Tumor Biol.
DOI 10.1007/s13277-015-3556-2

RESEARCH ARTICLE

Telomere shortening associated with increased genomic complexity in chronic lymphocytic leukemia

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Received: 9 March 2015 / Accepted: 11 May 2015

Abstract Telomeric dysfunction has been proposed as an emerging prognostic factor in chronic lymphocytic leukemia (CLL). We have explored the relationship between telomere length (TL) and chromosome alterations studied by fluorescence in situ hybridization (FISH) and conventional cytogenetics in 107 newly diagnosed CLL patients; 61 normal controls were also evaluated. Results were correlated with clinical parameters and outcome. Absolute TL measurement was carried out on DNA samples by real-time quantitative PCR. A significant telomere shortening in patients compared to controls was observed (p=0.0001). The analysis taking into account FISH risk groups showed shorter TLs in cases with del11q/17p compared to patients with 13q14 deletion as a single alteration (p=0.0037), no alterations (NA) (p=0.028), and cases with abnormal karyotypes (p=0.014). In addition, a significant TL reduction in cases with two or more anomalies with respect to those with NA (p=0.033) and with one alteration (p=0.045), and no differences compared to cases with deletions 11q/17p were observed. Patients with only one anomaly did not show statistical differences with respect to controls; meanwhile, a significant TL reduction in cases with two or more aberrations was observed (p=0.025). The shortest telomeres were associated to 11q/17p deletion with significant differences compared to the remaining groups ($p \leq 0.045$). Significantly shorter treatment free survival in patients with two or more alterations compared to those with NA plus one abnormality was observed (p=0.0006). Our findings support the association between short TL and chromosome alterations in CLL and indicate the importance of telomere dysfunction in driving genomic instability in this pathology.

Keywords Chronic lymphocytic leukemia · Telomere length · FISH · Cytogenetics

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Electronic supplementary material The online version of this article (doi:10.1007/s13277-015-3556-2) contains supplementary material, which is available to authorized users.

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Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common types of adult leukemia in the Western world [1]. The disease is characterized by a highly variable clinical course, with time to progression ranging from months to decades. Although the Rai [2] and Binet [3] staging systems are reliable predictors of outcome between staging groups, they do not distinguish patients who will evolve to a more aggressive disease from those who will remain indolent. Specific alterations in gene expression as well as the presence of different genomic aberrations are thought to underlie the variability in disease outcome [4]. In the last decades, several prognostic biomarkers have been identified, allowing the subdivision of this heterogeneous disease into clinical relevant subgroups. Genomic abnormality is one of

Published online: 26 May 2015

the subgroups that demonstrated to have predictive value for patient course and outcome, allowing the identification of distinct cytogenetic risk groups. Among them, cases with deletions 17p13 or 11q22 show the shortest median survival; patients with deletion 13q14 as a single alteration are associated with the better outcome, while cases with trisomy 12 have an intermediate prognosis [5]. In addition, cytogenetic studies in CLL have also proved to be an important tool in the biologic characterization of this disease. Specific cytogenetic patterns, particularly complex karyotypes, provide additional information for clinical outcome and have independent prognostic significance [6–8].

Telomeres are specialized protective structures at the end of eukaryotic chromosomes. They are essential to maintain chromosomal integrity and genome stability. Because of the end-replication problem, telomeres progressively shorten with repeated cell division, leading to telomere dysfunction and, ultimately, contributing to tumorigenesis. In tumor cells, short telomeres were associated with genetic instability and particularly with the origin of bridge-breakage events leading to genome reorganization [9]. Telomere erosion may be critical in tumor suppression as it impairs cell proliferation [10]. Telomeric dysfunction has been proposed as an emerging prognostic factor in CLL as short telomere length (TL) was associated with unmutated status of the immunoglobulin heavy-chain variable region gene, high-risk genomic aberrations, and poor outcome [11–15]. Besides, an association between short TL and genetic complexity measured by fluorescence in situ hybridization (FISH) analysis and/or the large of copy number aberrations detected by high resolution SNP-arrays was described [12, 16, 17]. In this report, we have explored the relationship between TL and chromosome alterations, studied by FISH and conventional cytogenetics, in newly diagnosed CLL patients. Results were also analyzed in relation to clinical parameters and outcome.

Materials and methods

Patients

This study included 107 patients with CLL at diagnosis (63 males; mean age 64.5 years, range 36–89 years) cytogenetically studied in our laboratory, from whom DNA samples were available. Patients were diagnosed according to the National Cancer Institute-Working Group CLL criteria [18]. Rai stage [2] was available in 85 cases with the following distribution: 0: 29 (34.1 %); I–II: 44 (51.8 %); and III–IV: 12 (14.1 %). Table 1 shows clinical and biological characteristics of patients. In addition, 61 normal controls (without a medical history of cancer) (21 males; mean age 56.5 years; range 21–91 years) were also studied. The study was approved by the Ethics Committee of our institution. All individuals provided their informed written consent.

Table 1 Clinical and biological characteristics of CLL patients

Characteristics	CLL patients
No. of patients (*n*)	107
Sex F/M	43/64
Mean age (years) (range)	64.5 (36–89)
Clinical stages (85 cases) (%)	
Rai 0	29 (34.1)
Rai I–II	44 (51.8)
Rai III–IV	12 (14.1)
Mean WBC count ($\times 10^9$/L) (range)	41.11 (5.4–210)
Mean lymphocyte (%) (range)	76.38 (34–97)
Mean Plt count ($\times 10^9$/L) (range)	213.3 (16.4–900)
Mean Hb (g/dL) (range)	12.6 (7–15.5)
Mean LDH (UI/L) (range)	371.1 (128–882)
Mean β2M (μg/mL) (range)	3.21 (1.17–15)

F female, *M* male, *WBC* white blood cells, *Plt* platelets, *Hb* hemoglobin, *LDH* lactate dehidrogenase, *β_2M* β_2-microglobulin

Cytogenetics and FISH analysis

Chromosome analyses were performed on peripheral blood lymphocytes, cultured for 96 h at 37 °C in F-12 medium supplemented with 15 % of fetal calf serum, stimulated with Pokeweed mitogen. Slides were prepared by conventional method. G-banding technique was used. For FISH analysis, slides were hybridized with SE 12, OLE13q14 D13S319, OLE11q22.3 (ATM), and 17p13.1 TP53 DNA probes (LiVE-LEXEL, Buenos Aires, Argentina) according to the manufacturer's protocol. Two hundred interphase nuclei were analyzed for each probe. The cut-offs for positive values (mean of normal control+3 standard deviations) determined from ten cytogenetically normal donors were as follows: 3, 10, 7.5, and 5.5 % for trisomy 12, monosomies of D13S319, ATM, and TP53, respectively.

Absolute telomere length analysis

High-molecular weight genomic DNA was obtained from peripheral blood mononuclear cells of CLL patients and controls, separated on a Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden) density gradient. Absolute TL measurement was carried out by real-time quantitative PCR (qPCR) in a LightCycler system (Roche Diagnostics)

according to Panero et al. [19]. For each DNA sample, two consecutive reactions were performed: the first to amplify a single copy gene, *RPLP0* (ribosomal phosphoprotein P0) (12q24.2), and the second for telomeric sequence. Primer sequences are shown in the supplementary online resource table S1. Briefly, both PCRs were performed in a final volume of 20 μl using 1X SYBRGreen Master Mix (Roche), 100 nmol/L of primers, and 20 ng of DNA from patients and controls. The PCR conditions were as follows: 95 °C 10 min, followed by 45 cycles of 95 °C 15 s, 60 °C 1 min. The melting curve was performed with 1 cycle of 95 °C 20 s, 50 °C 15 s, and 98 °C with a temperature ramp of 0.1 °C/s. All samples were analyzed in duplicate. No difference in cases with trisomy 12 using *RPLP0* or *β-globin* (on chromosome 11) as a single copy gene was previously observed [20], indicating that a disomy vs. trisomy DNA difference is of minor importance after PCR amplification.

In 46 CLL patients, the absolute qPCR technique was validated by terminal restriction fragments (TRF) analysis, performed according to previously described [21] ($p<0.0001$) (supplementary online resource figure S1). In order to convert the absolute TL (kb/diploid genome) into the equivalent Southern blot TL values (kb), the regression formula: $y=0.0746x+0.5285$, where $x=\text{Log [TL (qPCR)]}$ and $y=\text{Log [TL (TRF)]}$, was used.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0 (2008). Comparison of TL among groups was performed using the Mann-Whitney test and paired *t* test. Groupwise comparison of the distribution of clinical and laboratory variables was performed with the Student *t* test (for quantitative variables) and the χ^2 or Fisher's exact test (for categorical variables). Treatment free survival (TFS) calculated from the date of diagnosis to the first CLL-specific treatment were estimated by the Kaplan-Meier method and compared with the Log-rank test. For all tests, $p<0.05$ was considered statistically significant.

Results

In our series, the analysis of TL showed a significant telomere shortening in CLL patients (6.80±0.68kb) compared to the whole control group (7.26±0.9 kb) ($p=0.0001$) (Fig. 1) and particularly with respect to 40 age-matched (±2 years) control individuals (16 males; mean age 64.8 years; range 34–88 years) (7.09±0.48 kb) ($p<0.0002$). In line with previous studies [11, 22], TL in CLL patients did not correlate with age or sex ($p=0.13$ and $p=0.1$, respectively), supporting that these features have little or no effect in cancer cells (Fig. 2). On the contrary, a significant correlation between TL and age in the control group ($p<0.0001$; r^2: 0.4545) (Fig. 2) was found.

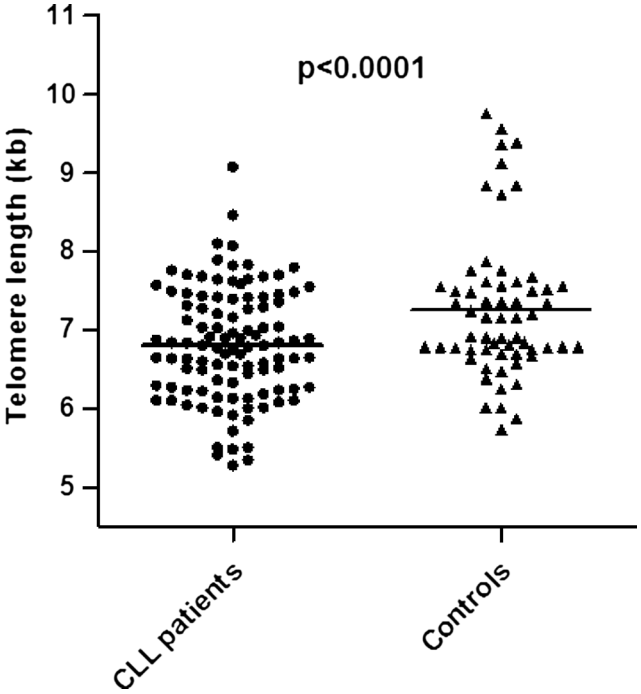

Fig. 1 Telomere length distribution in 61 controls and patients with chronic lymphocytic leukemia (CLL) ($p=0.0001$). The absolute TL (kb/diploid genome) data were converted into the equivalent Southern blot TL values (kb)

Our cohort included 107 CLL patients, 63 of them with normal karyotypes and the remaining 44 with clonal chromosome aberrations: 14 cases with trisomy 12 as a single alteration and 30 with structural and numerical anomalies (group CA: chromosome alterations). Supplementary online resource figure S2 shows the distribution of cytogenetic abnormalities

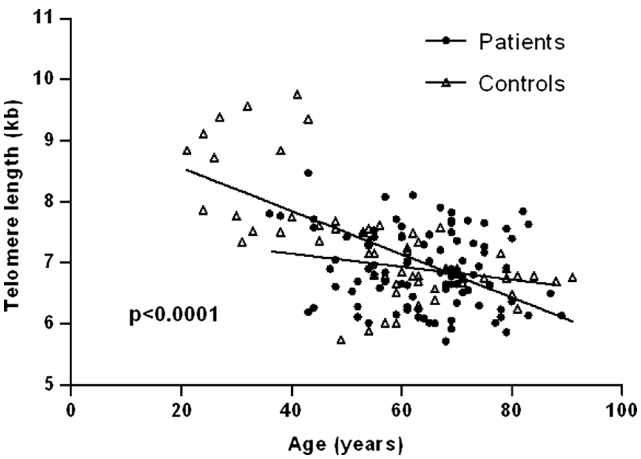

Fig. 2 Significant correlation between TL and age in the control group ($p<0.0001$; r^2: 0.4545) and no significant association in CLL patients ($p=0.13$). The absolute TL (kb/diploid genome) data were expressed as the equivalent Southern blot TL values (kb)

Table 2 Distribution of CLL patients according to cytogenetic and FISH groups

Group	No. of cases (%)	Age (years) (range)	Sex		Karyotype	Mean TL±SD (kb)
			F	M		
del13q14 single	24 (22.4)	64.3 (36–83)	8	16	Normal	6.99±0.57
+12	14 (13.1)	68.3 (54–76)	5	9	Trisomy 12	6.55±0.91
No alterations	18 (16.8)	60.6 (43–87)	12	6	Normal	6.96±0.77
del11q/17p	21 (19.6)	66.6 (43–89)	8	13	Normal	6.47±0.56*
Chromosome alterations	30 (28.0)	63.7 (44–82)	10	20	Abnormal	6.89±0.59

F female, *M* male, *TL* telomere length, *SD* standard deviation

*Significant differences between patients with del11q/17p with respect to cases with: del13q14 single (p=0.0037), no alterations (p=0.028), and with chromosome aberrations (p=0.014)

by chromosome of our series. Characteristics of patients in different FISH and cytogenetic groups are shown in Table 2. Seventeen cases showed more than one FISH alteration.

The analysis taking into account FISH risk groups showed a progressive TL reduction from del13q14 as a single alteration to del17p through cases with NA, +12, and del11q. Due to comparable TL among cases with del11q (n=10) (6.53±0.68 kb), del17p (n=7) (6.48±0.50 kb), and both alterations (n=4) (6.34±0.41 kb), they were evaluated together as a whole group named del11q/17p (6.47± 0.56 kb). Similar median TL levels were detected in patients with del13q14 as a single abnormality and those with NA compared to controls. Statistical differences in TL between patients with del11q/17p compared to cases with 13q14 deletion as a single alteration (p=0.0037) and NA (p=0.028) were observed; however, no difference with respect to cases with trisomy 12 (6.55±0.91 kb) was found. Patients with del11q/17p also showed shorter TL than cases with CA (p=0.014). Furthermore, the analysis considering the number of FISH alterations showed a significant TL reduction in patients with two or more anomalies (6.43±0.63 kb) compared to those with NA (6.96± 0.77 kb) (p=0.033) and with one alteration (6.82± 0.69 kb) (p=0.045) (Fig. 3). In addition, the distribution of FISH genomic alterations in relation to TL showed the shortest telomeres associated to poor risk abnormalities (del11q/17p), with significant differences with respect to cases with del13q14 and NA (p=0.042), CA (p=0.041), and the entire cohort (p=0.045) (Table 3).

In reference to the CA group, it showed shorter TL compared to the whole control population (p=0.046). When they were divided according to the number of alterations, a progressive TL reduction from cases with one alteration (6.92±0.66 kb), two alterations (6.83±0.79 kb), and three or more abnormalities (6.78±0.48 kb) were found. Patients with only one anomaly did not show statistical differences with respect to controls, meanwhile, a significant reduction was observed in cases with two or more alterations (6.79±0.56 kb) (p=0.025). Furthermore, a reduction of TL in cases with two or more alterations compared to cases with NA plus one alteration (6.96±0.74 kb) was detected but without significant differences (Fig. 4). No differences in mean TL between cases with two or more anomalies detected by FISH or cytogenetics and those with deletions 11q/17p were observed.

The analysis of clinico-biological characteristics in relation with TL was also performed. No significant differences according to known prognostic factors in CLL were observed

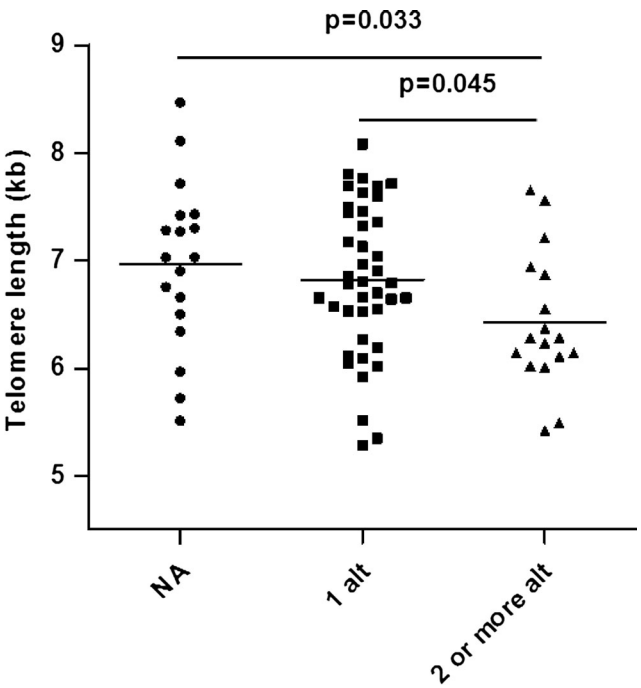

Fig. 3 Analysis of telomere length distribution expressed in the converted Southern blot values, considering the number of FISH alterations in CLL patients. Significant differences in the mean TL for cases with two or more alterations (2 or more alt) compared to those with no alterations (NA) (p=0.033) and with a single alteration (1 alt) (p=0.045)

Table 3 Distribution of FISH genomic alterations in relation to telomere length

Telomere length	NA	del13q14	+12	del 11q/17p	CA	Total patients
Short (<6.8 kb)	7	10	8	16*	13	53
Long (≥6.8 kb)	11	14	6	5	17	54

*Significant differences with respect to patients with: del13q14 single (p=0.042), no alterations (NA) (p=0.042), chromosome alterations (CA) (p=0.041), and the whole cohort (p=0.045)

either taking into account patients with short vs. long TL or when cases with two or more alterations by FISH or cytogenetics were compared to those with NA plus one abnormality. Nevertheless, a tendency to higher β_2-microglobulin levels in cases with two or more alterations by FISH or cytogenetics (5.29 μg/mL) than those with NA plus one abnormality (2.88 μg/mL) (p=0.07) was found. The analysis of TFS, indicated a significantly shorter survival in patients with two or more alterations, who also showed the shortest mean TL, compared to those with NA plus one abnormality (p=0.0006) (Fig. 5a). In addition, although no significant differences were found, reduced TFS in patients with short TL (40 months) than cases with long TL (96 months) was also observed (Fig. 5b).

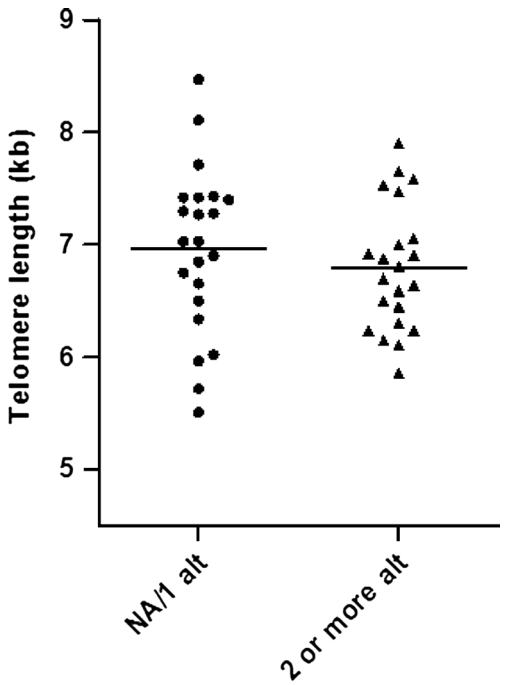

Fig. 4 Telomere length distribution expressed in the converted Southern blot values, taking into account the number of cytogenetic abnormalities (structural and numerical anomalies) in CLL patients. No differences in cases with two or more alterations (6.79±0.56 kb) compared to cases with NA plus one alteration (6.96±0.74 kb) were observed

Discussion

Dysfunctional telomeres have been related to genomic alterations, including loss of heterozigosity, gene truncation and aneuploidy, and also with the development of cell clones with an aggressive tumor phenotype. In this study, we have analyzed the relationship between TL and chromosome abnormalities detected by FISH analysis and conventional cytogenetics in patients with CLL. Different methodologies have been described for the study of TL, including TRF, FISH, flow cytometry, and the measurement of relative average telomere lengths by qPCR [23]. We have used for the first time qPCR assay to quantify absolute TL in CLL patients, confirming the usefulness of this technique for telomere measurements in this disease.

Our results showed an association between short telomeres and high-risk genomic aberrations and the presence of two or more cytogenetic alterations, supporting previous findings based in other methodologies [12, 13, 24]. Conversely, CLL cases with long TL were characterized by deletion 13q14, no cytogenetic alterations or only one abnormality. In agreement with our findings, a similar correlation between TL and the number of genomic aberrations was previously reported [12, 17], supporting dysfunctional telomeres may be a key event driving genomic instability in tumors and correlate with the emergence of increased genomic complexity [25].

However, there is scarce information about the association between TL and chromosome abnormalities detected by conventional cytogenetics. In lymphoid malignancies, an association between short TL and chromosomal abnormalities or increased frequency of telomeric associations was reported in multiple myeloma [21, 26], but no difference in TL between cases with normal and abnormal karyotypes or according to different genomic aberrations in patients with mantle cell lymphoma was found [27, 28]. Our results in CLL support a role for telomere shortening at the origin of genomic instability [24] and reinforce previous reports showing the adverse prognostic value of chromosome alterations in this pathology [6, 8, 29]. In this line, Mansouri et al. [17] found that an increased number of large copy number aberrations as detected by high resolution SNP-arrays correlated with decreasing TL in CLL patients. Rossi et al. [16] showed that short telomeres are an independent predictor of Richter's syndrome transformation, process associated to increased genetic complexity. Furthermore, Lin et al. [24] found a critical reduction in TL during progressive disease leading to loss of telomere end-capping function, chromosome end fusions and genomic instability, and demonstrated that they are common events in CLL patients. Most of our patients (86 %) were studied at an early stage of the disease. Different authors [22, 30, 31] found evidences of telomeric damage in early stage patients suggesting that short telomeres could be a possible marker for clinical progression.

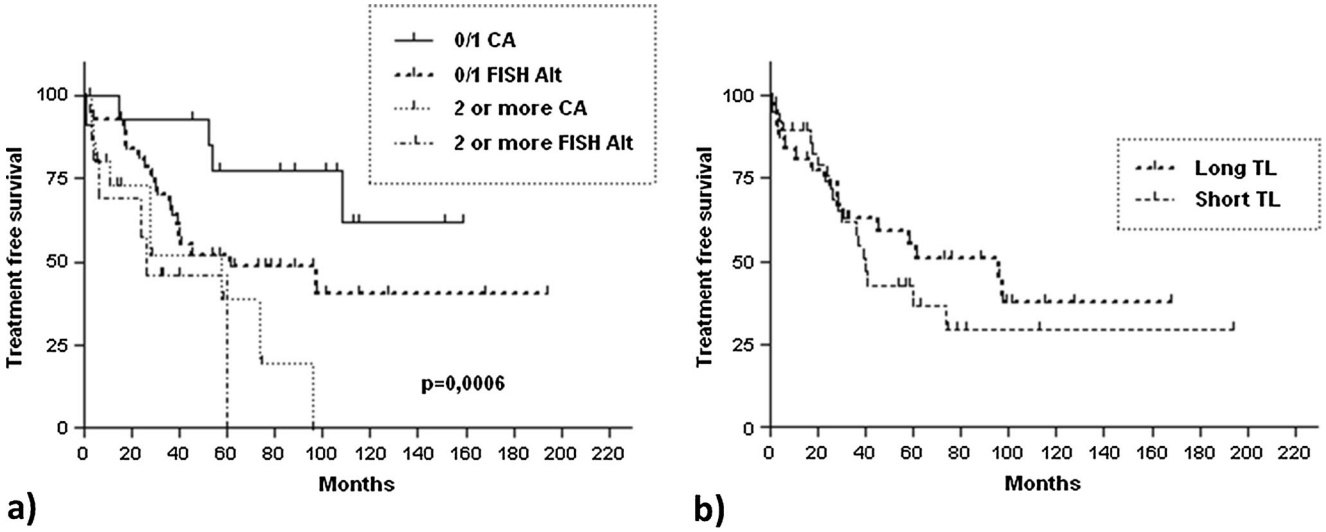

Fig. 5 Kaplan-Meier plot of treatment free survival (TFS) in CLL patients. **a** Significant differences between patients with two or more alterations compared to those with no alterations plus one abnormality (p=0.0006); **b** Shorter TFS in patients with short telomere length (TL) (40 months) compared to cases with long TL (96 months). *CA* chromosome alterations, *Alt* alterations

In addition, Ricca et al. [11] referred preliminary results indicating that TL is not always stable over time in CLL, and suggesting that TL is influenced by the natural history of the disease. In agreement with these findings, three patients of our series were studied both at diagnosis and during clonal evolution showing a clear reduction in their TL (data not shown), supporting telomere dysfunction as an important event in the accumulation of chromosome alterations during disease progression. Nevertheless, a recent report [17] also detected modifications in TL during the course of the disease, showing that cases with long TL at diagnosis had the highest telomere attrition, whereas patients with short telomeres could show elongated TL. More cases will be evaluated to clarify these findings.

In addition, no differences in TLs in cases with two or more anomalies compared to patients with deletions 11q/17p were observed, supporting the association of short TL with chromosome alterations in CLL. As known, 11q/17p genomic alterations determine survival advantage and increased cell proliferation that promote telomere attrition [32]. Similarly, a rapid kinetics of progression, as documented by short time to lymphocyte doubling, time to progression to a more advanced stage and increased levels of proliferation markers, was observed in CLL cases with genomic complexity, indicating the importance of telomere dysfunction in driving genomic instability and clonal evolution in this pathology. In this context, Brugat et al. [33] found that a subset of CLL cells from patients with unfavorable clinical outcome harbor a novel type of telomeric abnormalities resulting from telomere dysfunction, which were associated with the appearance of multiple cytogenetic aberrations and shorter mean TL.

In reference to clinical characteristics, a significant shorter TFS in patients with two or more alterations compared to those with NA plus one anomaly was found, supporting a strong correlation between short telomeres and genetic complexity, and showing their impact on clinical outcome. These findings are in agreement with previous studies including those of our group, showing the adverse clinical significance of CA and complex karyotypes in CLL [6, 8, 29], and reinforce the importance to identify this subset of patients in the design of risk-adapted treatment strategies [34]. In addition, although no significant differences were found, shorter TFS for patients with less than the median TL value compared to cases with higher telomere lengths was observed. These results are in concordance to those previously found [15, 16, 22, 31], supporting the participation of telomere dysfunction in disease progression. However, in multivariate analysis, TL was not demonstrated to be an independent prognostic factor for overall survival and time to first treatment [17, 22], suggesting that it could be probably related to its strong association with poor risk factors in CLL.

In conclusion, our findings support the association of short TL with genetic aberrations and genomic complexity, detected by FISH or conventional cytogenetics, in CLL and indicate the importance of telomere dysfunction in driving genomic instability in this pathology. Thus, the assessment of telomere length becomes important not only in understanding the biological characteristics of the disease but also in providing useful prognostic information and the opportunity to choose the best patient population for new therapeutic approaches with telomere inhibitors.

Acknowledgments This work was supported by grants from the National Research Council (CONICET) (PIP N°: 517), the National Agency of Scientific and Technical Promotion (ANPCyT) (PICT No: 2008–0465), and the Alberto J. Roemmers Foundation.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individuals included in the study.

Conflicts of interest None

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