Telomere length and expression of human telomerase reverse transcriptase splice variants in chronic lymphocytic leukemia

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Telomerase activity and telomere length (TL) are prognostic markers in chronic lymphocytic leukemia (CLL). The rate-limiting component of telomerase is human telomerase reverse transcriptase (hTERT), for which multiple transcripts exist. Two splicing sites, \(\alpha\) and \(\beta\), have been described that generate deleted transcripts. Only the full-length (FL; \(\alpha^{+}\beta^{+}\)) transcript translates into a functional protein. The aim of this work was to characterize hTERT splice variants in CLL in relation to disease activity, clinical stage, immunoglobulin heavy chain variable (IGHV) genes mutational status, and TL. Real-time polymerase chain reaction assays were validated for quantification of the hTERT transcripts with either \(\alpha\) deletion (del-\(\alpha\); \(\alpha^{-}\beta^{+}\)), \(\beta\) deletion (del-\(\beta\); \(\alpha^{+}\beta^{-}\)) or both \(\alpha\) and \(\beta\) deletions (del-\(\alpha\beta\); \(\alpha^{-}\beta^{-}\)). The splice variant expression pattern was studied in 97 patients with CLL, 6 healthy control subjects, and one CD34\textsuperscript{+} cell sample. TL was assessed with real-time polymerase chain reaction in 71 of 97 samples. Thirty-two percent of the cases did not express any of the splice variants. Average FL expression was 5.5-fold higher in IGHV-unmutated (\(n = 35\)) compared with mutated (\(n = 59\)) patients \((p < 0.0001)\). FL levels correlated directly with the percentage of IGHV homology \((r = 0.34; p = 0.0007)\) and inversely with TL \((r = -0.44; p = 0.0001)\). Overall, FL expression correlated significantly with that of the other splice variants. All transcripts were more frequently expressed in progressive compared with nonprogressive patients \((p < 0.0001\) for FL and del-\(\alpha\); \(p = 0.01\) for del-\(\beta\); and \(p = 0.006\) for del-\(\alpha\beta\)). This study provides a detailed insight into the hTERT transcript pattern in CLL, highlighting the necessity of subgrouping patients according to IGHV mutation status when analyzing hTERT expression. © 2013 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Chronic lymphocytic leukemia (CLL) is a malignant lymphoproliferative disorder of mature B lymphocytes expressing a distinct cell surface phenotype (CD19\textsuperscript{+}, CD5\textsuperscript{+}, and CD23\textsuperscript{+} with diminished surface membrane immunoglobulin). It is the most common leukemia in adults in the Western world and typically affects elderly people. CLL is characterized by an extremely heterogeneous clinical course, with some patients living for decades without requiring treatment and others progressing rapidly despite therapy. Currently, there is a dearth of proactive interventions during the indolent phase to identify patients with the greatest risk of developing aggressive disease.

Over the past decade, considerable effort has been directed to understanding the mechanisms underlying the heterogeneous clinical course of this disease and finding prognostic biomarkers for clinical classification. Molecular patterns have been identified that define patient subgroups with different prognosis or response to therapy. Among these, one of the best established prognostic (but not predictive) factors is the mutational status of the immunoglobulin heavy chain variable (IGHV) gene locus, with the IGHV unmutated status being associated with a worse prognosis [1,2]. The replicative potential of eukaryotic cells is regulated through specialized DNA structures called telomeres, which cap the ends of the chromosomes. Telomere length is a key determinant of the normal lifespan of a cell and is maintained by two different processes: the activity of the
enzyme telomerase [3–5] and the alternative lengthening of telomere (ALT) pathway [6].

Telomerase is a ribonucleoprotein complex composed of two essential components: a catalytic subunit with reverse transcriptase activity (human telomerase reverse transcriptase [hTERT]) and an RNA subunit human telomerase RNA (hTR). In the adult organism, telomerase expression is restricted to a few cell types, most notably germ cells and stem/progenitor cells.

Telomerase has been a target of increasing interest because high telomerase activity is one of the mechanisms that sustain the unlimited growth of cancer cells [7,8] and has been described in 85% of human cancers [9,10]. Several studies have investigated telomerase activity in CLL and have shown an association of high telomerase activity with progressive disease [11] and with an unmutated IGHV profile [12].

Telomere length has also been shown to be an independent predictor of survival and need for therapy in CLL [13]. In addition, an association between short telomeres and genetic complexity, high-risk genomic aberrations, and short survival has also been reported [14]. A comprehensive telomere dysfunction in CLL was suggested based on aberrant telomere structure [15] and altered expression of telomere maintenance genes [16]. Telomerase activation and telomere shortening occurs during the classical T cell–mediated germinal center (GC) reaction [17–20]. In a recently published study, Damle et al. [21] studied the effect of B cell receptor (BCR) stimulation on modulating telomerase activity and found that T cell–independent BCR-mediated signaling also induces telomerase activity preferentially in IGHV unmutated CLL cases [21]. This observation indicates that the fate of the CLL cell can be driven by the quality of the antigen that interacts with the BCR, strengthening the hypothesis that CLL is an antigen-driven disease and that this factor is a key determinant for the clinical features of the disease.

Telomerase activity is regulated by a number of different mechanisms. Among these mechanisms, regulation of hTERT messenger RNA (mRNA) expression seems to be the most important and rate-limiting step for telomerase activation [22]. Accordingly, expression of hTERT was also found to correlate with poor clinical outcome in most cancer types and hematologic malignancies [23,24], including CLL [25].

To date, 10 alternative splicing sites of the hTERT transcript have been described, including splicing at two main sites: α and β [26–29]. The α splice site causes a 36-bp deletion within the conserved reverse transcriptase motif A [27,28] and has been shown to act as a dominant negative modulator of telomerase activity [30,31]. The β splice site generates a 182-bp deletion leading to a nonsense mutation [27,28]. Only the full-length (FL) transcript (αβ) containing the A and B reverse transcriptase motifs, translates into a functional protein [27–29] (Fig. 1).

However, previous studies investigating hTERT expression in CLL by reverse-transcription polymerase chain reaction (RT-PCR)–based assays used primers designed either to the region upstream [32] or downstream [16] of the α- and β-subunits and so did not discriminate between the deletion variants.

Tchirkov et al. [32] showed that hTERT expression increased with advancing stage and in patients with unmutated IGHV genes [32]. However, the quantitative PCR (qPCR) assay used by these authors did not distinguish between the different deletion variants. This might have contributed to an overestimation of hTERT expression because only the FL hTERT transcript leads to the translation of a functional enzyme. Moreover, an imbalance in the proportions of IGHV mutated versus unmutated cases in the patient population studied might also have contributed to underestimating or overestimating hTERT expression levels. Poncet et al. [16] reported a lower expression of the telomerase enzyme complex genes (i.e., hTERT, hTR, DYSKERIN) and genes of the sheltering complex (TRF1, hRAP1, POT1) in CLL patients compared with healthy donors. Of 42 patients tested, only 10 had unmutated IGHV genes. In addition, the qPCR assay used for hTERT did not discriminate between the deletion variants.

The existence of multiple transcripts suggests that telomerase activity might be regulated by transcriptional control mechanisms as well as alternative splicing of hTERT [27,33–35].

The aim of the present study was to characterize hTERT splice variants in CLL cells, as well as study the expression of hTERT splice variants and TL in relation with disease activity and clinical stage. Expression of hTERT splice variants was also characterized in a group of healthy control donors and in CD34+ cells. hTERT is indeed known to be upregulated in hematopoietic stem cells, with telomerase being responsible for the self-renewal properties of these cells [36].

Methods

Patient characteristics

The study was performed in keeping with the Helsinki declaration on research with human subjects and approved by the regional ethics committee. Peripheral blood was obtained from 97 patients fulfilling the diagnostic criteria for CLL [37] and from six healthy donors with informed consent. The clinical characteristics of the patients with CLL are shown in Table 1.

Total RNA isolation and cDNA synthesis

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of patients with CLL and healthy donors by Ficoll separation (GE Healthcare, Uppsala, Sweden). Total RNA was extracted from 5–10 × 10^6 PBMCs using the Trizol reagent (Invitrogen, Gaithersburg, MD, USA). Total RNA was also extracted from 5–10 × 10^6 purified CD34+ cells obtained from a patient with non-Hodgkin lymphoma who had undergone...
apheresis for autologous stem cell transplantation. RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). One microgram of total RNA was used to perform reverse transcription of RNA with the SuperScript II Kit (Invitrogen) in a total volume of 20 μL, according to the manufacturer’s instructions. Complementary DNA (cDNA) samples were stored at −20°C.

Quantification of hTERT transcripts by qPCR

Primers and probes. Quantification of the functional hTERT (α+β+) transcript was performed using the FL1/FL2 primers and FL hydrolysis probe, as described by Terrin et al. [25]. For the quantification of the other three transcripts (α+β−, del-α; α−β+, del-β; α−β−, del-αβ) newly designed primers, FL3 (5′-TTGTCAAGGACAGGCTCAC-3′) and FL4 (5′-TGGACGTAG GACGTGGCTC-3′), and hydrolysis probe ST (FAM 5′-CC AGAACAGTACTGCTGC-3′ MGBNFQ) were used. Primer FL3 binds specifically to the mRNA with deletion in the α site (del-α). Primer FL4 binds specifically to the mRNA splice variant with the deletion in the β site (del-β). Primer combinations for each splice variant were as follows: FL1-FL2 (α+β+, FL), FL3-FL2 (α+β−, del-α), FL1-FL4 (α−β+, del-β), FL3-FL4 (α−β−, del-αβ; Fig. 1). RT-PCR products were visualized in a 3% agarose gel, excised, extracted with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced using ABI PRISM 310 (Applied Biosystems, Foster City, CA, USA) with respective forward and reverse primers and Big Dye terminator V1.1 Cycle Sequencing (Applied Biosystems) to confirm the correct transcript sequence. The hTERT sequences are available under GenBank accession number AF015950.

Real-time qPCR conditions. All qPCR reactions were performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). For detection of the α+β+ (FL) and of the

Figure 1. Structure of hTERT deletion variants and principle of the proposed qPCR assay for the quantification of the four hTERT splice variants. In the upper part, seven conserved Reverse transcriptase domain motifs (1, 2, A’, B, C, D, and E), exons 3-13, and deletion sites (α, β, γ) are shown. In the lower part, the four alternative splice variants transcripts (FL; del-α, del-β, del-αβ) are represented with corresponding primer (arrows) combinations used for specific amplification.
RNA extracted from K562 cells, which strongly express the hTERT gene, and used as template for the amplification of hTERT-FL by real-time PCR using primers FL1 and FL2. For RPLP0, external standards were constructed using RPLP0 specific primers.

**Immunoglobulin VH family usage and mutational status of CLL cells**

The IGHV gene family of the CLL clone was identified using a sense, family-specific (VH1-VH6) framework region primer in conjunction with the appropriate antisense CH primer as described previously [39]. IGHV gene sequences were considered mutated if the deviation from the corresponding germline gene was greater than 2%.

**Telomere length analysis**

Average telomere length was assessed by monochrome multiplex quantitative PCR (MMQPCR) method of Cawthon [40]. Dual annealing temperature PCR amplification of telomere repeats (Tel) and the chromosome 4, single copy gene ALB were performed in a single reaction in the presence of a fluorescent DNA-intercalating dye (SYBR green). Tel signals were collected in early cycles, before ALB signals rise above baseline, and ALB signals were collected at a temperature that fully melts the Tel product. Five concentrations of a reference DNA sample (standard DNA), spanning a 10-fold range of DNA concentration, were analyzed in triplicate in each run to provide a standard curve used for relative quantification. Each patient sample was assayed in triplicate, giving three T/S results; the final reported result is the average of the three T/S values. Samples with T/S > 1.0 have an average telomere length greater than that of the standard DNA; samples with T/S < 1.0 have an average telomere length shorter than that of the standard DNA.

**Statistical analysis**

Statistical analyses were performed using the JMP software 5.1.2 and the StatView software 5.0.1 (SAS Institute, Cary, NC, USA). Comparisons of transcript expression levels between different groups of patients were done with the nonparametric Mann-Whitney U test. Simple regression analyses and the Spearman rank test were used to examine the relationship between the levels of expression of the different transcripts and the percentage of mutation of the IGHV gene sequences. All tests were two-sided, and p < 0.05 was considered statistically significant.

**Results**

**Expression of the full-length hTERT transcript**

Twenty-seven of the 85 cases (32%) for which all splice variants could be analyzed did not express any of the splice variants. All these patients were nonprogressive and, except for two, had mutated IGHV genes. The functional transcript of hTERT-FL (α+β-) was expressed at higher levels in patients with unmutated than mutated IGHV genes. Average hTERT-FL expression was 5.5-fold higher in unmutated (n = 35) versus mutated (n = 59) patients (median MNE, 4.69 × 10⁻⁴ [0.00–9.89 × 10⁻³] vs. 0.00 [0.00–4.00 × 10⁻³]; p < 0.0001; Fig. 2A). MNE levels of FL
transcript detected in CD34+ cells and in the K562 cell line were $6.03 \times 10^{-4}$ and $1.85 \times 10^{-3}$, respectively. FL levels directly correlated with the percentage of IGHV homology ($r = 0.33; p = 0.001; n = 94$).

The expression of FL was sixfold higher in progressive (n = 35) compared with nonprogressive patients (n = 62; median, $6.39 \times 10^{-4}$ [0.00–9.89 $\times 10^{-3}$] vs. 0.00 [0.00–4.00 $\times 10^{-3}$]; $p < 0.0001$; Fig. 2B). In the IGHV mutated group, progressive patients (n = 11) were found to have significantly higher FL levels compared with nonprogressive patients (n = 48; median, 0.00 [0.00–3.18 $\times 10^{-3}$] vs. 0.00 [0.00–4.00 $\times 10^{-3}$]; $p = 0.02$). The same finding was observed in the IGHV unmutated group (median, $7.99 \times 10^{-4}$ [0.00–9.89 $\times 10^{-3}$]) in the progressive patients group vs. 0.00 [0.00–2.10 $\times 10^{-3}$] in the nonprogressive group; $p = 0.02$; Fig. 2D).

When the patients were subgrouped by clinical stage, increasing FL expression was noted with increasing clinical stage (Fig. 2C). Higher FL expression in high-stage compared with low-stage patients was also observed when the IGHV mutated and unmutated groups were analyzed separately. No difference, however, was seen between low- and intermediate-stage patients in the mutated group and between intermediate- and high-stage patients in the unmutated group (Fig. 2E). Among all patients, FL expression was found to be higher among pretreated compared with nonpretreated patients (median, $4.5 \times 10^{-4}$ [0.00–9.89 $\times 10^{-3}$] vs. 0.00 [0.00–4.00 $\times 10^{-3}$]; $p = 0.003$). All pretreated patients had either intermediate (13 of 26) or high (13 of 26) clinical stage, and 60% had progressive disease at the time of testing; 50% had unmutated IGHV genes.

Figure 2. Mean normalized expression (MNE) values of hTERT functional transcript (FL) in healthy control donors, IGHV mutated and IGHV unmutated CLL patients (A); in healthy control donors, nonprogressive and progressive CLL patients (B); in CLL patients subgrouped by stage (C); in nonprogressive and progressive CLL patients within the IGHV mutated and unmutated subgroups (D); and in CLL patients subgrouped by stage within the IGHV mutated and unmutated subgroups (E). Scatter plots display individual values with line at median. Only significant statistical values are reported.
Expression of the other hTERT splice variants

The del-α transcript was more highly expressed in IGHV unmutated (n = 35) than mutated (n = 58) patients (median $1.01 \times 10^{-3}$ [0.00–1.73 $\times 10^{-2}$] vs. median 0.00 [0.00–4.59 $\times 10^{-3}$]; $p < 0.0001$; Fig. 3A). del-α levels directly correlated with the percentage of VH homology ($r = 0.38$, $p = 0.00021$, n = 92). The expression of this transcript was also significantly higher in progressive (n = 35) compared to non-progressive (n = 61) patients (median 1.01 $\times 10^{-3}$ [0.00–1.73 $\times 10^{-2}$] vs. 0.00 [0.00–5.08 $\times 10^{-3}$]; $p < 0.0001$) (Fig. 3B). When IGHV mutated and unmutated patients were analyzed separately, this difference was observed only in the unmutated group (Fig. 3E). del-α expression was found to be higher in pretreated versus nonpretreated patients (median $8.7 \times 10^{-4}$ [0.00–1.73 $\times 10^{-2}$] vs. 0.00 [0.00–5.08 $\times 10^{-3}$]; $p = 0.001$).

For the del-β (α$^{-}$β$^{-}$) transcript, no difference was observed between unmutated (n = 34) and mutated (n = 53) patients, whereas both patient groups had significantly lower expression levels compared with healthy donors. Three of six healthy controls were indeed found to express the del-β transcript, whereas only 1 of 53 mutated patients and 5 of 34 unmutated patients did so (median $4.52 \times 10^{-4}$ [0.00–1.13 $\times 10^{-3}$] vs. 0.00 [0.00–7.18 $\times 10^{-3}$]; $p = 0.045$, and vs. 0.00 [0.00–1.76 $\times 10^{-2}$]; $p = 0.0002$, respectively; Fig. 4A). No correlation was seen between del-β expression and the percentage of IGHV homology ($r = 0.18$; $p = 0.09$; n = 87). Moreover, nonprogressive patients expressed significantly lower levels of del-β compared with healthy donors (median, 0.00 [0.00–1.76 $\times 10^{-2}$] vs. $4.52 \times 10^{-4}$ [0.00–1.13 $\times 10^{-3}$]; $p < 0.0001$). This difference between nonprogressive patients and healthy donors was also noticed when the IGHV mutated and unmutated subgroups were analyzed separately (Fig. 4D). The expression of this transcript was also significantly higher in progressive compared with nonprogressive patients (median, 0.00 [0.00–7.18 $\times 10^{-3}$] vs. 0.00 [0.00–1.76 $\times 10^{-2}$]; $p = 0.01$). The del-β transcript was not detected in CD34$^{+}$ cells, and the expression in K562 cell line was $2.7 \times 10^{-3}$. Significantly lower del-β expression was noted in both low- and intermediate-stage patients compared with healthy donors (Fig. 4C). When the IGHV mutated and unmutated groups were analyzed separately, this difference was observed only in the mutated group (Fig. 4E). No difference was seen between pretreated and nonpretreated patients.

The del-αβ (α$^{-}$β$^{-}$) transcript was more highly expressed in IGHV unmutated (n = 32) compared with mutated (n = 55) patients (median, $1.16 \times 10^{-3}$ [0.00–1.74 $\times 10^{-2}$] vs. 0.00 [0.00–4.89 $\times 10^{-2}$]; $p = 0.0025$; Fig. 5A). No correlation was observed with the percentage of IGHV homology ($r = 0.19$; $p = 0.08$; n = 85). The expression of this transcript was also significantly higher in progressive compared with nonprogressive patients (median, 9.40 [0.00–9.94 $\times 10^{-4}$] vs. 0.00 [0.00–4.89 $\times 10^{-2}$]; $p = 0.006$; Fig. 5B). This difference was also noticed when the IGHV mutated subgroup was analyzed separately (median, 6.72 [0.00–9.94, $\times 10^{-2}$] vs. 0.00 [0.00–4.89 $\times 10^{-2}$]; $p = 0.04$; Fig. 5D). No difference was seen between CLL patients and healthy donors (Fig. 5A).

The levels of del-αβ transcript were $1.67 \times 10^{-3}$ in CD34$^{+}$ cells and $4.93 \times 10^{-3}$ in the K562 cell line. When the patients were subgrouped by clinical stage, no significant difference was observed between patients with different clinical stages (Fig. 5C) in either the mutated or unmutated subgroups (Fig. 5E). No difference was seen between pretreated and nonpretreated patients.

Correlation between levels of expression of the different hTERT splice variants

The levels of expression of the FL transcript significantly correlated with those of the other splice variants: del-α ($r = 0.58$; $p < 0.0001$; n = 96), del-β ($r = 0.52$; $p < 0.0001$; n = 91), del-αβ ($r = 0.33$; $p = 0.02$; n = 89). Accordingly, the two unmutated progressive patients expressing the highest FL levels (Fig. 2) are also those expressing the highest del-α levels (Fig. 3). Both patients had chemorefractory disease, and one had 17p deletion in 90% of the leukemia cells as detected by interphase fluorescence in situ hybridization.

Alterations in transcript levels at disease progression

Two patients were tested in two different disease phases (i.e., when the disease was early-stage and asymptomatic and then at disease progression). Both patients had mutated IGHV genes. When tested the first time, patient #09 was in clinical remission after chlorambucil treatment: the clinical stage was Rai I with a white blood cell count of $69 \times 10^9$ cells/L. The patient remained clinically stable for almost 2 years, when the lymphocytosis quickly progressed to a value of $247 \times 10^9$ cells/L and the patient developed both anemia and thrombocytopenia (Rai stage IV). In this patient, the hTERT functional transcript slightly increased in expression at disease progression, whereas the del-α transcript expression levels decreased.

The other patient (#67) was first tested at diagnosis, when the clinical stage was Rai 0 with a white blood cell count of $41 \times 10^9$ cells/L. After 1 year, the lymphocytosis had progressed to a value of $189 \times 10^9$ cells/L, and the patient had developed constitutional disease-related symptoms and autoimmune anemia, which led to initiation of
treatment. In this patient, the functional transcript was expressed neither during the indolent phase nor at disease progression, whereas the del-α transcript, which was expressed during the indolent phase, was found to be no longer expressed at disease progression (Fig. 6).

Telomere length

Telomere length was found to be significantly shorter in patients with unmutated compared with mutated IGHV genes (median relative TL, 0.67 vs. 1.6; \( p < 0.0001 \); \( n = 71 \)) and correlated inversely with the percentage of
IGHV homology to germline ($r = -0.46; p < 0.0001; n = 71$). Despite the fact that we did not adapt for age, this observation is unlikely to be ascribable to age difference between the two groups, because the age of the patients in the two groups was comparable (median, 69 vs. 67 years; $p = 0.7$).

TL was also found to be higher in nonprogressive versus progressive patients (median, 1.54 vs. 0.53; $p < 0.0001$). When subgrouping the patients based on IGHV mutational status, TL was found to be greater in nonprogressive compared with progressive patients in both groups (median, 1.67 vs. 0.44 [$p = 0.0009$] and 0.98 vs. 0.59 [$p = 0.034$] for the mutated and unmutated groups, respectively).

Expression of the FL, del-α, del-β, and del-αβ transcripts inversely correlated to TL ($r = -0.44, p = 0.0001, n = 71; r = -0.37, p = 0.001, n = 71; r = -0.27, p = 0.03, n = 64; and r = -0.28, p = 0.03, n = 63$, respectively).

**Discussion**

To our knowledge, this study is the first to investigate the full hTERT expression pattern, including both the functional and the deleted variants in different disease phases and clinical stages of CLL.

In a previous study, Terrin et al. [25] studied hTERT in 134 CLL cases and evaluated the prognostic values in...
relation to other known prognostic markers, as IGHV mutation status, CD38 and ZAP-70 expression. They developed a real-time PCR assay to quantify the amount of hTERT functional transcript (hTERT-FL) and of all the other transcripts (hTERT-AT). The majority of the analyzed samples were collected at diagnosis. The authors observed that the cumulative expression of all hTERT transcripts was higher in patients with unmutated IGHV genes.

Figure 5. Mean normalized expression (MNE) values of the hTERT del-αβ (α–β) transcript in healthy control donors, IGHV mutated and IGHV unmutated CLL patients (A); in healthy control donors, nonprogressive and progressive CLL patients (B); in CLL patients subgrouped by stage (C); in nonprogressive and progressive CLL patients within the IGHV mutated and unmutated subgroups (D); and in CLL patients subgrouped by stage within the IGHV mutated and unmutated subgroups (E). Scatter plots display individual values with a line at the median. Only significant statistical values are reported.
Our study confirms the observation that TL is significantly shorter in patients with unmutated compared with mutated IGHV genes [12,41,42] and that the functional transcript of hTERT is expressed at higher levels in patients with unmutated IGHV genes. This study also highlights the necessity of subgrouping patients according to this prognostic factor when analyzing hTERT expression. We found the expression of FL to be higher in progressive compared with nonprogressive patients. Such difference in FL expression between patients in various disease phases was noted both in the mutated and unmutated IGHV subgroups. The results indicate that the higher expression of the functional transcript seen in the progressive patients may be due to a higher frequency of unmutated patients in the progressive disease patient subgroup. Moreover, when the patients were subgrouped by clinical stage, increasing FL expression was noted with increasing clinical stage. This difference was also observed when the IGHV mutated and unmutated patients were analyzed separately, but appeared more remarkable in the unmutated group. This observation, together with the knowledge that pretreated patients express higher FL levels, is in accordance with the higher FL expression observed in the progressive patient group; that is, it likely reflects the fact that most progressive patients gather in the intermediate to high clinical stage group and are often pretreated.

We observed that the del-α (αβ) transcript is also expressed at a higher level in unmutated compared with mutated patients, whereas no difference was seen for the del-β transcript. Regarding the del-β transcript, unmutated and mutated patients were found to have significantly lower expression levels compared with healthy donors. A significant difference was also observed between healthy donors and nonprogressive patients who expressed lower del-β transcript levels in the mutated and unmutated subgroups. Healthy donors expressed higher del-β levels when compared with patients with low-stage and intermediate-stage CLL, which applied only to the unmutated group, once the patients were analyzed separately. Caution is nevertheless warranted in interpreting these findings because PBMCs from healthy donors contain both T and B lymphocytes, and likely T cells in higher percentage.

However, the del-αβ transcript was found to be more expressed in mutated compared with unmutated patients.

A relative increase of the hTERT-FL levels can occur in individual patients during disease progression. We performed serial analysis in two patients, both with mutated IGHV genes, and noted that at disease progression (increase in lymphocytosis and bone marrow failure in the first case; progression of lymphocytosis and appearance of constitutional symptoms in the second), FL levels increased and del-α levels decreased. The results suggest an ongoing process favoring the expression of the functional transcript and abating the dominant negative modulator variant del-α during progression of CLL.

In conclusion, our study highlights the necessity of focusing on the functional transcript when analyzing hTERT expression in patients with CLL and of interpreting the results in various phases and clinical stages of the disease in the light of the IGHV mutational status. The function and biological role of hTERT splice variants remains to be clarified. A few assays have been developed to discriminate between deletion variants by qPCR. These assays have been used to analyze hTERT mRNA splicing patterns in immortal human cells [43], benign and malignant breast tumors [44], non–small cell lung cancer specimens [45], melanoma [46], and thyroid neoplasms [47]. To our knowledge, this study is the first to perform such analysis in CLL. Predominant splice variance patterns in tumor cells could be used as a diagnostic or prognostic biomarker [48] or possibly as targets for therapy. We demonstrated previously that patients with CLL with hTERT-positive leukemic cells have hTERT-specific T cells [39]. Cancer-specific splicing events can generate novel epitopes that serve as targets [49]. Because the correlation between hTERT-FL transcript expression and telomerase
activity indicates that the hTERT-FL mRNA is translated in leukemic cells, it can give rise to tumor-specific antigens that may be targeted with immunotherapy or telomerase inhibitors.

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Conflict of interest disclosure
No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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