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Relevance of Telomere/Telomerase System Impairment in Early Stage Chronic Lymphocytic Leukemia

Mirjam Hoxha,[1†] Sonia Fabris,[2†] Luca Agnelli,[2] Valentina Bollati,[1] Giovanna Cutrona,[3] Serena Matis,[3] Anna Grazia Recchia,[4] Massimo Gentile,[4] Agostino Cortelezzi,[2] Fortunato Morabito,[4] Pier Alberto Bertazzi,[1] Manlio Ferrarini,[5] and Antonino Neri[2*]

[1]Department of Clinical Sciences and Community Health, Center of Molecular and Genetic Epidemiology, University of Milan, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[2]Department of Clinical Sciences and Community Health, Hematology I CTMO, University of Milan, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[3]SS Molecular Diagnostics, IRCCS S. Martino IST, Genoa, Italy
[4]U.O.C. Hematology, Azienda Ospedaliera di Cosenza, Cosenza, Italy
[5]Direzione Scientifica IRCCS, San Martino IST, Genoa, Italy

Several studies have proposed telomere length and telomerase activity as prognostic factors in chronic lymphocytic leukemia (CLL), whereas information addressing the role of telomere-associated genes is limited. We measured relative telomere length (RTL) and *TERT* expression levels in purified peripheral $CD19^+$ B-cells from seven healthy donors and 77 untreated CLLs in early stage disease (Binet A). Data were correlated with the major biological and cytogenetic markers, global DNA methylation (*Alu* and *LINE-1*), and clinical outcome. The expression profiles of telomere-associated genes were also investigated. RTL was decreased in CLLs as compared with controls ($P < 0.001$); within CLL, a progressive and significant RTL shortening was observed in patients from 13q- through +12, 11q-, and 17p- alterations; short telomeres were significantly associated with unmutated *IGHV* configuration and global DNA hypomethylation. Decreased RTL was associated with a shorter time to first treatment. A significant upregulation of *POT1*, *TRF1*, *RAP1*, *MRE11A*, *RAD50*, and *RPA1* transcript levels was observed in CLLs compared with controls. Our study suggests that impairment of telomere/telomerase system represents an early event in CLL pathogenesis. Moreover, the correlation between telomere shortening and global DNA hypomethylation supports the involvement of DNA hypomethylation to increase chromosome instability.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in Western countries (Chiorazzi et al., 2005). It is a heterogeneous disorder characterized by highly variable clinical course. Classical staging systems introduced by Rai et al. (1975) and Binet et al. (1977) help to predict survival in CLL; however, they do not distinguish patients who will evolve to a more advanced, aggressive disease from those who will remain indolent. Despite the established prognostic value of several molecular indicators such as genomic aberrations, *IGHV* mutational status, CD38, and ZAP-70 expression, a stratification based on these parameters fails to cover the complex heterogeneity of CLL (Damle et al., 1999; Dohner et al., 1999; Crespo et al., 2003). Therefore, the identification of factors that could predict the clinical course of early stage CLL represents a crucial objective in this malignancy. Several studies have focused on the potential prognostic significance of telomere length (TL) and telomerase activity in CLL, in addition to the aforementioned better established prognostic markers (Damle et al., 2004; Grabowski et al., 2005; Ricca et al., 2007; Terrin et al., 2007; Roos et al., 2008; Rossi

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†Mirjam Hoxha and Sonia Fabris contributed equally to this work.

*Correspondence to: Antonino Neri, Department of Clinical Sciences and Community Health, Hematology 1 CTMO, University of Milan, Foundation IRCCS Ca' Granda, Ospedale Maggiore Policlinico, F. Sforza 35, 20122 Milan, Italy.
E-mail: antonino.neri@unimi.it

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et al., 2009; Sellmann et al., 2011; Rampazzo et al., 2012); however, a comprehensive analysis in uniform and representative series of early stage CLL patients (Binet A) is still lacking.

Telomeres, which are specialized protective structures at the end of eukaryotic chromosomes, are progressively shortened during each round of cell replication because of end-replication problems of DNA polymerase, leading to a process thought to contribute to senescence and cell death (Harley et al., 1990; Blackburn, 2011). In contrast to normal somatic cells, immortalized cells that have overcome the cellular senescence blockade acquire the ability to stabilize their TL by expressing high telomerase levels (Counter et al., 1992). Telomerase is a multisubunit ribonucleoprotein enzyme (TERT, TERC, and DYSKERIN) that adds telomeric repeats to the chromosomal ends, thus, compensating the progressive telomere erosion (Morin, 1989). Telomerase activity is regulated by the human telomerase reverse transcriptase (*TERT*) gene, which encodes the catalytic subunit of telomerase (Poole et al., 2001).

While the telomere structure and function depend on the telomerase for elongation (Cohen et al., 2007), telomere maintenance involves the shelterin complex including TRF1, TRF2, TIN2, RAP1, TPP1, and POT1 factors which play an essential role in telomere protection and telomerase activity regulation. In addition to shelterin complex, a large number of factors have an important contribution to the maintenance and the protection of chromosome ends; among these, EST1A is involved in the telomerase recruitment, whereas RPA1, KU70/KU80, and the MRN complex (MRE11, RAD50, and NBS1) are thought to have an important role in DNA damage recognition and repair (Matsutani et al., 2001; Givalos et al., 2007; Palm and de Lange, 2008; Salhab et al., 2008).

The aim of this study was to assess the TL and the expression levels of *TERT* in a cohort of highly purified B cells from 77 untreated CLLs in early stage disease (Binet A), and to evaluate their relationship with major biological and cytogenetic risk factors and their prognostic impact on therapy-free survival (TFS) defined as time from diagnosis to first treatment. Data on the TL were correlated with the methylation levels of long interspersed nuclear elements-1 (*LINE-1*), *Alu*, and satellite alpha (*SAT-α*) repetitive genomic sequences, known to be associated with chromosomal instability. Given the importance of telomere-associated proteins in the control of TL, stability and integrity, we additionally investigated whether the transcriptional levels of these genes were modulated in CLL patients in comparison with normal B cells.

MATERIALS AND METHODS

Study Design

This study was based on a retrospective series of 77 early stage CLL patients included in a database from a collaborative Italian study (Morabito et al., 2009). Peripheral blood samples from seven normal healthy age-matched controls (age range 29–81 years, mean age 61 years) were properly selected for this study. An independent cohort of 62 prospective early stage (Binet A) CLL patients enrolled in the Gruppo Italiano Studio Linfomi O-CLL1 multicenter trial (registered at ClinicalTrials.gov, accession #NCT00917540) (Fabris et al., 2013) was selected for quantitative real-time PCR (Q-RT-PCR) analysis validation (Supporting Information Table S1). Written informed consent was obtained from all patients in accordance with the declaration of Helsinki and the study was approved by the local Ethics Review Committee (Comitato Etico Provinciale, Modena, Italy). For Q-RT-PCR validation five healthy donors samples, different from previously controls, were used. DNA and RNA samples preparation, immunophenotype, and biological and cytogenetic markers analyses were done as previously described by us (Fabris et al., 2011). All analyses were performed in highly purified (>90%) $CD19^+$ peripheral blood mononuclear B-cells.

TL Measurement by Quantitative PCR

TL was measured using the quantitative real-time method described by Cawthon (2002). This method measures the relative telomere length (RTL) in genomic DNA by determining the ratio of telomere repeat (T) copy number to single copy gene (S) copy number (T/S ratio) in experimental samples relative to a reference sample. The T PCR mix was: iQ SYBR Green Supermix (Bio-Rad) 1x, tel 1b 100 nM, tel 2b 900 nM, DMSO 1%, EDTA 1x. The S human beta-globin (*hbg*) gene PCR mix was:iQ SYBR Green Supermix (Bio-Rad) 1x, hbg1 300 nM, hbg2 700 nM, DMSO 1%, DTT 2.5 mM, EDTA 1x. We used the PCR primer sets previously described by McGrath et al. (2007). All samples containing *Escherichia coli* DNA were heated at 96°C for 10 m and cooled at

room temperature. DNA (15 ng) samples were added to each reaction (final volume 20 μl). A six-point standard curve, derived from serially diluted DNA pool, ranging from 40 to 1.25 ng/μl was included in each PCR plate, so that relative quantities of T and S could be determined. All PCRs were performed on a DNA Engine thermal cycler Chromo4 (Bio-Rad, Hercules, CA). The thermal cycling profile for both amplicons began with a 95°C incubation for 3 min to activate the hot-start iTaq DNA polymerase. For telomere PCR, 25 cycles followed at 95°C for 15 sec, then annealing/extension at 54°C for 49 sec. For *hbg*, 35 cycles followed at 95°C for 15 sec, annealing at 58°C for 1 sec, extension at 72°C for 15 sec. At the end of each reaction, a melting curve was used to define the specificity for both T and S PCRs. All samples were run in triplicate and the average of the three T measurements was divided by the average of the three S measurements to calculate the average T:S ratio or RTL.

Gene Expression Profiling Analysis

Forty-two of 77 CLL patients and six of seven normal healthy age-matched controls were previously profiled with the GeneChip® Human Genome U133A (HG-U133A) arrays according to manufacturer's protocols; data were publicly available on the Gene Expression Omnibus website under accession number GSE16746. The major genetic lesions and *IGHV* mutational status, CD38, and ZAP-70 expression of the 42 patients analyzed by microarray analysis are shown in Supporting Information Table S2.

Q-RT-PCR Validation of the Microarray Expression Data

High-capacity cDNA reverse transcription kit (Applied Biosystems) was used for the reverse transcription step. Q-RT-PCR assays were developed to validate expression levels of four candidate genes in purified $CD19^+$ cells using TaqMan gene expression assays (Applied Biosystems): *TERT* (Hs00972656_ml), *POT1* (Hs00209984_ml), *TRF1* (Hs00819517_mH), and *MRE11A* (Hs00967443_ml). Analysis of *GAPDH* expression, selected as the housekeeping gene, was carried out using Hs02758991_gl TaqMan gene expression assay (Applied Biosystems). All PCR runs were performed in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan® Universal PCR Master Mix following the protocol provided by the manufacturer. High-precision MICROLAB STARlet Robot (Hamilton Life Science Robotics, Bonaduz AG, Switzerland) was used for transferring a volume of 5.5-μl reaction mix and 4.5-μl cDNA in a 384-well plate. Data were presented as relative quantity of target mRNA, normalized to housekeeping gene and to a calibrator derived by a pool of six random CLL samples. RQ MANAGER (Version 1.2) Software (Applied Biosystems) provided relative quantification of mRNA targets.

Statistical Analysis

All data were statistically analyzed in R software (www.r-project.org). Kendall's τ correlation was used to assess associations among different variables. Wilcoxon rank sum test was used to test for differences among two distributions, and the Kruskal–Wallis test if more than two groups of patients were compared. Survival analysis was conducted with *survcomp* package in R software, using the Kaplan–Meier estimator and log-rank test, and *P* values were calculated according the standard normal asymptotic distribution. The prognostic impact of the biological and molecular variables in terms of TFS was investigated by Cox proportional hazard models. The regression was evaluated in terms of hazard ratio (HR) and 95% confidence interval (C.I.) either on any single predictor alone or testing the predictors (TL, *IGHV* mutational status and presence of 11q22.3 and *TP53* deletions) in multivariate model. Cox proportional hazard model was also used in the *globaltest* function (Goeman et al., 2006) (under 100,000 permutation) to test the positive or negative association between covariates (mRNA expression levels of telomere-associate genes), assumed as continuous variable, and clinical outcome as response variable (in terms of TFS). Jonckheere–Terpstra test in *clinfun* package was used to investigate the significance of distribution trends. *P* values were adjusted for multiple testing through Benjamini and Hochberg's correction method. A value of $P < 0.05$ was considered significant for all statistical calculations.

RESULTS

Molecular Characterization of the CLL Patients

The molecular and biological characteristics of the 77 patients included in the study were described in a previous study (Fabris et al., 2011).

TABLE 1. RTL Related to Different Biological Prognostic Factors in CLL

	Number of patients	RTL median [IQR]	*P*-value
CD38 expression			
Negative (<30%)	36	0.5 [0.4–0.9]	Ns
Positive (≥30%)	35	0.4 [0.3–0.6]	
ZAP-70 expression			
Negative (<30%)	46	0.4 [0.3–0.9]	Ns
Positive (≥30%)	28	0.4 [0.3–0.5]	
IGHV mutational status			
Unmutated (≥98% homology)	48	0.4 [0.3–0.5]	<0.001
Mutated (<98% homology)	28	0.8 [0.4–1.0]	

RTL, relative telomere length; IQR, interquartile range: 25th and 75th percentiles are shown; Ns, not significant.

Briefly, 48 patients had unmutated *IGHV* gene (*IGHV*-UM) while ZAP-70 and CD38 were positive in 28 and 35 cases, respectively. Deletion of 13q14.3 was present as sole abnormality in 21 of the 33 patients in which it has been detected, while in the remaining cases it was combined with 17p13.1 ($n = 4$) or 11q22.3 deletions ($n = 7$) or both ($n = 1$). The 11q22.3 and 17p13.1 deletions were identified as sole abnormality in six and seven patients, respectively. Finally, trisomy 12 occurred in 17 CLLs, as sole abnormality in all cases. Overall, based on FISH analyses, at least one abnormality was found in 63/77 (81.8%) cases.

TL Determination and Association with Prognostic Parameters

RTL values in CLLs were compared with those obtained from $CD19^+$ purified peripheral B-cells from seven healthy donors. Significantly lower RTL values were found in CLL patients [median RTL = 0.4; interquartile range (IQR): 25th and 75th percentiles, 0.3–0.6] than controls (median RTL = 1.0 IQR 0.9–1.3; $P < 0.001$). Based on multivariate analysis, the telomere shortening was not age dependent. *IGHV* unmutated patients showed significantly shorter telomeres than those with mutated *IGHV* ($P < 0.001$), while no significant association was found between TL and both CD38 and ZAP-70 expression (Table 1). As regards genomic aberrations, a progressive and significant RTL decrease occurred from 13q (as a sole abnormality) to 17p- through +12 and 11q-alterations, 17p- cases showing the shortest telomeres (*P* for differences among categories and *P* for trend < 0.0001, Fig. 1), whereas similar median TL levels were detected in normal karyotype (0.58) and 13q deleted cases (0.64).

Association Between TL and Global DNA Methylation

Based on previously available data (Fabris et al., 2011), significantly decreased methylation levels of *Alu*, *LINE-1*, and *SAT-α* repetitive elements were detected in the same cohort of CLL patients when compared with healthy donors. In this study, we found that telomere shortening was associated with hypomethylation of *Alu* (tau = 0.15, $P < 0.05$) and *LINE-1* (tau = 0.24, $P = 0.001$) repetitive elements, but not with *SAT-α* (Fig. 2).

TERT Levels and Association with Prognostic Parameters and TL

To investigate the relationship between *TERT* expression and the major prognostic markers as well as RTL values, we evaluated the expression levels of the *TERT* gene in 42 out of 77 CLLs and six healthy donors for whom gene expression profiles were available in the proprietary database.

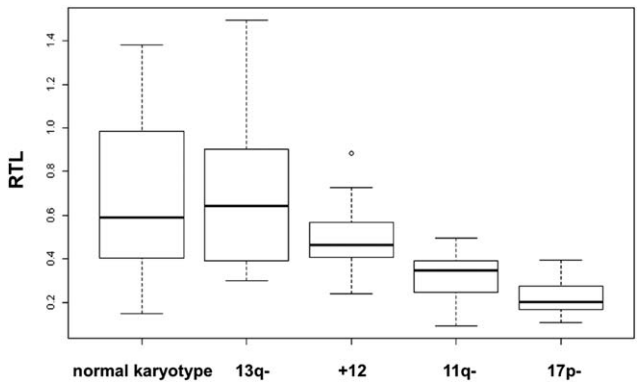

Figure 1. Boxplot representation of RTL in CLL samples according to different cytogenetic groups (*P* for differences among categories and *P* for trend < 0.0001).

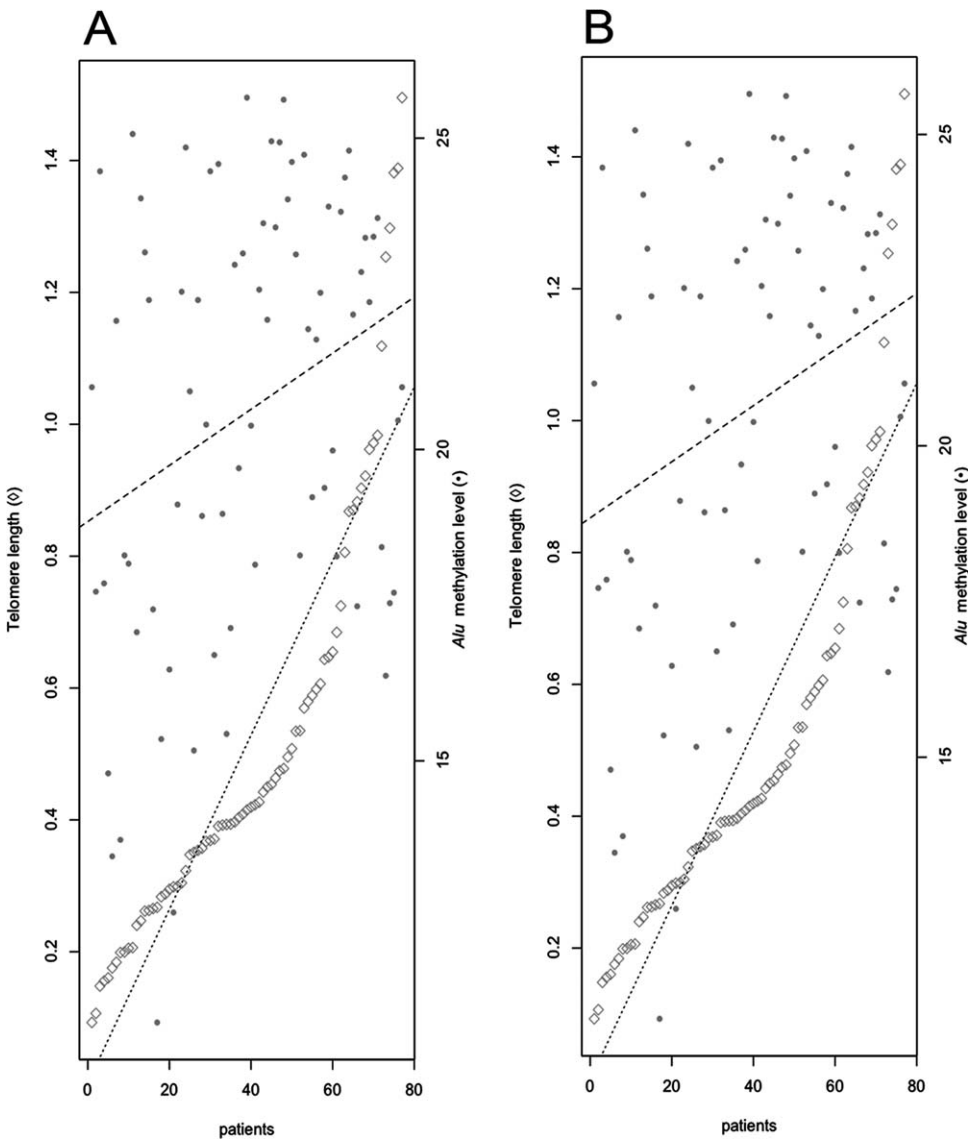

Figure 2. Relationships between *Alu* and *LINE-1* methylation levels and telomere length. Squares (◇) represent telomere length, whereas dots (•) indicate *Alu* (A) and *LINE-1* (B) methylation levels.

CLL samples displayed absolute median *TERT* expression higher than controls (median: 64.8, range: 45.5–90.6; *Padj* = 0.0301; Fig. 3). No statistically significant association between *TERT* expression levels and genomic aberrations, *IGHV* mutational status, CD38, or ZAP-70 expression was observed (data not shown).

Prognostic Value of TL and *TERT* Levels in CLLs

Clinical data were available for 59 patients. The median follow-up was 2 years (range 1–10 years) and 39 patients had received treatment by the end of the study. TFS was considered as marker for time to disease progression. The analysis of RTL revealed significantly lower risk of starting treatment for patients included in the highest RTL quartile (HR = 0.23, 95% C.I. 0.07–0.73; $P = 0.0124$) (Fig. 4). However, based on multivariate analysis that considered as covariates the deletion of 11q and 17p deletion and the *IGHV* mutational status, TL did not represent an independent risk factor for TFS. Finally, as regards *TERT* mRNA levels, no association with clinical outcome has been found in our dataset (data not shown).

Expression Profiling Analysis of Telomere Maintenance Genes

To identify changes in the mRNA expression of genes encoding telomere-associated proteins

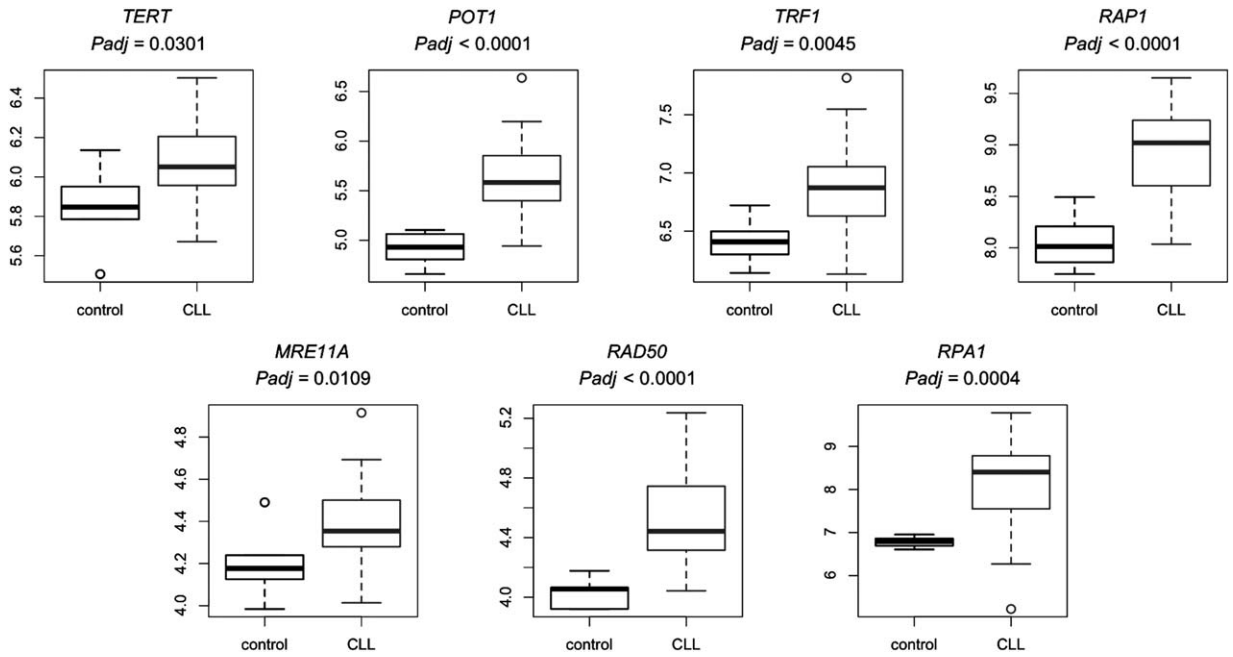

Figure 3. Boxplot representation of absolute RNA expression levels of telomerase and telomere-associated genes in healthy subjects and CLL patients as assessed by microarray analysis. A significantly increased absolute median RNA expression level in CLLs vs. healthy donors was found for *TERT, POT1*, *TRF1*, *RAP1*, *MRE11A*, *RAD50*, and *RPA1*.

influencing TL regulation, we evaluated their absolute expression levels in 42 of 77 CLLs available in our microarray gene expression profiling database and in six healthy donors (Fig. 2). As regards shelterin components, a significant increase was observed for *POT1*, *TRF1*, and *RAP1* mRNA expression ($Padj < 0.0001$, $Padj = 0.0045$, and $Padj < 0.0001$, respectively) while no significant differences were found for *TRF2*, *TPP1*, and *TIN2* mRNA levels. Among genes belonging to the MRE11 complex, *MRE11A* and *RAD50* showed a significant upregulation ($Padj = 0.0109$, $Padj < 0.0001$, respectively) but no statistically significant difference was observed for *NBS1*. Finally, we found a significant increase of *RPA1* expression in CLL samples compared with healthy donors ($Padj = 0.0004$).

There was no statistically significant correlation between mRNA levels of these upregulated genes and TL (data not shown).

To confirm the results from the microarray analysis, *TERT*, *POT1*, *TRF1*, and *MRE11A* genes were selected for Q-RT-PCR validation. The Q-RT-PCR analyses were performed on an independent prospective series of 62 early stage CLL samples. CLLs showed a statistically significant increase of *POT1*, *TRF1*, and *MRE11A* expression levels as compared with controls ($P = 0.0004$, 10-fold; $P = 0.00025$, 4.2-fold; and $P = 0.00047$, 7-fold, respectively), whereas

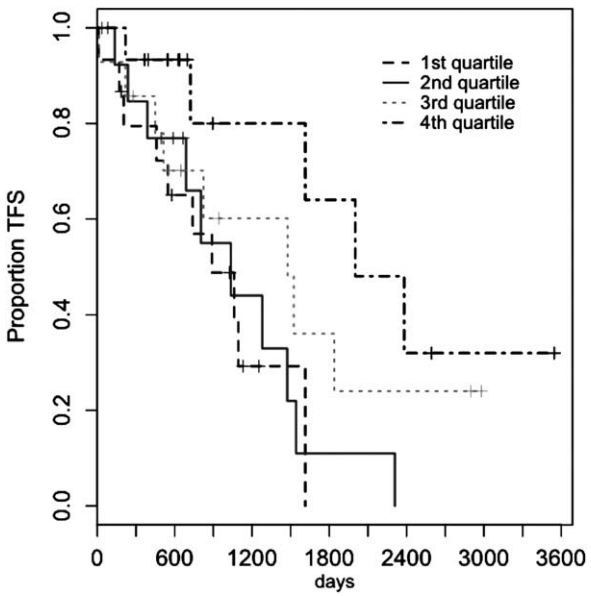

Figure 4. Cox-derived estimated curves according to the RTL levels. The analysis of RTL revealed a significantly lower risk of starting treatment for patients belonging to the fourth quartile (HR = 0.23, 95% C.I. 0.07–0.73, $P = 0.0124$).

no significant differences were observed for *TERT* mRNA expression (Fig. 5).

DISCUSSION

In recent years, several biomarkers have been used for outcome prediction in CLL patients.

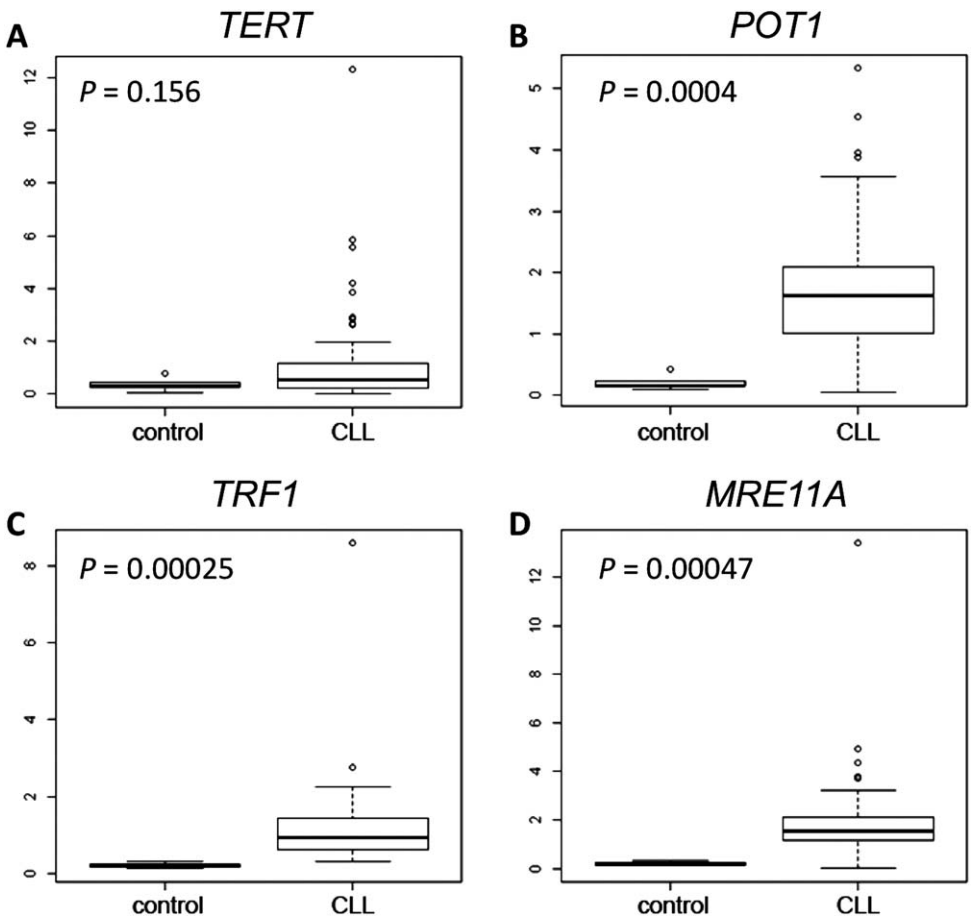

Figure 5. Q-RT-PCR validation of RNA expression levels of telomere-associated genes *TERT* (A), *POT1* (B), *TRF1* (C), and *MRE11A* (D). Box plots of mRNA expression levels obtained by means of Q-RT-PCR analysis in the independent cohort of 62 CLL patients and five healthy donors. Expression levels are given as $2-\Delta\Delta Ct$.

However, the stratification according to these currently used parameters including *IGHV* mutational status, cytogenetics, and ZAP-70 and CD38 expression, does not fully explain the heterogeneity of this disease (Damle et al., 1999; Hamblin et al., 1999; Dohner et al., 2000; Hamblin et al., 2002; Crespo et al., 2003). Several studies have indicated TL and telomerase levels as prognostic factors in the risk stratification of CLL (Damle et al., 2004; Grabowski et al., 2005; Ricca et al., 2007; Terrin et al., 2007; Roos et al., 2008; Rossi et al., 2009; Sellmann et al., 2011; Rampazzo et al., 2012; Mansouri et al., 2013). Furthermore, recent articles have addressed the deregulation of telomerase and telomeric proteins leading to a better comprehension of the pattern of telomere dysregulation in CLL (Poncet et al., 2008; Augereau et al., 2011; Veronese et al., 2013). However, a comprehensive and detailed evaluation of TL, expression levels of telomere-related genes, and well-established biological and cytogenetic prognostic factors is still limited in uniform and representative series of early stage (Binet A) CLLs. Our study was, therefore, aimed at providing insights into the possible involvement of telomere/telomerase system impairment in the asymptomatic phase of the disease.

First, we showed a significant telomere shortening in CLL patients compared with healthy donors. As our series involved a cohort of 77 untreated CLLs in early stage disease, this finding strongly suggests that telomere shortening may represent an early event, as previously demonstrated by Augereau et al. (2011). Herein, we have also found that *IGHV*-UM CLLs have significantly shorter telomeres than mutated *IGHV* cases, as described in previous studies (Damle et al., 2004; Grabowski et al., 2005; Ricca et al., 2007; Rossi et al., 2009; Rampazzo et al., 2012; Mansouri et al., 2013). Concerning ZAP-70 and CD38 expression,

controversial data were reported so far. In our study, we did not find any association between TL and ZAP-70/CD38 expression. Similar results for both ZAP-70/CD38 and for only ZAP-70 were described by Ricca et al. (2007) and Rampazzo et al. (2012), respectively; conversely, other studies (Roos et al., 2008; Brezinova et al., 2010; Mansouri et al., 2013) found that TL correlates with ZAP-70 and/or CD38. However, the controversial definition of the cut-off values for these parameters could in all likelihood explain the different results.

Conflicting results have been reported as well in regard of genomic aberrations, when individual cytogenetic prognostic groups (good, intermediate, and poor) were considered (Ricca et al., 2007; Roos et al., 2008; Brezinova et al., 2010; Sellmann et al., 2011; Rampazzo et al., 2012). Our findings were consistent with recent reports describing a correlation between TL and high-risk genomic aberrations including 11q- and 17p- (Roos et al., 2008; Sellmann et al., 2011; Rampazzo et al., 2012). In particular, the gradual and significant telomere shortening from 13q (as a sole abnormality) to +12, 11q-, and 17p- alterations, the latter showing the shortest telomeres, confirmed recently published data (Mansouri et al., 2013) and the notion that erosion of telomeres may impair their function in protecting chromosome ends, resulting in genetic instability (Hackett and Greider, 2002; Rampazzo et al., 2012).

With regard to clinical data, in a multivariate analysis TL was not an independent prognostic factor for time to first treatment, as also recently reported by Mansouri et al. (2013). This could be explained by the strong association between *IGHV* mutational status and high-risk genomic aberrations including 11q- and 17p and TL (Ricca et al., 2007; Roos et al., 2008).

Global hypomethylation mechanisms involving repetitive sequences may be one of the crucial factors that facilitate clonal expansion of malignant cells (Counts and Goodman, 1995). This mechanism is considered to result in increased genome rearrangement, chromosome instability, and mutation events (Wilson et al., 2007). Based on a previous study performed by us on the same cohort as in this investigation, significantly lower methylation levels of *Alu*, *LINE-1*, and *SAT-α* repetitive elements were detected in CLL than in healthy donors (Fabris et al., 2011). Here, we found a significant correlation of telomere shortening with global hypomethylation of *Alu* and *LINE-1* repetitive sequences. Albeit short telomeres should not be considered per se a measure for genomic instability and could be a consequence of other causes (e.g., higher proliferation rate), our data suggest that tumor TL and integrity can be influenced by the epigenetic status, namely increased hypomethylation, in early stage CLL (Vera et al., 2008).

In addition to telomerase, various telomere-binding proteins are involved in the regulation of telomere structure and functions (Stansel et al., 2001). To date, the investigations evaluating the involvement of telomere-associated genes in CLL pathogenesis are limited and mainly focused on shelterin proteins and a set of multifunctional factors, including RPA1, EST1A, KU70/KU80, and the MRE11 complex (Poncet et al., 2008; Augereau et al., 2011; Veronese et al., 2013).

Concerning these genes, our microarray analysis showed a significant increase in the expression levels of the *TRF1*, *POT1*, and *RAP1*, all of them belonging to the shelterin complex. With regard to MRE11 complex, we found significantly higher mRNA levels of *MRE11A* and *RAD50* in CLLs as compared with controls. A significant overexpression of replication protein A1 (*RPA1*) was also detected. Finally, increased mRNA levels of *TERT* was observed.

On the basis of their role in the maintenance of telomere functions in several hematological and solid tumors, *TERT*, *POT1*, *TRF1*, and *MRE11A* were selected for Q-RT-PCR validation in an independent prospective series. Microarray data were confirmed for all the genes with the exception of *TERT* for which no significant differences were shown. These findings suggest that telomere-associated genes might be involved in the regulation of telomere structure at the early stage of the disease independently of telomerase levels, as recently reported in human astroglial brain tumors (La et al., 2013). Previous studies indicated TRF1, POT1, and RAP1 as negative regulators of the TL (Oh et al., 2005; La et al., 2013); while TRF1 and POT1 compete with telomerase to prevent telomere access (Loayza and De Lange, 2003; Chang, 2013), RAP1 acts to modulate the recruitment of other negative regulators (O'Connor et al., 2004). Consistent with our findings, the upregulation of these factors could be involved in a progressive telomere shortening, playing a role in the immortalization of cancer cells by promoting genomic instability as recently reported for *TFR1* in brain tumors (La et al., 2013). Significantly increased levels of *MRE11A*, *RAD50*, and *RPA1* were also observed in our study; in addition to DNA replication, these factors were

reported to be involved in DNA damage recognition and repair, also in concerted fashion (Matsutani et al., 2001; Bochkarev and Bochkareva, 2004; Givalos et al., 2007). It has also been suggested that the upregulation of these genes, in particular RPA1, occurs as a result of the increasing need for DNA repair (Givalos et al., 2007). We should mention that our results are not fully concordant with previous data (Poncet et al., 2008; Veronese et al., 2013), reporting a downregulation in CLL cases of some of the genes investigated in this study. However, we cannot exclude that such results were affected by the small number of patients included in their cohorts (in particular of early stage CLL) compared with our series or, not less importantly, by the presence of advanced stage patients. These aspects could likely explain conflicting data. Nonetheless, our data were further validated and clearly confirmed by Q-RT-PCR in an independent representative cohort of Binet A CLLs, thus strongly suggesting that modulation of telomere-associated genes, together with telomere shortening, represent early event in the disease.

Overall, our study extends previous evidence of the association between telomere status and genomic instability in CLL and indicates for the first time a significant correlation between telomere shortening and global DNA hypomethylation. Our data provide a further contribution to the notion that impairment of the telomere/telomerase system represents an early event in CLL pathogenesis.

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