia correlates with shelterin dysregulation

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