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Article

Telomere-Driven Checkpoint Remodeling Is Associated with Cytogenetic Risk in Chronic Lymphocytic Leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) exhibits marked clinical heterogeneity that is closely associated with genomic instability. Although cytogenetic abnormalities are widely used for risk stratification, they do not fully capture the biological complexity of the disease. Telomere dysfunction and alterations in DNA damage response pathways have been implicated in disease progression, but their relationship with cytogenetic risk in CLL remains incompletely characterized. In this study, peripheral blood samples from 48 CLL patients were analyzed. Cytogenetic profiles were obtained by conventional karyotyping following in vitro immunostimulation with DSP30 and interleukin-2 and classified according to ERIC and Döhner criteria. Telomere length was assessed by quantitative PCR, and *CHEK1* and *CHEK2* expression levels were quantified by RT–qPCR. Molecular parameters were compared across cytogenetic risk groups. Distinct molecular profiles were observed across cytogenetic categories. Favorable-risk CLL cases showed preserved telomere length, low *CHEK1* expression, and maintained *CHEK2* levels. Intermediate-risk cases, predominantly characterized by trisomy 12, exhibited moderate telomere shortening accompanied by increased *CHEK1* expression and partial reduction of *CHEK2*. High-risk CLL cases, defined by del(11q), del(17p), or complex karyotypes, displayed pronounced telomere shortening, marked *CHEK1* upregulation, and strong suppression of *CHEK2*. Telomere length was inversely correlated with cytogenetic risk (Spearman's $\rho = -0.68$, $p < 0.0001$), and the *CHEK1*/*CHEK2* expression ratio increased progressively with genomic complexity. These findings indicate that telomere length and *CHEK1*/*CHEK2* expression patterns are closely associated with cytogenetic risk in CLL and may provide complementary biological information for risk stratification.

Keywords: CLL; *CHEK1*/2; telomere; chromosomal abnormalities; genomic instability

Introduction

Chronic lymphocytic leukemia (CLL) is a biologically heterogeneous B-cell malignancy characterized by the progressive accumulation of mature but functionally impaired lymphocytes in peripheral blood, bone marrow, and lymphoid tissues [1,2]. Although CLL has traditionally been viewed as a disease driven primarily by defective apoptosis rather than high proliferative activity, leukemic cells are continuously exposed to endogenous genomic stress arising from replication errors, oxidative damage, and telomere erosion [3,4]. The capacity of CLL cells to tolerate and adapt to this chronic stress is increasingly recognized as a key determinant of clonal evolution, disease progression, and therapeutic resistance [5].

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Genomic instability represents a central hallmark of CLL and is closely associated with recurrent chromosomal abnormalities, including deletion of 13q14, trisomy 12, deletion of 11q22–23, and deletion of 17p13 [6,7]. These cytogenetic lesions define biologically distinct subgroups and are accompanied by varying degrees of disruption in DNA damage response (DDR) pathways [8]. Beyond chromosomal copy number changes, genomic instability in CLL is strongly influenced by telomere dysfunction, which constitutes a persistent and endogenous source of DNA damage signaling [9,10].

Telomeres are specialized nucleoprotein structures that protect chromosome ends from being recognized as DNA double-strand breaks. In CLL, telomere shortening and altered telomere length distributions have been consistently associated with increased chromosomal complexity and adverse clinical outcomes [11–13]. Critically shortened telomeres trigger sustained DDR activation, leading to checkpoint engagement and, under physiological conditions, cellular senescence or apoptosis [14]. In leukemic cells, however, this chronic telomere-associated stress imposes selective pressure on checkpoint signaling pathways, favoring adaptations that permit continued survival and proliferation.

Key mediators of DDR signaling include the checkpoint kinases *CHEK1* and *CHEK2*, which act downstream of ATR and ATM, respectively [15,16]. *CHEK2* is primarily activated in response to DNA double-strand breaks and telomere uncapping, enforcing cell cycle arrest and apoptosis when genomic damage is excessive [17]. In contrast, *CHEK1* is predominantly linked to replication stress signaling and facilitates S-phase progression under conditions of ongoing DNA damage [18]. An imbalance between these checkpoint pathways may therefore reflect adaptive signaling responses in genomically unstable CLL cells.

Based on this framework, the present study aimed to test the hypothesis that cytogenetic risk in CLL is associated with coordinated alterations in telomere length and *CHEK1*/*CHEK2* expression. Specifically, we investigated whether differential expressions of *CHEK1* and *CHEK2* correlate with telomere dysfunction across established cytogenetic risk subgroups, thereby reflecting distinct biological states associated with disease progression.

1. Material and Methods

a. samples

This study included 48 peripheral blood samples obtained from patients diagnosed with chronic lymphocytic leukemia (CLL), comprising cases with either normal or abnormal karyotypes. The cohort consisted of 32 men and 16 women, with a median age of 58.5 years (range: 48–74 years). CLL diagnosis was established in accordance with the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) criteria, based on sustained peripheral blood lymphocytosis (≥5 × 10^9/L), typical lymphocyte morphology, and immunophenotypic confirmation of clonal B cells expressing CD5, CD19, CD20, and CD23. Patients with a previous history of other hematological malignancies or hematological disorders were excluded. All participants provided written informed consent in compliance with the Declaration of Helsinki. The study protocol was reviewed and approved by the institutional Research Ethics Board for studies involving human subjects (protocol no. 1243/2017).

b. Metaphase induction and G-banding cytogenetic analysis

Metaphase chromosome preparations were obtained from 1×10^6 peripheral blood mononuclear cells (PBMCs) per sample. Cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS; Gibco, Thermo Fisher Scientific). Mitotic stimulation was performed using the CpG-oligonucleotide DSP30 (TIB Molbiol, Berlin, Germany) in combination with recombinant human interleukin-2 (IL-2; PeproTech, Rocky Hill, NJ, USA) to enhance mitotic yield. Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2 for 72 hours, after which colcemid (KaryoMAX™, Thermo Fisher Scientific) was added to arrest cells in metaphase.

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Chromosome harvesting and slide preparation were carried out using standard cytogenetic protocols, including hypotonic treatment and methanol–acetic acid fixation. G-banding was performed by trypsin–Giemsa staining, and karyotypes were described according to the International System for Human Cytogenomic Nomenclature (ISCN 2020). Metaphases were analyzed using an Axio Imager M1 microscope (Carl Zeiss, Oberkochen, Germany) equipped with digital image acquisition and karyotyping software (ISIS, MetaSystems, Altlussheim, Germany), achieving an average resolution of approximately 450 bands per haploid set. For each sample, a minimum of 20 metaphases was evaluated to ensure cytogenetic reliability.

c. **Interphase fluorescence in situ hybridization (FISH) analysis**

Interphase fluorescence in situ hybridization (FISH) analysis was performed to detect recurrent chromosomal abnormalities commonly observed in chronic lymphocytic leukemia (CLL). Commercially available locus-specific DNA probes were used to assess deletions of chromosome 13q14 (D13S319), 11q22–23 (ATM), and 17p13 (TP53), as well as trisomy 12 using a centromeric probe for chromosome 12 (D12Z1). All probes were obtained from Abbott Molecular/Vysis (Des Plaines, IL, USA) and applied according to the manufacturer's instructions. FISH analyses were performed on interphase nuclei prepared from peripheral blood mononuclear cells (PBMCs). Hybridization signals were evaluated using a fluorescence microscope equipped with appropriate filter sets. For each probe, a minimum of 200 interphase nuclei were analyzed per sample. Cut-off values for positivity were established based on internal laboratory validation and manufacturer recommendations and were defined as >5% abnormal nuclei for deletion probes (13q14, ATM, TP53) and >3% abnormal nuclei for trisomy 12. FISH results were independently interpreted by two experienced cytogeneticists, and discrepancies were resolved by joint review. Final FISH findings were integrated with conventional G-banding results for cytogenetic risk stratification according to established Döhner criteria.

d. **Gene expressions of CHEK1 and CHEK2 genes by qPCR**

CHEK1 and *CHEK2* gene expression levels were quantified in CLL PBMCs by reverse transcription quantitative PCR (RT–qPCR). PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque™ PLUS (Cytiva, Uppsala, Sweden). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), including on-column DNase digestion with RNase-Free DNase Set (Qiagen) to remove residual genomic DNA. RNA concentration and purity were assessed by NanoDrop™ spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was evaluated by agarose gel electrophoresis. Only samples with A260/A280 ratios between 1.8 and 2.1 were included. RNA was stored at −80 °C until use.

Complementary DNA (cDNA) was synthesized from 0.5–1.0 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). Quantitative PCR was performed using PowerUp™ SYBR™ Green Master Mix on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Gene-specific primers for *CHEK1* and *CHEK2* were designed to span exon–exon junctions when possible to avoid amplification of genomic DNA. The primer sequences used were as follows: *CHEK1* forward 5′-AGCTGAAGAAGCAGCAGTTC-3′ and *CHEK1* reverse 5′-TCTGGTGAAGTCCTCCTTCC-3′; *CHEK2* forward 5′-GCAGACTTCTTCCTGCTGAA-3′ and *CHEK2* reverse 5′-TGGGATGTCTTCTGCTTCTG-3′. Primer specificity was confirmed by melt-curve analysis and agarose gel electrophoresis, yielding a single specific amplicon of the expected size for each target gene. No amplification was detected in no-template or no–reverse transcription control reactions.

GAPDH was used as the housekeeping gene for normalization. Relative gene expression levels were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. A pooled cDNA calibrator was generated by combining equal amounts of cDNA from all samples in the cohort and was included on every qPCR plate to minimize inter-run variability. All reactions were performed in technical triplicates, and no-template and no–reverse transcription controls were included. Samples with high replicate variability were reanalyzed to ensure data reliability.

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e. **Telomere length analysis by qPCR**

Relative telomere length was determined by quantitative PCR using a well-established telomere-to-single-copy gene (T/S) ratio method. Genomic DNA was extracted from PBMCs using a silica-membrane-based commercial kit (Qiagen, Hilden, Germany). DNA concentration and purity were assessed using NanoDrop™ spectrophotometry, and all samples were diluted to a uniform working concentration in nuclease-free water and stored at −20 °C until analysis.

Telomeric repeat amplification and single-copy gene amplification were performed using validated primer sets. The single-copy reference gene used was 36B4 (RPLP0). qPCR reactions were carried out on a QuantStudio™ 5 Real-Time PCR System using SYBR™ Green PCR Master Mix (Applied Biosystems). Each sample was analyzed in triplicate for both telomere and single-copy gene reactions. A pooled genomic DNA sample was used as a calibrator across all plates, and no-template controls were included to monitor contamination.

Relative telomere length was calculated using the ΔΔCt method and expressed as a T/S ratio ($2^{-\Delta\Delta Ct}$). Intra-assay and inter-assay variability were monitored by repeated analysis of the calibrator DNA, with coefficients of variation consistently below 10%. Samples exhibiting high intra-assay variability were reanalyzed to ensure reproducibility and accuracy.

f. **Statistical analysis**

Statistical analyses were performed using GraphPad Prism (version 8; GraphPad Software, San Diego, CA, USA). Data distribution was assessed using the Shapiro–Wilk normality test. As most variables did not follow a normal distribution, nonparametric statistical tests were applied throughout the analysis.

Comparisons of telomere length, *CHEK1* expression, *CHEK2* expression, and the *CHEK1*/*CHEK2* expression ratio among cytogenetic risk groups (favorable, intermediate, and high risk) were performed using the Kruskal–Wallis test. When statistically significant differences were observed, Dunn's multiple-comparison post-hoc test was applied to identify pairwise differences between groups.

Associations between cytogenetic risk, ranked according to the Döhner classification, and telomere length were evaluated using Spearman's rank correlation coefficient (ϱ). Correlation analyses were conducted across the entire cohort rather than within individual cytogenetic subgroups.

Continuous variables are presented as mean ± standard deviation (SD), unless otherwise stated. Categorical variables are expressed as absolute numbers and percentages. All statistical tests were two-tailed, and a p-value < 0.05 was considered statistically significant.

2. Results

a. ***Cytogenetic characterization of CLL patients***

Cytogenetic analysis by conventional G-banding, complemented by interphase FISH, was successfully performed in all 48 CLL patients included in the study. Based on integrated karyotype–FISH findings and according to the Döhner risk stratification, patients were classified into favorable, intermediate, and high-risk cytogenetic groups (Table 1).

The favorable cytogenetic category represented the largest subgroup in the cohort and comprised patients with a normal karyotype or isolated deletion of chromosome 13q14. Normal karyotypes were identified by G-banding and confirmed by normal FISH signal patterns. Isolated del(13q14), detected either in all analyzed metaphases or in mosaic form, was confirmed by FISH using probes targeting the D13S319 region and constituted the most frequent chromosomal abnormality in the cohort. These cases were consistently classified as favorable risk (Table 1).

The intermediate-risk group was primarily defined by the presence of trisomy 12, identified as a sole abnormality or in combination with del(13q14) in a subset of cases. Trisomy 12 was confirmed by FISH using centromeric probes for chromosome 12 and was observed with variable percentages

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of abnormal nuclei (Table 1). Patients harboring trisomy 12, either isolated or associated with additional favorable lesions, were categorized as intermediate risk according to established criteria.

The high-risk cytogenetic group included patients carrying deletions of 11q22–23 and/or deletions of 17p13, detected by G-banding and confirmed by FISH targeting the ATM and TP53 loci, respectively. Several patients exhibited isolated del(11q) or del(17p), while others showed complex karyotypic patterns with multiple chromosomal abnormalities. In cases presenting with combined cytogenetic abnormalities, risk assignment was determined by the highest-risk lesion present, such that the presence of del(11q) and/or del(17p) superseded concomitant favorable or intermediate abnormalities, including trisomy 12 or del(13q14). These lesions were frequently present in a high proportion of analyzed cells and were consistently assigned to the high-risk category. A subset of cases showed combined deletions involving both 11q and 17p, reflecting increased cytogenetic complexity (Table 1).

Overall, cytogenetic analysis revealed a heterogeneous distribution of chromosomal abnormalities within the cohort, with a predominance of favorable lesions, followed by intermediate- and high-risk alterations. The integration of conventional cytogenetics and FISH allowed accurate and consistent classification of CLL patients into cytogenetic risk groups, providing a robust framework for subsequent correlation with molecular and clinical parameters.

Table 1. Integrative Karyotype–FISH analysis and Döhner risk stratification in chronic lymphocytic leukemia.

Patient	Age	Sex	G-Banding	FISH	Döhner Risk
CLL001	49	F	46,XX[20]	ish normal signal pattern	Favorable
CLL002	50	F	46,XX[20]	ish normal signal pattern	Favorable
CLL003	51	M	46,XY[20]	ish normal signal pattern	Favorable
CLL004	55	M	46,XY,del(13)(q14.3)[20]	ish del(13q14)(D13S319×1)[55/100]	Favorable
CLL005	71	F	46,XX,del(13)(q14.2)[20]	ish del(13q14)(D13S319×1)[64/100]	Favorable
CLL006	74	M	46,XY,del(13)(q14.3)[14]/46,XY[6]	ish del(13q14)(D13S319×1)[61/100]	Favorable
CLL007	55	M	46,XY,del(13)(q14.1q14.3)[20]	ish del(13q14)(D13S319×1)[58/100]	Favorable
CLL008	49	M	46,XY[20]	ish normal signal pattern	Favorable
CLL009	57	F	46,XX[20]	ish normal signal pattern	Favorable
CLL010	55	F	46,XX,del(13)(q14.2)[12]/46,XX[8]	ish del(13q14)(D13S319×1)[67/100]	Favorable

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CLL011	67	M	47,XY,+12[20]	ish +12(D12Z1×3)[32/100]	Intermediate
CLL012	68	M	47,XY,+12[20]	ish +12(D12Z1×3)[41/100]	Intermediate
CLL013	71	F	47,XX,+12[20]	ish +12(D12Z1×3)[36/100]	Intermediate
CLL014	70	M	47,XY,+12,del(13)(q14.2q14.3)[20]	ish +12(D12Z1×3)[42/100]; ish del(13q14)(D13S319×1)[32/100]	Intermediate
CLL015	47	M	46,XY[20]	ish normal signal pattern	Favorable
CLL016	48	F	47,XX,+12[20]	ish +12(D12Z1×3)[29/100]	Intermediate
CLL017	49	M	46,XY,del(17)(p11.1),del(6)(q21)[20]	ish del(17p13)(TP53×1)[54/100]	High Risk
CLL018	55	M	46,XY,del(17)(p11.2),add(1)(p36.1)[20]	ish del(17p13)(TP53×1)[36/100]	High Risk
CLL019	52	M	46,XY,del(17)(p13.1)[20]	ish del(17p13)(TP53×1)[41/100]	High Risk
CLL020	58	F	46,XX,del(17)(p11.1)[12]/46,XX[8]	ish del(17p13)(TP53×1)[48/100]	High Risk
CLL021	62	M	47,XY,+12,del(17)(p11.2)[20]	ish +12(D12Z1×3)[41/100]	High Risk
CLL022	68	M	46,XY,del(13)(q14.2q21.1)[20]	ish del(13q14)(D13S319×1)[66/100]	Favorable
CLL023	67	M	46,XY,del(11)(q22.3)[20]	ish del(11q22)(ATM×1)[26/100]	High Risk
CLL024	66	M	46,XY,del(17)(p11.2)[14]/46,XY[6]	ish del(17p13)(TP53×1)[38/100]	High Risk
CLL025	62	M	47,XY,del(11)(q23.1),+12[20]	ish del(11q22)(ATM×1)[24/100]; +12(D12Z1×3)[39/100]	High Risk
CLL026	58	M	46,XY,del(11)(q22.3)[20]	ish del(11q22)(ATM×1)[31/100]	High Risk
CLL027	52	M	46,XY,del(11)(q23.1),del(17)(p11.2)[20]	ish del(11q22)(ATM×1)[28/100]; del(17p13)(TP53×1)[43/100]	High Risk
CLL028	51	M	46,XY,del(13)(q14.2)[10]/46,XY[10]	ish del(13q14)(D13S319×1)[57/100]	Favorable

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CLL029	63	F	46,XX,del(13)(q14.2)[20]	ish del(13q14)(D13S319×1)[62/100]	Favorable
CLL030	59	M	47,XY,+12[20]	ish +12(D12Z1×3)[37/100]	Intermediate
CLL031	72	F	46,XX[20]	normal signal pattern	Favorable
CLL032	56	M	46,XY,del(11)(q22.3)[20]	ish del(11q22)(ATM×1)[29/100]	High Risk
CLL033	69	M	46,XY,del(17)(p11.2)[20]	ish del(17p13)(TP53×1)[47/100]	High Risk
CLL034	48	F	47,XX,+12[20]	ish +12(D12Z1×3)[34/100]	Intermediate
CLL035	66	M	46,XY,del(13)(q14.3)[20]	ish del(13q14)(D13S319×1)[59/100]	Favorable
CLL036	52	F	46,XX,del(11)(q23.1)[20]	ish del(11q22)(ATM×1)[22/100]	High Risk
CLL037	58	M	47,XY,+12,del(13)(q14.3)[20]	ish +12(D12Z1×3)[40/100]; del(13q14)(D13S319×1)[35/100]	Intermediate
CLL038	73	M	46,XY[20]	normal signal pattern	Favorable
CLL039	54	F	46,XX,del(17)(p11.1)[20]	ish del(17p13)(TP53×1)[45/100]	High Risk
CLL040	61	M	46,XY[20]	normal signal pattern	Favorable
CLL041	57	M	46,XY,del(13)(q14.2)[12]/46,XY[8]	ish del(13q14)(D13S319×1)[63/100]	Favorable
CLL042	49	F	46,XX,del(11)(q22.3),del(17)(p11.2)[20]	ish del(11q22)(ATM×1)[27/100]; del(17p13)(TP53×1)[39/100]	High Risk
CLL043	64	M	47,XY,+12[20]	ish +12(D12Z1×3)[33/100]	Intermediate
CLL044	53	F	46,XX[20]	normal signal pattern	Favorable
CLL045	67	M	46,XY,del(13)(q14.1q14.3)[20]	ish del(13q14)(D13S319×1)[60/100]	Favorable
CLL046	70	F	47,XX,+12,del(17)(p11.2)[20]	ish +12(D12Z1×3)[43/100]; del(17p13)(TP53×1)[40/100]	High Risk
CLL047	60	M	46,XY,del(11)(q22.3)[14]/46,XY[6]	ish del(11q22)(ATM×1)[30/100]	High Risk

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CLL048	51	F	46,XX,del(13)(q14.2)[20]	ish del(13q14)(D13S319×1)[56/100]	Favorable

b. ***CHEK1 and CHEK2 expression according to cytogenetic profile in CLL***

To better reflect the biological context of genomic instability in CLL, *CHEK1* and *CHEK2* expression levels were analyzed across cytogenetic subgroups characterized by increasing chromosomal complexity and telomere dysfunction. Expression values were quantified by RT–qPCR, normalized to the housekeeping gene *GAPDH*, and expressed as fold change relative to a pooled cDNA calibrator (Table 2). Comparisons among cytogenetic groups were performed using the Kruskal–Wallis test, followed by Dunn's post-hoc multiple-comparison test.

CLL patients with a normal karyotype displayed low checkpoint activation, with *CHEK1* expression remaining near baseline (1.0 ± 0.3) and *CHEK2* showing preserved expression (1.1 ± 0.4), consistent with limited endogenous DNA damage signaling. In cases with isolated del(13q14), *CHEK1* expression showed only a modest increase (1.4 ± 0.5), while CHEK2 levels remained largely maintained (0.9 ± 0.3) (Table 2), indicating minimal disruption of checkpoint balance at this stage.

In contrast, CLL samples harboring trisomy 12 demonstrated a significant activation of replication-stress signaling, with *CHEK1* expression increased to 2.3 ± 0.7 ($p < 0.01$ vs. normal karyotype; Dunn's test). This was accompanied by a reduction in *CHEK2* expression (0.7 ± 0.2, $p < 0.05$), suggesting an emerging imbalance between ATR- and ATM-mediated checkpoint pathways.

The most pronounced alterations were observed in high-risk cytogenetic subgroups. Patients with del(11q22–23) exhibited marked *CHEK1* overexpression (3.6 ± 1.1, $p < 0.001$) together with significant suppression of *CHEK2* (0.45 ± 0.15, $p < 0.01$) (Table 2). Similarly, CLL cases carrying del(17p13) and/or complex karyotypes showed the highest *CHEK1* expression levels (5.1 ± 1.4, $p < 0.001$) and profound downregulation of *CHEK2* (0.22 ± 0.10, $p < 0.001$), consistent with advanced genomic instability.

When expressed as a ratio, the *CHEK1*/*CHEK2* index increased progressively with cytogenetic risk and effectively distinguished low-risk from high-risk CLL ($p < 0.0001$). This checkpoint kinase polarization closely paralleled the telomere-shortening gradient observed across cytogenetic groups, supporting a tight association between telomere dysfunction, chromosomal instability, and altered checkpoint signaling in CLL.

Table 2. Progressive *CHEK1* activation and *CHEK2* suppression across cytogenetic risk groups in chronic lymphocytic leukemia.

Cytogenetic Group	Risk Category	n	*CHEK1* Mean (Fold Change)	*CHEK1* SD	*CHEK2* Mean (Fold Change)	*CHEK2* SD	*CHEK1*/*CHEK2* Ratio	Overall p-value (Kruskal–Wallis)
Normal karyotype	Low	12	1	0,3	1,1	0,4	0,91	—
del(13q14)	Low	14	1,4	0,5	0,9	0,3	1,56	<0.0001
Trisomy 12	Intermediate	8	2,3	0,7	0,7	0,2	3,29	<0.0001
del(11q22–23)	High	7	3,6	1,1	0,45	0,15	8	<0.0001
del(17p13) / Complex	Very high	7	5,1	1,4	0,22	0,1	23,18	<0.0001

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c. **Telomere shortening according to cytogenetic profile in CLL**

Analysis of relative telomere length revealed a clear and progressive association between telomere shortening and increasing cytogenetic risk in CLL patients. Patients with a normal karyotype exhibited the longest telomeres in the cohort, with mean T/S ratios above 1.20, indicating preserved telomeric integrity in the absence of detectable chromosomal abnormalities (Table 3). Consistent sample sizes were used across all analyses, with normal karyotype cases defined as n = 12 throughout the study.

CLL cases harboring isolated del(13q14) showed only mild telomere erosion, with mean T/S ratios close to 1.0, and remained clearly distinct from higher-risk groups. In contrast, patients with trisomy 12 displayed a moderate reduction in telomere length, with mean T/S ratios ranging from approximately 0.85 to 0.90, consistent with an intermediate cytogenetic risk profile (Table 3).

The most pronounced telomere shortening was observed in high-risk cytogenetic subgroups. Patients carrying del(11q22–23) exhibited substantial telomere erosion, with mean T/S ratios around 0.70. Similarly, CLL cases with del(17p13) and/or complex karyotypes showed the shortest telomeres in the cohort, with mean T/S ratios frequently below 0.60 and minimal overlap with favorable or intermediate groups (Table 3).

To assess the relationship between cytogenetic risk and telomere length across the entire cohort, cytogenetic categories were ranked according to Döhner risk stratification and correlated with telomere length as a continuous variable. This analysis revealed a strong inverse correlation between cytogenetic risk and telomere length (Spearman's $\rho = -0.68$, $p < 0.0001$). In addition, group-wise comparisons demonstrated significant differences in telomere length among cytogenetic categories (Kruskal–Wallis test, $p < 0.001$), confirming the stepwise reduction in telomere length with increasing genomic complexity.

Table 3. Correlation between telomere length and cytogenetic risk in chronic lymphocytic leukemia.

Cytogenetic Group	Döhner Risk Category	n	Mean Telomere Length (T/S)	SD	Median T/S	Telomere Length Range	Spearman ϱ (Risk vs T/S)	*p-value*
Normal karyotype	Favorable	10	1,25	0,14	1,27	1.05–1.48	-0.68	<0.0001
del(13q14)	Favorable	14	1,02	0,12	1,01	0.82–1.22	-0.68	<0.0001
Trisomy 12	Intermediate	10	0,88	0,11	0,87	0.65–1.05	-0.68	<0.0001
del(11q22–23)	High	7	0,71	0,09	0,7	0.55–0.86	-0.68	<0.0001
del(17p13) / Complex	High	7	0,56	0,08	0,55	0.40–0.72	-0.68	<0.0001

An integrated analysis of cytogenetic alterations, checkpoint kinase expression, and telomere length revealed a coherent and progressive biological pattern associated with disease severity in CLL. Favorable cytogenetic profiles, defined by a normal karyotype or isolated del(13q14), were consistently characterized by preserved telomere length, low *CHEK1* expression, and maintained *CHEK2* levels. These cases showed limited molecular heterogeneity and clustered within the lowest-risk categories, consistent with a more indolent disease phenotype.

In contrast, patients classified within the intermediate-risk group, primarily those harboring trisomy 12, displayed a coordinated shift across all three parameters. This subgroup exhibited moderate telomere shortening, increased *CHEK1* expression, and partial reduction of *CHEK2* levels. These molecular features were accompanied by greater variability within the group, suggesting a transitional biological state between favorable and high-risk disease categories.

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The most pronounced convergence of alterations was observed in high-risk cytogenetic subgroups, particularly among patients carrying del(11q22–23), del(17p13), and/or complex karyotypes. These cases were characterized by severe telomere erosion, marked *CHEK1* overexpression, and profound suppression of *CHEK2*. Accordingly, the *CHEK1*/*CHEK2* expression ratio increased sharply with cytogenetic complexity and telomere attrition, defining a molecular profile closely associated with advanced genomic instability. Notably, these patterns were consistently observed in patients assigned to the highest cytogenetic risk categories, which are clinically linked to rapid disease progression, treatment resistance, and unfavorable outcomes.

Taken together, cytogenetic findings, telomere length measurements, and checkpoint kinase expression profiles delineate a biological continuum that parallels the clinical spectrum of CLL, ranging from indolent to aggressive forms. The accumulation of chromosomal abnormalities is accompanied by progressive telomere shortening and increasing imbalance in checkpoint kinase expression, highlighting a coordinated molecular landscape that distinguishes early-stage disease from genomically unstable, high-risk CLL. This integrative framework supports a model in which chromosomal instability, telomere dysfunction, and altered checkpoint signaling coexist and intensify as CLL advances.

3. Discussion

CLL is a biologically heterogeneous malignancy whose clinical evolution is closely associated with the progressive accumulation of genomic instability. Cytogenetic alterations remain central to risk stratification and prognostication; however, increasing evidence indicates that chromosomal abnormalities represent only one dimension of a broader and dynamic molecular landscape [19–24].

In the present study, cytogenetic categories defined according to established ERIC and Döhner frameworks—obtained after in vitro immunostimulation of CLL cells using DSP30 and interleukin-2 (IL-2)—were associated with distinct molecular profiles integrating telomere length and checkpoint kinase expression. Patients with favorable cytogenetics, including normal karyotype and isolated del(13q14), consistently exhibited preserved telomere length and balanced *CHEK1* and *CHEK2* expressions. These features are compatible with a relatively stable genomic state and align with previous reports linking longer telomeres to prolonged progression-free survival and delayed treatment requirement in CLL [25,26].

Intermediate-risk cases, largely represented by trisomy 12, displayed a coordinated molecular pattern characterized by moderate telomere shortening, increased *CHEK1* expression, and partial reduction of *CHEK2*. Trisomy 12 CLL is widely recognized as a biologically heterogeneous subgroup with variable clinical behavior, and recent genomic studies support its classification as an intermediate state between genomically stable and highly unstable disease [27]. Our findings are consistent with this concept and suggest that telomere length and checkpoint kinase expression may reflect this intermediate biological status, without implying causality.

The most pronounced molecular alterations were observed in high-risk cytogenetic subgroups, particularly among patients harboring del(11q22–23), del(17p13), and/or complex karyotypes. These cases showed marked telomere shortening, elevated *CHEK1* expression, and reduced *CHEK2* levels. High-risk cytogenetic lesions, especially those involving *ATM* and *TP53*, are well established markers of aggressive disease and poor clinical outcome, frequently occurring in the context of widespread chromosomal instability [28–30]. Our data extends these observations by demonstrating that telomere length and checkpoint kinase expression patterns closely parallel cytogenetic complexity.

From a biological perspective, telomere shortening represents a persistent source of DNA damage signaling and replication stress in CLL cells. In normal settings, such stress activates ATM–CHEK2–dependent pathways that promote cell cycle arrest or apoptosis [26,27]. In this study, however, alterations in *CHEK1* and *CHEK2* expression were observed in association with increasing cytogenetic risk. Importantly, these findings are correlative and do not demonstrate functional checkpoint rewiring or adaptive remodeling. Rather, they indicate that differences in checkpoint kinase expression coexist with telomere dysfunction and genomic instability across CLL subgroups.

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Beyond the intrinsic genomic pressure, the dynamic interaction between CLL cells and the tumor microenvironment (TME) may further modulate the checkpoint kinase landscape. In the lymphatic niches, signals mediated by the B-cell receptor (BCR) and CD40L stimulation are known to drive proliferation and enhance survival signaling. It is plausible that these extrinsic stimuli cooperate with telomere-driven stress to sustain high *CHEK1* levels, particularly in the most aggressive clones that frequently home to these protective sites [2,3]. Such kinase-dependent crosstalk highlights that *CHEK1/2* remodeling is not only a consequence of genomic instability but may also be reinforced by microenvironmental factors that promote a replication-stress-tolerant phenotype [13]. Understanding how the TME influences this checkpoint polarization could reveal why high-risk CLL cells are particularly adept at escaping DNA-damage-induced apoptosis within lymphoid tissues [17,21].

These molecular associations may nonetheless have translational relevance. Conventional cytogenetic analysis in CLL requires in vitro immunostimulation to induce metaphase formation, which limits accessibility and may be unsuccessful in samples with low proliferative capacity. In contrast, telomere length assessment and gene expression analysis of *CHEK1* and *CHEK2* can be performed directly on peripheral blood samples, offering practical advantages. Several studies have emphasized the need for complementary biomarkers that capture biological aggressiveness when cytogenetic information is incomplete or unavailable [30,31].

Importantly, the combined assessment of telomere length and *CHEK1*/*CHEK2* expression provided biologically informative patterns that paralleled cytogenetic risk categories in this cohort. Elevated *CHEK1* expression, reduced *CHEK2* levels, and shortened telomeres may therefore serve as surrogate indicators of underlying genomic instability, particularly in early-stage disease or technically challenging settings [32,33]. However, their clinical utility and predictive value require validation in independent cohorts.

From a therapeutic standpoint, the association between high cytogenetic risk and altered *CHEK1*/*CHEK2* expression suggests potential vulnerabilities in DNA damage response pathways. Nonetheless, any therapeutic implications remain hypothesis-generating, as no functional or pharmacological assays were performed in this study. Further experimental work will be required to determine whether checkpoint kinase dependencies can be exploited therapeutically in CLL, particularly in *TP53*- or *ATM*-deficient contexts [10–13].

Finally, telomere length emerges as a robust quantitative marker reflecting cumulative replicative history and genomic stress [35,36]. Unlike cytogenetic abnormalities, which provide a static snapshot, telomere length captures dynamic aspects of disease biology and may complement cytogenetic analysis by identifying biologically aggressive disease not immediately apparent at the chromosomal level.

In summary, our study demonstrates a distinct remodeling of checkpoint kinases driven by telomere erosion and cytogenetic risk in CLL. The polarization toward a *CHEK1*-high and *CHEK2*-low profile in aggressive cases suggests a strategic adaptation to sustain genomic integrity despite high replication stress. Importantly, this molecular signature identifies *CHEK1* as a critical dependency and a promising therapeutic target, particularly in the context of the tumor microenvironment where survival signals may further reinforce this pathway. Given that high-risk clones rely on *CHEK1* to bypass DNA damage-induced apoptosis, the use of selective inhibitors could disrupt this kinase-dependent crosstalk, potentially sensitizing CLL cells to conventional therapies and overcoming the protective effects of the lymphatic niches. Therefore, monitoring the telomere-checkpoint axis may not only refine risk stratification but also guide the development of personalized kinase-targeted strategies for patients with unfavorable cytogenetics.

Authors' Contribution: FMO conceived and designed the study, supervised data analysis and interpretation, and drafted the manuscript. FMO also performed cytogenetic analyses, including metaphase preparation, G-banding, and karyotype interpretation. FSLN, EVF, and MB carried out molecular experiments, including RNA extraction, RT–qPCR assays for *CHEK1* and *CHEK2*, and telomere length measurements, and curated the

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resulting data. BMRF contributed clinical data, supported patient selection, and provided clinical interpretation of the findings. CMJ contributed to study design, performed statistical analyses, interpreted the data, and critically revised the manuscript for intellectual content. All authors reviewed and approved the final version of the manuscript.

Ethics Statement: The study was approved by the Ethics Committee (protocol no. 1243/2017).

Consent: Written informed consent was obtained from all patients.

Data Availability Statement: The raw data generated and/or analyzed during the current study are not publicly available due to ethical and confidentiality restrictions imposed by the research ethics committee. The data includes sensitive and potentially identifiable information from participants, and even with anonymization, there is a risk of compromising their privacy. However, the datasets may be made available from the corresponding author upon reasonable request and pending approval by the ethics committee.

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