Quantification of ZAP70 mRNA in B Cells by Real-Time PCR Is a Powerful Prognostic Factor in Chronic Lymphocytic Leukemia

Basile Stamatopoulos, Nathalie Meuleman, Benjamin Haibe-Kains, Hughes Duvillier, Martine Massy, Philippe Martiat, Dominique Bron, and Laurence Lagnequx

Background: Chronic lymphocytic leukemia (CLL) is heterogeneous with respect to prognosis and clinical outcome. The mutational status of the immunoglobulin variable heavy chain region (IGHV) has been used to classify patients into 2 groups in terms of overall survival (OS) and clinical characteristics, but the labor-intensive nature and the cost of this time-consuming analysis has prompted investigations of surrogate markers.

Methods: We developed a standardized quantitative real-time reverse transcription-PCR (qPCR) method to measure zeta-chain (TCR)-associated protein kinase (ZAP70) mRNA in purified CD19+ cells. We evaluated this and other methods (flow cytometry analyses of ZAP70 and CD38 proteins and qPCR analysis of lipoprotein lipase mRNA) in a cohort of 108 patients (median follow-up, 82 months) to evaluate any associations with IGHV mutational status, OS, and treatment-free survival (TFS).

Results: The association between qPCR-measured ZAP70 and IGHV mutational status was statistically significant ($\chi^2$ (1) = 50.95; $P < 0.0001$), and the value of Cramer’s $V$ statistic (0.72) indicated a very strong relation. This method also demonstrated sensitivity, specificity, and positive and negative predictive values of 87.8%, 85.7%, 87.5%, and 86%, respectively. ZAP70 expression was significantly associated with OS ($P = 0.0021$) and TFS ($P < 0.0001$). ZAP70+ patients had significantly shorter median TFS (24 months) than ZAP70− patients (157 months) ($P < 0.0001$). Moreover, qPCR-measured ZAP70 expression has greater prognostic power than IGHV mutational status and the other prognostic markers tested.

Conclusions: ZAP70 mRNA quantification via qPCR is a strong surrogate marker of IGHV mutational status and a powerful prognostic variable.

B-cell chronic lymphocytic leukemia (CLL) is the most common leukemia in western countries. CLL mainly affects elderly individuals, but about one-third of patients are younger than 60 years at diagnosis (1). CLL has an extremely variable clinical course, with overall survival (OS) times ranging from months to decades. The disease runs an indolent clinical course in some patients, and life expectancy is not shortened. For others, the disease is aggressive and progresses rapidly, with survival times after diagnosis of 2–3 years (2). Therefore, it is very important to identify variables that can predict a poor prognosis and the patients who will benefit from intensive therapy at an early disease stage.

Several prognostic markers have been identified in the last 3 decades. Initially, Rai et al. (3) and Binet et al. (4) developed clinical staging systems based on patient clinical characteristics, but these systems were unable to prospectively distinguish early-stage CLL that progresses...
rapidly to aggressive disease from disease destined to remain in an early stage for a long time (5). This difficulty prompted the increasing use of other genetic and biological variables for predicting the prognosis of CLL (6). Many prognostic variables have recently been found to predict clinical outcome, with one of the most important molecular genetic variable markers being the mutational status of the immunoglobulin variable heavy chain region (IGHV) (7). This new marker can be used to separate patients into 2 groups: a group with unmutated IGHV and a worse outcome, and a group with mutated IGHV associated with a good prognosis. However, this analysis is laborious, costly, and is inaccessible for most clinical laboratories. Identifying a surrogate marker for IGHV mutational status is therefore an important goal. CD38 was the first marker to be correlated with IGHV mutational status (8), but the relationship is not absolute. In 2001, a comparison of gene expression profiles for the 2 patient groups revealed a small number of differentially expressed genes (9, 10), of which the lipoprotein lipase gene (LPL)\(^5\) was closely related to IGHV mutational status. Further studies confirmed the predictive value of this gene (11, 12). In 2003, Wiestner et al. (13) found that zeta-chain (TCR)-associated protein kinase (ZAP70) expression, which is usually found in T lymphocytes and natural killer cells, was correlated with IGHV mutational status in 93% of cases. Patients with <20% ZAP70\(^+\) B cells as measured by flow cytometry (FC) generally had a mutated IGHV status, and patients with ≥20% ZAP70\(^+\) cells had an unmutated IGHV status. Further clinical studies confirmed the prognostic value of the ZAP70 protein (14-20); however, FC measurement of ZAP70 status is often inaccurate at the positivity limit (21) because of low resolution of the positive and negative populations, and can also be influenced by the gating procedure and antibody choice (22). To offset these drawbacks, investigators have proposed absolute quantification of ZAP70 mRNA (23, 24).

We describe the validation of a new standardized quantitative real-time reverse transcription-PCR (qPCR) analysis for measuring ZAP70 mRNA in purified B lymphocytes and its power as both a surrogate for IGHV mutational status and a prognostic marker for survival and treatment-free time in CLL.

### Materials and Methods

**PATIENTS, SAMPLE COLLECTION, AND RNA EXTRACTION**

This study was approved by the Bordet Institute Ethics Committee and was based on peripheral blood samples obtained with informed consent from 108 CLL patients. Patients either were untreated or had received no treatment for at least 6 months before the study. The 63 male and 45 female patients had a typical CD19\(^+\)CD5\(^−\)CD23\(^+\) phenotype and blood leukocyte counts between 1 \(\times\) 10\(^3\) and 25 \(\times\) 10\(^3\) cells/L. The median age at diagnosis was 65 years (range, 46–90 years). The median follow-up duration was 82 months (range, 8–299 months). Table 1 summarizes other patient features. Some information is unavailable because of limited biological material or the absence of clinical data. Treatment-free survival (TFS) and disease-related mortality were calculated from the time of diagnosis. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation over Linfosep (Biomedics). B cells were purified with a CD19\(^+\) magnetic-bead system (MidImacs, Miltenyi Biotec) according to the manufacturer’s instructions. Mean B-cell purity as measured by FC was >99%. Total RNA was extracted from purified CD19\(^+\) cells in a single step with TriPure Isolation Reagent (Roche Applied Science) (25).

**FC MEASUREMENT OF ZAP70 AND CD38 EXPRESSION**

We measured the expression of cytoplasmic ZAP70 protein by FC with the Fix and Perm Permeabilization Kit (ImTec Diagnostics), a ZAP70 phycoerythrin-conjugated antibody (clone 1E7.2, eBioscience), fluorescein-isothiocyanate–conjugated CD3, and phycoerythrin-Cy5–conjugated CD19 (Immunotech) (16). Because the choice of the threshold for ZAP70 positivity can critically affect the decision regarding ZAP70 status (see Text 1 and Figs. 1 and 2 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue10), we defined it on the basis of the lower limit of the region which included 99% of ZAP70\(^+\)CD3\(^−\) cells. This threshold maximized the concordance between ZAP70 status and mutational status. After establishing the appropriate gating on CD3\(^−\) cells, we fixed the cutoff for ZAP70 positivity and measured ZAP70 in CD19\(^+\) cells. We evaluated the expression of CD38 on cell surfaces by FC in a CD19\(^−\) gate with a panel of fluorochrome-labeled monoclonal antibodies (phycoerythrin-conjugated CD38, Immunotech). CD38 expression was deemed positive if 7% of the cells stained positive in a standard 3-color FC analysis (8).

**QPCR ANALYSIS OF ZAP70 AND LPL EXPRESSION**

We used 25 ng cDNA (produced by a standard reverse transcription) in a qPCR reaction with SYBR\(^®\) Green PCR Master Mix (Applied Biosystems) and 0.32 \(\mu\)mol/L of gene-specific forward and reverse primers (Invitrogen). The sequences of the ZAP70, PPIA [peptidylprolyl isomerase A (cyclophilin A)], and LPL primers have been published [(26), (27), and (11), respectively]. We also tested 5 housekeeping genes [LMNB1, lamin B1; EIF1AX, eukaryotic translation initiation factor 1A, X-linked; CASC3, cancer susceptibility candidate 3; IGHV, immunoglobulin variable heavy chain region; PGK1, phosphoglycerate kinase 1; CD38, CD38 molecule.\(^5\)]

\(^5\) Human genes: LPL, lipoprotein lipase; ZAP70, zeta-chain (TCR) associated protein kinase 70 kDa; PPIA, peptidylprolyl isomerase A (cyclophilin A); LMNB1, lamin B1; EIF1AX, eukaryotic translation initiation factor 1A, X-linked; CASC3, cancer susceptibility candidate 3; IGHV, immunoglobulin variable heavy chain region; PGK1, phosphoglycerate kinase 1; CD38, CD38 molecule.
as endogenous controls (data not shown). Finally, we standardized all results using PPIA gene expression, which was the most stable. Standard real-time PCR was performed on an ABI Prism 7900 HT (Applied Biosystems). A calibrator sample (cDNA from the Namalwa cell line, a human B-lymphoid leukemia cell line that expresses ZAP70 at a low level; ATCC) was included as a control in each experiment. In all cases, we created dissociation curves to confirm PCR specificity. We analyzed the data with the comparative Ct method (for details, see Text 2 in the online Data Supplement).

**IGHV GENE MUTATIONAL ANALYSIS**
We conducted IGHV gene mutational analysis as previously described (28) and aligned sequences with those in the international ImMunoGeneTics information system database (http://imgt.cines.fr). Sequences with \( \pm 2\% \) deviation from any germ line IGHV sequence were considered unmutated (7).

**STATISTICAL ANALYSIS**
We analyzed ROC curves with GraphPad Prism 5.0 (GraphPad Software) to determine the ZAP70, LPL, and CD38 expression cutoff values that best distinguished mutated and unmutated cases. We generated time-dependent ROC curves with the R package ROC survival (29). We used \( \chi^2 \) Pearson statistics (with the Yates continuity correction for 2 \( \times \) 2 tables) to describe associations between clinical markers and used Cramer’s \( V \) statistic to quantify the strength of association between 2 variables (information unobtainable from the \( P \) value). Values of 0.20–0.35 indicate a moderate relation, 0.36–0.49 indicate a substantial relation, and values \( \geq 0.50 \) a strong relation. We plotted OS and TFS distributions with the Kaplan–Meier method and used the log-rank test with GraphPad Prism 5.0 to compare the distributions. Univariate and multivariate Cox regression analyses evaluated the effects of the different prognostic variables on TFS and/or OS. We included clinical stage [early (Binet A) vs advanced (Binet B and C)], CD38 expression (\( \geq 7\% \) vs \(< 7\% \)), FC-measured ZAP70 expression (\( \geq 20\% \) vs \(< 20\% \)), and/or OS. We included clinical stage [early (Binet A) vs advanced (Binet B and C)].

**Table 1. Cross-tabulations of prognostic markers vs IGHV mutational status and ZAP70 mRNA measured by qPCR.**

<table>
<thead>
<tr>
<th></th>
<th>Unmutated IGHV, n (%)</th>
<th>Mutated IGHV, n (%)</th>
<th>( P )</th>
<th>( \chi^2 (1) )</th>
<th>CVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pts, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>105</td>
<td>51 (49)</td>
<td>54 (51)</td>
<td>NS</td>
<td>1.83 0.13</td>
</tr>
<tr>
<td>Female</td>
<td>62</td>
<td>34 (55)</td>
<td>28 (45)</td>
<td>63</td>
<td>37 (59) 26 (41)</td>
</tr>
<tr>
<td>Binet stage A</td>
<td>72</td>
<td>29 (40)</td>
<td>43 (60)</td>
<td>45</td>
<td>18 (40) 27 (60)</td>
</tr>
<tr>
<td>Binet stage B</td>
<td>20</td>
<td>13 (65)</td>
<td>7 (35)</td>
<td>20</td>
<td>16 (80) 4 (20)</td>
</tr>
<tr>
<td>Binet stage C</td>
<td>10</td>
<td>9 (90)</td>
<td>1 (10)</td>
<td>10</td>
<td>9 (90) 1 (10)</td>
</tr>
<tr>
<td>Mutational status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmutated IGHV</td>
<td>51</td>
<td>45 (88)</td>
<td>6 (12)</td>
<td>108</td>
<td>55 (51) 53 (49) NS</td>
</tr>
<tr>
<td>ZAP70 (qPCR)</td>
<td>54</td>
<td>9 (17)</td>
<td>45 (83)</td>
<td></td>
<td>2.34 0.17</td>
</tr>
<tr>
<td>&gt;115 (Positive)</td>
<td>54</td>
<td>45 (83)</td>
<td>9 (17)</td>
<td></td>
<td>&lt;0.0001 50.95 0.72</td>
</tr>
<tr>
<td>&lt;115 (Negative)</td>
<td>51</td>
<td>6 (12)</td>
<td>45 (88)</td>
<td></td>
<td>&lt;0.0001 24.08 0.50</td>
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<tr>
<td>CD38 (FC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;7% (Positive)</td>
<td>53</td>
<td>32 (60)</td>
<td>21 (40)</td>
<td>53</td>
<td>32 (60) 21 (40)</td>
</tr>
<tr>
<td>&lt;7% (Negative)</td>
<td>38</td>
<td>9 (24)</td>
<td>29 (76)</td>
<td>39</td>
<td>11 (28) 28 (72)</td>
</tr>
<tr>
<td>Pts requiring no treatment</td>
<td>46</td>
<td>14 (30)</td>
<td>32 (70)</td>
<td>48</td>
<td>13 (27) 35 (73)</td>
</tr>
<tr>
<td>Pts requiring treatment</td>
<td>52</td>
<td>35 (67)</td>
<td>17 (33)</td>
<td>53</td>
<td>38 (72) 15 (28)</td>
</tr>
<tr>
<td>Pts still alive</td>
<td>83</td>
<td>36 (43)</td>
<td>47 (57)</td>
<td>85</td>
<td>37 (44) 48 (56)</td>
</tr>
<tr>
<td>Pts died during study</td>
<td>15</td>
<td>13 (87)</td>
<td>2 (13)</td>
<td>16</td>
<td>13 (81) 3 (19)</td>
</tr>
</tbody>
</table>

\( a \) Pts, Patients; CVS, Cramer’s \( V \) statistic; NS, not statistically significant. 
\( b \) IGHV mutational status is based on a 98% cutoff value.
\( c \) The cutoffs in ROC curve analyses are expressed as the fold expression of the target gene over that in the calibrator Namalwa cell line.
\( d \) The 20% cutoff for CD19\(^+\) cells expressing ZAP70 (at the same level as T cell) in FC analyses is the cutoff commonly accepted in the literature.
<0.05 was considered statistically significant. All analyses were performed with SPSS 13.0 software.

**Results**

**Association Between Binet Stage and IGHV Mutational Status**

Cross-tabulations showed that mutational status was significantly associated with Binet stage ($P = 0.004$), treatment status ($P < 0.0001$), and being alive or dead ($P = 0.004$) (Table 1). Cramer’s $V$ values for association strength were 0.33 (moderate), 0.40 (substantial relation), and 0.31 (moderate), respectively. We also evaluated TFS with respect to Binet stage (Kaplan–Meier data not shown). Patients presented median TFS times of 88 months, 29 months, and 19 months for stages A, B, and C, respectively ($P < 0.0001$).

**Strong Association of qPCR-Measured ZAP70 Expression with Mutational Status**

Table 1 and Fig. 1 summarize cross-tabulations for mutational status and other prognostic variables. Mutational status was significantly associated with all markers tested. The value for Cramer’s $V$ statistic (0.72) indicated a very strong relation between qPCR-measured ZAP70 expression and mutational status; the other markers showed a less good relation (0.33 to 0.56) (Table 1). Table 1 in the online Data Supplement presents rates of concordance with mutational status and association by cross-tabulation. To estimate the powers of these markers and their ability to correctly predict mutational status, we evaluated sensitivity, specificity, and positive and negative predictive values (see Table 2 in the online Data Supplement). These values for qPCR-measured ZAP70 expression were 87.8%, 85.7%, 87.5%, and 86%, respectively, and were better than for the other markers. Moreover, we compared the areas under the ROC curve (AUCs) with a nonparametric statistical test (30) (Fig. 2A). The AUC reflects the probability of correctly discriminating between true-positive and true-negative findings. The AUC for qPCR-measured ZAP70 expression is significantly different from the AUCs for LPL expression ($P = 0.014$) and CD38 expression ($P = 0.007$) but not significantly different from FC-measured ZAP70 expression ($P = 0.424$), indicating that ZAP70 expression is a better variable for predicting mutational status. Moreover, the AUCs for FC-measured ZAP70 expression and LPL expression are not significantly different ($P = 0.165$), indicating that these 2 methods are globally good predictors of mutational status, independently of the chosen cutoff value. With respect to the optimal cutoff, however, qPCR-measured ZAP70 expression gives better results.

**Prognostic Value of qPCR-Measured ZAP70 Expression**

**TFS and OS Analyses**

Need for treatment and patient death are clearly associated with both IGHV mutational status and qPCR-measured ZAP70 expression (Table 1). The median TFS times for qPCR-measured $ZAP70^+$ and $ZAP70^-$ patients were 24 months and 157 months, respectively ($P < 0.0001$). Moreover, OS was significantly associated in log-rank tests with IGHV mutational status ($P = 0.0034$) and qPCR- and FC-measured analyses of ZAP70 expression ($P = 0.0021$ and 0.0006, respectively), but not with LPL or CD38 expression ($P = 0.1972$ and 0.2267). Table 3 in the online Data Supplement and Fig. 3 summarize the effects of other prognostic variables on TFS and OS.

**Patients Exhibiting Discordance between IGHV Mutational Status and Other Prognostic Variables**

We also investigated TFS for patients who presented discordance between mutational status and other prognostic variables (see Table 4 in the online Data Supplement). We evaluated TFS for each marker and compared the 2 groups with a log-rank test. We only observed a significant difference in TFS only for qPCR-measured ZAP70 expression. Unmutated IGHV/ZAP70$^-$ and mutated IGHV/ZAP70$^+$ patients have median TFS times of 178 months and 67 months, respectively ($P = 0.0395$). To evaluate whether qPCR-measured ZAP70 expression is the predominant prognostic variable for TFS, we also compared patients with discordance between qPCR-measured ZAP70 expression and other prognostic variables. Although there were no significant differences because of the small number of patients, patients evaluated as ZAP70$^+$ by qPCR had an apparently shorter median TFS time (see Table 4 in the online Data Supplement).

**Univariate and Multivariate Cox Regression**

We used univariate Cox regression to evaluate the impact of the binarized data (using Table 1 cutoffs). All of the tested markers were significant univariate predictors of TFS, but IGHV mutational status and qPCR- and FC-measured ZAP70 expression were the only significant predictors of OS ($P = 0.011$, $0.008$, and 0.004, respectively; see Table 5 in the online Data Supplement). A multivariate Cox regression analysis that included ZAP70 measurement (either by qPCR or by FC; because the 2 variables are highly correlated, fitting of the Cox model may become unstable if both are used), qPCR-measured LPL expression, mutational status, and CD38 expression also indicated that ZAP70 expression (by qPCR, $P = 0.0209$; by FC, $P = 0.0068$) better predicts TFS than mutational status and the other markers (see Table 5 in the online Data Supplement).

**Time-Dependent ROC Curves**

We generated time-dependent ROC curves to evaluate the power of the tested markers at 1 and 2 years after diagnosis. The AUC for ZAP70 expression (by either method) was higher than for any of the other prognostic variables, including mutational status (Table 2 and Fig. 2, B and C).
Discussion

Clinical staging systems fail to identify early-stage patients most likely to experience disease progression, and this limitation has prompted a search for new prognostic markers. IGHV mutational status was recently identified as one of the most predictive variables for time from diagnosis to disease progression. The diagram illustrates the correlations between IGHV mutational status and other prognostic variables, with optimal cutoff values based on ROC curve analyses and indicated by horizontal lines. Closed circles represent CLL-related deaths.
diagnosis to initial treatment. CD38, ZAP70, and LPL have been shown to have similar prognostic values and have been considered surrogate markers of mutational status (8,11–13,31). The aim of this study was to develop a qPCR assay for ZAP70 expression in B cells isolated from CLL patients. We also evaluated IGHV mutational status, LPL mRNA expression, CD38 expression, and FC-measured ZAP70 expression in our cohort of 108 patients (median follow-up, 82 months). Because the initial method for measuring ZAP70 expression by FC was promising but imprecise, we also investigated the use of a qPCR assay. Furthermore, FC measurement of ZAP70 expression requires cytoplasmic staining and an internal control, such as T cells or natural killer cells, to evaluate the positivity limit. These 2 variables plus antibody choice, the conjugated fluorochrome, and the subjective choice of the positivity limit can influence the final result and may lead to incorrect patient classification. The requirement of a standardized methodology therefore led us to develop a standardized qPCR assay with an absolute positivity limit, which was normalized with a stable endogenous control tested among 5 housekeeping genes.

Fig. 2. ROC curve analysis of the different prognostic variables vs IGHV mutational status and time-dependent ROC curves.

(A) AUC values are in Table 2. AUC values calculated for all markers were compared with a nonparametric test. The qPCR-measured ZAP70 AUC was significantly different from the LPL AUC (P = 0.014) and the CD38 AUC (P = 0.007). The AUC for FC-measured ZAP70 was not significantly different from the AUCs for qPCR-measured ZAP70 and LPL (P = 0.424 and 0.165, respectively). (B) Time-dependent ROC curves at 1 year after diagnosis. (C) Time-dependent ROC curves at 2 years after diagnosis.
Fig. 3. Kaplan–Meier survival curves for TFS and OS.

(A and F), indicated are TFS and OS curves for IGHV mutational status, (B and G), ZAP70 by qPCR, (C and H), ZAP70 by FC, (D and I), LPL by qPCR, and (E and J), CD38 by FC. Assessment of mutational status was based on a 98% cutoff value (n = 11005), ROC curve analyses were used to determine cutoffs for ZAP70 (n = 101) and LPL (n = 101) in qPCR measurements of mRNA in purified B cells, and 20% and 7% cutoffs for positive cells were used in FC analyses of ZAP70 expression (n = 87) and CD38 expression (n = 82), respectively.
and expressed as the fold expression of ZAP70 over that in the calibrator Namalwa cell line.

We also evaluated LPL and CD38 expression because these markers have been associated with more aggressive disease and have been correlated with IGHV status (11, 32, 33). qPCR measurement of ZAP70 mRNA shows sensitivity, specificity, and positive and negative predictive values that better predict mutational status than the other markers tested. Similarly with other markers, qPCR-measured ZAP70 expression was associated with IGHV mutational status but with a higher concordance rate (86%). AUC analysis indicated that measurement of ZAP70 expression by either method is an appreciably better predictor of IGHV status than LPL or CD38 expression. Evaluation of Cramer’s V statistic, a measure of the strength of these associations, indicated a very strong relation between mutational status and qPCR-measured ZAP70 expression and indicated substantial to strong relationships for FC-measured ZAP70 expression, LPL expression, and CD38 expression. We conclude that qPCR measurement of ZAP70 mRNA is a strong surrogate marker for IGHV mutational status and is more powerful than the other tested markers. Other researchers have also reported a clear correlation between mutational profile and ZAP70 expression, with concordance rates of 83% and 81% for qPCR and 77% for FC analysis (24, 34). The degree of concordance between ZAP70 expression and mutational status thus varied, depending on the method. We therefore calculated all cutoff values for each method independently to maximize the concordance with mutational status, the most robust biological prognostic variable. After this operation we considered all correlations optimal.

Several clinical studies have shown mutational status to be a good predictor of TFS and OS (7, 35). We confirmed these findings with our patient cohort. All markers tested were significant predictors of TFS in log-rank tests [ZAP70 expression measured by either method (P <0.0001), LPL expression (P = 0.0063), and CD38 expression (P = 0.0017)]. ZAP70+ patients by qPCR had a significantly shorter median TFS time (24 months) than ZAP70− patients (157 months). Measurement of ZAP70 expression by FC has been identified as a significant predictor of disease progression and OS in CLL (15, 19). The Kaplan–Meier estimates of the survival function for ZAP70+ and ZAP70− patients were significantly different (by qPCR, P = 0.0021; by FC, P = 0.0006), but no significant differences were apparent for LPL or CD38 expression despite the numbers of patients included in the analyses. Our results for the predictive value of CD38 expression with the 7% cutoff are in accord with those of Domingo-Domenech et al. (36). The 7% threshold is apparently not the best cutoff for identifying patients with a poor outcome, given that the use of 20% and 30% cutoff thresholds have shown higher survival rates for CD38− patients than for CD38+ patients (8). In our study, however, even CD38 expression with a 20% or 30% cutoff was unable to predict OS. Furthermore, CD38 expression may vary over time (37). Recent studies have suggested that IGHV mutations and CD38 expression are independent prognostic variables (38), with CD38 expression probably reflecting the disease’s proliferative potential (39).

A recent study demonstrated that LPL expression was a predictor of CLL survival as IGHV and more reliable than ZAP70 expression (11); however, the median follow-up in this study was 17 months (range, 0–57 months). Our results are in line with those of Heintel et al. (12), who reported high LPL expression to be significantly associated with a shorter TFS time; however, the median OS times of the 2 groups were not significantly different. The median follow-up time in the Heintel et al. (12) study was 48 months, compared with 82 months (range, 8–299 months) for our cohort. In contrast, we found the difference in OS survival times for ZAP70 expression to be highly significant (P = 0.0021). The median OS time for ZAP70+ patients was 12.7 years, which is similar to previously reported findings (14, 16, 18). Only 3 studies have demonstrated a correlation between ZAP70 expression and OS; the other studies evaluated ZAP70’s prognostic value only in terms of TFS.

Univariate Cox regression analysis indicated that only IGHV mutational status and ZAP70 expression evaluated by either method were good predictors of OS. In the multivariate analysis, only ZAP70 expression was a significant independent variable for predicting TFS. These data suggest that ZAP70 expression is the best of the prognostic variables tested and that the qPCR method can offset the limitations of FC.

Regarding the patients who displayed discordance (i.e., unmutated IGHV and negative for qPCR- or FC-

<table>
<thead>
<tr>
<th>Association with MS</th>
<th>Strength of association with MS</th>
<th>AUC prediction of MS</th>
<th>Concordance with MS</th>
<th>Association with TFS</th>
<th>Assoc with OS</th>
<th>TFS in case of discordance with MS</th>
<th>Univariate Cox predictor of TFS</th>
<th>Univariate Cox predictor of OS</th>
<th>Multivariate Cox predictor of TFS</th>
<th>1-Year AUC predictor of TFS</th>
<th>2-Year AUC predictor of TFS</th>
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<td>MS</td>
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<td></td>
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<tr>
<td>ZAP70 (qPCR)</td>
<td>S</td>
<td>Very strong</td>
<td>89%</td>
<td>86%</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NS</td>
<td>70%</td>
<td>77%</td>
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<td>ZAP70 (FC)</td>
<td>S</td>
<td>Strong</td>
<td>85%</td>
<td>78%</td>
<td>S</td>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>74%</td>
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</tr>
<tr>
<td>LPL (qPCR)</td>
<td>S</td>
<td>Strong</td>
<td>76%</td>
<td>75%</td>
<td>S</td>
<td>S</td>
<td>NS</td>
<td>S</td>
<td>NS</td>
<td>69%</td>
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<tr>
<td>CD38 (FC)</td>
<td>S</td>
<td>Substantial</td>
<td>70%</td>
<td>67%</td>
<td>S</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>63%</td>
<td>66%</td>
</tr>
</tbody>
</table>

a MS, IGHV mutational status; S, significant (P <0.05); NS, nonsignificant.
b values of >0.36–0.49 a substantial relation, and values ≥0.50 indicate a strong relation, whereas a value of 1 indicated a perfect relation.
measured ZAP70, LPL expression, or CD38 expression), only qPCR-measured ZAP70 status discordant for mutational status was able to predict TFS (P = 0.0395). We thus conclude that ZAP70 expression is the strongest predictor of the need for treatment and that ZAP70 expression is a better predictor than IGHV mutational status. When we plotted discordant cases for qPCR- and FC-measured ZAP70 expression by the Kaplan–Meier method, the qPCR+/FC− TFS curve showed a clear trend toward a shorter TFS time (median TFS, 57 months) compared with the qPCR−/FC+ TFS curves (median TFS, 80 months), but the difference was not significant (P = 0.23) because of the small number of patients (n = 11) with discordance.

Furthermore, time-dependent ROC curves and values for Cramer’s V statistic confirmed the superior clinical impact of ZAP70 compared with mutational status and other tested markers. These results agree with Del Principe et al. (15), who reported that FC measurement of ZAP70 protein better predicts outcome than mutational status or CD38 expression. On the contrary, others have found LPL to be more reliable than ZAP70 for predicting mutational status (11) and survival or to be at least as powerful as ZAP70 (32). However, when we analyzed discordant cases for ZAP70 and LPL with the Kaplan–Meier method, the TFS curve showed a clear trend to shorter TFS in the ZAP70+/LPL− group but the difference was not statistically significant (126 months vs 157 months, P = 0.056), probably because of the small sample size (n = 25).

We conclude that ZAP70 is the most powerful prognostic marker among those tested (Table 2). The choice of ZAP70 method is more complicated, but qPCR-measured ZAP70 is strongly associated with mutational status, prevails over mutational status in discordant cases, and clearly trends to prevailing in cases of discordance with FC-measured ZAP70 expression or LPL expression (see Table 4 in the online Data Supplement). Moreover, the qPCR method is more accurate than the FC method.

In conclusion, we have demonstrated that quantifying ZAP70 mRNA in B cells by real-time PCR is a strong surrogate marker of IGHV mutational status and that this marker is highly associated with TFS and OS. This straightforward and standardized assay can be routinely used in laboratories to better evaluate the outcomes and therapeutic needs of CLL patients.

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References


