hTERT expression and prognosis in B-chronic lymphocytic leukemia

A. Tchirkov1,2*, C. Chaleteix1, C. Magnac3, Y. Vasconcelos3,4, F. Davi5, A. Michel5, F. Kwiatkowski6, O. Tournilhac1, G. Dighiero3 & P. Travade1

1Service d'Hématologie Clinique, CHU, Clermont-Ferrand; 2Département de Radiothérapie and 6Service Statistiques et Communications Médicales, Centre Jean Perrin, Clermont-Ferrand; 3Unité d’Immunohématologie et d’Immunopathologie, Institut Pasteur, Paris; 4Département d’Hématologie, Hôpital Pitie-Salpétrière, Paris, France; 5Division of Hematology, Universidade Federal de São Paulo, São Paulo, Brazil

Received 23 November 2003; revised 16 May 2004; accepted 19 May 2004

Background: In B-chronic lymphocytic leukemia (B-CLL), there is a need for molecular markers to predict the evolution of this heterogeneous disease in individual patients. The level of expression of the human telomerase reverse transcriptase (hTERT) gene has been associated with disease aggressiveness in human cancers. The purpose of the present study was to examine the prognostic significance of hTERT expression in B-CLL.

Patients and methods: We used real-time reverse transcription-PCR to quantitate the amount of hTERT transcripts in mononuclear blood cells from 90 B-CLL patients. In addition, samples were analyzed for somatic mutations in the immunoglobulin V (IgV) genes.

Results: The expression of hTERT gene was detected in 59% of patients. The level of expression increased with advancing B-CLL stage (P = 0.0064). Patients expressing hTERT showed significantly shorter survival than hTERT-negative patients (P = 0.000034), irrespective of the disease stage. On average, the level hTERT mRNA expression was seven-fold higher in the poor-prognosis B-CLL group with unmutated IgV than in the Ig-mutated group (P < 10−7). The level of hTERT expression discriminated the Ig-unmutated from Ig-mutated B-CLL in 89% of cases.

Conclusion: Our data indicate that hTERT expression in B-CLL may serve as a molecular prognostic marker.

Key words: B-CLL, hTERT, prognosis, real-time reverse transcription-PCR

Introduction

Human cancer cells are believed to acquire immortality through the activation of telomerase that is repressed in the majority of normal somatic cells (reviewed in [1]). Telomerase is a ribonucleoprotein complex that maintains telomeres by adding hexameric TTAGGG repeats to the chromosomal ends, thus compensating for the continued replicative loss of telomeres [2]. Telomerase activity is regulated at the expression level of the human telomerase reverse transcriptase (hTERT) gene coding for the catalytic subunit of telomerase [3]. The acquisition of expression of hTERT seems to be an essential step in the development and progression of a majority of human tumors [4]. Recent studies have related the level of hTERT expression to clinical aggressiveness and poor prognosis in a variety of malignancies, including leukemia and lymphoma [5–10].

The identification of reliable prognostic markers is essential in B-cell chronic lymphocytic leukemia (B-CLL), which is clinically heterogeneous. Clinical staging systems enable physicians to divide patients into low-, intermediate- and high-risk groups, but they do not accurately predict disease evolution within the low-risk group, which contains 65% of B-CLL cases [11, 12] Among known biological indicators of prognosis, the presence or absence of somatic mutations in the immunoglobulin V (IgV) gene regions is considered to be the best discriminator between stable and progressive disease. Within all clinical B-CLL stages, an Ig-unmutated gene profile is associated with an aggressive clinical course [13–15] However, this analysis is difficult to perform.

In B-CLL, one study has shown that high telomerase activity may be a predictor of a shorter survival [16]. Very recently, higher telomerase activity was reported in a poor-prognosis Ig-unmutated B-CLL subgroup, as compared with an Ig-mutated subgroup [17]. However, in this study, telomerase activity evaluated using telomeric repeat amplification protocol (TRAP) technology was not significantly related to patient outcome. Thus, the prognostic value of telomerase activity needs to be further investigated.
analysis in B-CLL needs to be confirmed. Moreover, the prognostic significance of hTERT analysis at the gene expression level has not yet been investigated in B-CLL.

To address these issues, we measured the relative amount of hTERT mRNA using real-time reverse transcription (RT)-PCR in mononuclear blood cells obtained from 90 patients with B-CLL at various stages of the disease. The results were correlated to overall patient survival and IgV mutational status. We found that hTERT expression was significantly correlated to short patient survival and Ig-unmutated B-CLL subtype. These results suggest that hTERT expression may serve as a molecular prognostic marker in B-CLL.

Patients and methods

Patients and samples

Ninety B-CLL patients (60 men and 30 women with a mean age of 61.5 years (range 36–96)) were included in this study. Median follow-up was 6 years (range 1–22). Patients were seen at the Clermont-Ferrand Hospital, the Pasteur Institute and the Pitié Salpêtrière Hospital (France), and at the Division of Hematology, Universidade Federal de São Paulo (Brazil). After informed consent was given, peripheral blood samples were obtained for the study. Mononuclear cells were isolated from whole blood by means of a standard Ficoll procedure and cryopreserved until RNA extraction. At the time of sampling, 55 patients were in stage A, 22 in stage B and 13 in stage C according to the Binet classification, and 86% of them were untreated. In addition, mononuclear cells obtained from 15 age-matched healthy individuals were included as controls.

Real-time RT-PCR for hTERT mRNA

The amount of hTERT mRNA was assessed using real-time RT-PCR in the LightCycler system (Roche Diagnostics, Meylan, France) as reported previously, with minor modifications [10]. Total RNA was extracted, reverse transcribed with random hexamers and amplified using: hTERT-specific primers 5'-GGAGCAAGTGGCAAGAGCATTG-3' (forward) and 5'-TCCCCACGACGTAGTCCATGTT-3' (reverse); and probes 5'-CTGCCGGGAGCTGTCGG-3'FITC (probe 1) and 5'LCRed 640-GCAGAGGTCAGGCAGCA-3'Ph (probe 2). In addition, the amount of ABL mRNA was quantified in all samples as an internal control using the forward primer 5'-GCCGCTCGTTGGAACTCCAAGG-3', reverse primer 5'-TGACTGGCGTGATGTAGTTTGCTT-3' and SYBRGreen I as a detection format. The results of real-time RT-PCR were given as normalized hTERT expression, i.e. the ratio between hTERT and ABL transcripts multiplied by 1000. All experiments were performed in triplicate, with good consistency of results (the mean coefficient of variation was 9.4%).

Analysis of IgV mutational status

A cDNA sample was amplified using primers for the V_H gene, and cloned and sequenced as reported previously [15]. The sequence was aligned to the DDBJ/EMBL/GenBank and V-BASE databases. Homology of ≥98% to the germ line sequence was used to define the absence of IgV mutations.

Statistical analysis

The Kruskal–Wallis and Spearman rank tests were used to determine the significance of associations between characteristics. A receiver operating characteristic (ROC) analysis was used to determine which hTERT level best discriminated between unmutated and mutated B-CLL cases [18]. Overall survival was calculated using the Kaplan–Meier method and survival curves were compared using the log-rank test. Deaths not attributable to B-CLL were censored. Univariate and multivariate analyses were performed using Cox regression study.

Results

Of 90 B-CLL cases, expression of the hTERT gene was detected using real-time RT-PCR in 53 (59%). In positive samples, the normalized amounts of hTERT mRNA transcripts varied between 0.9 and 155.8, with a median of 10.0 (interquartile range 3.7–20.6). The percentage of hTERT-positive cases was essentially identical in stage A (56%) and B/C patients (63%). In contrast, the level of hTERT expression significantly increased with advancing B-CLL stage. The mean normalized hTERT transcript numbers were 5.8 in stage A, 14.4 in stage B, and 23.1 in stage C. The results are shown in Figure 1.

Agreement with the literature

The results of the present study confirm that hTERT expression is significantly associated with short survival and Ig-unmutated B-CLL subtype. These findings support the hypothesis that hTERT expression may serve as a molecular prognostic marker in B-CLL.

Figure 1. Comparison of patient survival in the human telomerase reverse transcriptase gene (hTERT)-positive and -negative B-chronic lymphocytic leukemia (B-CLL) groups. Patients expressing hTERT exhibited significantly reduced survival within the whole patient population (A, P = 0.000034), and among stage A (B, P = 0.0025) and stage B/C patients (C, P = 0.0041).
10.7 in stage B and 26.0 in stage C patients (Kruskal–Wallis test, \(P = 0.0064\)).

Overall patient survival was analyzed as a function of \(hTERT\) expression. As shown in Figure 1A, \(hTERT\)-positive patients have significantly shorter survival than those with \(hTERT\)-negative B-CLL (log-rank test, \(P = 0.000034\)), irrespective of the disease stage (Figure 1B and C).

In addition, we compared the levels of \(hTERT\) transcripts with unmutated and mutated IgV genes. Data concerning IgV mutation status were available in 83 patients. Of these, 42 (51\%) showed unmutated IgV profiles. On average, the level of \(hTERT\) mRNA expression was seven-fold higher in Ig-unmutated than in Ig-mutated B-CLL (\(P < 10^{-7}\)). Mean \(hTERT\) values (horizontal bars) were 18.1 in the Ig-unmutated and 2.6 in the Ig-mutated groups.

![Figure 2](chart.png)

**Figure 2.** Levels of \(hTERT\) expression were significantly higher in B-CLL patients with unmutated IgV genes than in those with mutated IgV genes (Kruskal–Wallis test, \(P = 0.0064\)).

Table 1. Cox analysis for overall survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>Relative risk (95% confidence interval)</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(hTERT) (positive versus negative)</td>
<td>1.93</td>
<td>6.7 (2.0–23.2)</td>
<td>0.0018</td>
</tr>
<tr>
<td>IgV gene profile (unmutated versus mutated)</td>
<td>0.75</td>
<td>4.5 (1.7–12.1)</td>
<td>0.0032</td>
</tr>
</tbody>
</table>

\(hTERT\), human telomerase reverse transcriptase gene; IgV, immunoglobulin V.

Discussion

This study was performed to investigate the potential prognostic significance of \(hTERT\) expression in B-CLL. Using real-time RT-PCR, we quantified the levels of \(hTERT\) mRNA in mononuclear blood cells from 90 patients with B-CLL. The results were correlated with clinical and biological data. We found that patients expressing \(hTERT\) have significantly shorter survival than \(hTERT\)-negative patients, regardless of disease stage. In addition, the levels of \(hTERT\) expression were significantly increased in advanced B-CLL stages and, within all stages, in the clinically aggressive Ig-unmutated B-CLL subtype. Expression of \(hTERT\) was a significant prognostic factor when evaluated in a univariate analysis and, simultaneously with IgV mutations, in a multivariate analysis. These results suggest that \(hTERT\) expression in B-CLL may be a molecular marker of progressive clinical course and adverse prognosis.

Bechter et al. [16] showed previously that high telomerase activity evaluated using TRAP assay in bone marrow from B-CLL patients was associated with a shorter median survival. However, this relationship was not seen in a more recent study of telomeres and telomerase in blood-derived B-CLL.
cells, although high telomerase activity was found in the poor-outcome, Ig-unmutated B-CLL subgroup [17]. Here, we inves-
tigated telomerase by using real-time RT-PCR for hTERT
mRNA, an approach offering enhanced sensitivity and more
precise quantitation in comparison with the TRAP assay [19].

The possibility to study telomerase activation in B-CLL at the
transcriptional level is supported by a recent report showing
that B-CLL patients with high telomerase activity had low
methylation of the hTERT gene promoter, which may contrib-
ute to up-regulation of hTERT transcription [20]. The signifi-
cant relationship between hTERT expression and decreased patient survival may be viewed as confirmation of
the prognostic implication of telomerase in B-CLL. The advan-
tage of hTERT analysis is its capacity to detect clinically
relevant differences in telomerase expression in blood-derived
B-CLL cells.

The results of the present study indicated that the level of
hTERT mRNA was significantly higher in Ig-unmutated than
that in Ig-mutated B-CLL, which is in line with a recent report
by Damle et al. [17]. This difference suggests that hTERT
expression may also be useful as a simple surrogate for IgV
mutations. As mutation analysis is technically difficult, many
efforts have recently been made to identify markers that are
easy to detect and are predictive of IgV gene status. The most
sensitive (91%) and specific (100%) distinction was achieved
by immunofluorescence detection of ZAP-70 expression,
which is higher in Ig-unmutated than in Ig-mutated B-CLL
[21]. Since ZAP-70 is normally expressed in T-lymphocytes,
the analysis should be performed selectively on leukemic
cells. In contrast, our results showed that the presence of non-
malignant cells in unselected B-CLL blood samples does not
significantly influence the specificity of hTERT analysis.

Eighty-nine per cent of patients were correctly assigned an
Ig-unmutated or Ig-mutated profile on the basis of hTERT
expression level.

The difference in hTERT expression between Ig-unmutated
and Ig-mutated B-CLL subtypes might be related to their telo-
mere status. Telomeres were found to be significantly shorter
in Ig-unmutated than in Ig-mutated B-CLL [17, 22]. In
addition, B-CLL cases with shorter telomeres were shown to
exhibit higher levels of telomerase and hTERT mRNA [16,
22]. This association may be the result of a greater need for
telomeric end maintenance in B-CLL cells with shortened telo-
meres to extend their proliferative lifespan. To prevent criti-
cal telomere attrition during expansion, Ig-unmutated B-CLL
cells with short telomeres are likely to up-regulate hTERT and
telomerase. In contrast, Ig-mutated B-CLL cells are less lim-
ited in their expansion potential because of their increased
telomere reserve, at least in the ‘early’ stage of the disease.

In summary, hTERT expression appears to be a novel mol-
ecular prognostic marker in B-CLL. Further assessment of this
approach awaits validation in a larger series of patients in com-
parison with other known prognostic parameters such as lym-
phocyte doubling time [23], levels of β2-microglobulin [24]
and soluble CD23 [25], serum thymidine kinase levels [26],
genetic abnormalities [27, 28] and IgV mutations [13–15].

Acknowledgements

The authors are grateful to Dr J. Chassagne for providing
B-CLL samples and to F. Aıt-Ouaret for expert technical
assistance.

References

1. Hahn WC, Counter CM, Lundberg AS et al. Creation of human
tumour cells with defined genetic elements. Nature 1999; 400:
464–468.
2. Morin GB. The human telomere terminal transferase enzyme is a
ribonucleoprotein that synthesizes TTAGGG repeats. Cell 1989; 59:
521–529.
3. Pool JC, Andrews LG, Tollefsbol TO. Activity, function, and gene
regulation of the catalytic subunit of telomerase (hTERT). Gene
2001; 269: 1–12.
5. Wang L, Soria JC, Kemp BL et al. hTERT expression is a prognostic
factor of survival in patients with stage I non-small cell lung cancer.
telomerase reverse transcriptase mRNA expression are correlated with
clinical aggressiveness in soft tissue tumors. Cancer 2002; 95:
1127–1133.
subunits gene expression patterns in neuroblastoma: a molecular and
immunohistochemical study establishing prognostic tools for fresh-fro-
8. Xu D, Gruber A, Peterson C, Pisa P. Telomerase activity and the
expression of telomerase components in acute myelogenous leukaem-


