ment options. We observed that those of our patients treated with rituximab-based regimens who were assigned to the high risk group had a median overall and cause-specific survival of less than four years. When such patients were treated with alkylating agents or with nucleoside analogs median overall survival was also less than four years.[9] This observation indicates that rituximab-based regimens, as well as nucleoside analog/alkylating agent based regimens, may be a suboptimal treatment for such high risk patients. For these patients new treatment approaches are needed.[12]

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ZAP-70 mRNA expression provides clinically valuable information in early-stage chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with a variable clinical course and overall survival times ranging from months to decades.[1] Mutational status of immunoglobulin heavy chain variable region (IGHV) correlates with clinical behavior and is a powerful prognostic factor in CLL. Unmutated IGHV patients have a reduced survival and poor responsiveness to chemotherapy,[2,3] however, IGHV sequencing is difficult to perform in a routine diagnostics laboratory. Gene profiling studies indicated that *ZAP-70* was the gene that best distinguished between IGHV groups[4] and that it could serve as an independent prognostic factor that is expressed in a stable manner during the course of the disease.[5-7] On the other hand, assessment of *ZAP-70* by flow cytometry (FC) presents some technical difficulties since T and NK cells express *ZAP-70* and must be excluded from the analysis.

Our objective was to evaluate the prognostic significance of *ZAP-70* determined by real-time PCR (RTqPCR) in early-stage CLL patients and compare its performance with FC analysis and IGHV mutational status to identify patients at risk of progression.

We studied 70 samples from untreated CLL patients (Binet stage A) after obtaining their informed consent. Rearranged *IGHV* genes were amplified by PCR using a standard protocol.[8] We considered unmutated those samples with >98% homology with the closest germinal line.

FC analysis of *ZAP-70* was performed on fresh samples (n= 69) according to Crespo *et al.*[6] with some modifications. An isotype control was used as negative control. Results ≥20% were considered positive.

For RTqPCR assays, RNA was prepared from 7-10×10^6 $CD19^+$ selected cells and amplification was carried out using Hs00277148_m1 primers and probe sets (TaqMan® Gene Expression Assays, Applied Biosystems). Amplification of GUS gene was performed in all cases to normalize gene expression.

Time to progression (TTP) was calculated from the date of diagnosis to the date of disease progression (based on NCI guidelines) or last follow-up. All statistical calculations were performed using the SPSS 13.0 software. Out of the 70 patients studied, 20 (28.6%) were unmutated. The most common families used in mutated samples were $V_{H3\text{-}7}$ (10.0%), $V_{H2\text{-}5}$ (10.0%), $V_{H3\text{-}30}$ (8.0%), and $V_{H1\text{-}3}$ (8.0%), whereas unmutated patients were $V_{H1\text{-}69}$ (30%) and $V_{H3\text{-}33}$ (15%).

The mean ± 2 SEM values of *ZAP-70* measured by

RTqPCR and by FC in IGHV unmutated and mutated samples were as follows: RTqPCR: 0.5115±0.284 and 0.1000±0.040; FC: 40.36+ 12.3 and 14.44±4.44 respectively (p<0.0001 in both cases, Mann Whitney test).

The area under the ROC curve for *ZAP-70* expression measured by RTqPCR was higher (0.879) than for FC (0.849), indicating that RTqPCR was a better method for predicting mutational status than FC. A global concordance between RTqPCR or FC results and IGHV mutational status was almost equal (84.28% and 84.06% respectively). We obtained 11 discordant patients for both assays; however, the distributions of the discordances were different. By RTqPCR we found discordant results only in IGHV mutated group, whereas 100% of the IGHV unmutated patients show ZAP-70 positive values. By FC we had 11 discordant patients, 7 were IGHV mutated and ZAP-70 positive, and 4 were IGHV unmutated and ZAP-70 negative. In ZAP-70/IGHV discordant cases, there were 3 patients showing FC and RTqPCR concordant results. These patients were ZAP-70 positive and had other prognostic factors of poor outcome: 2 of them were CD38 positive (one had del11q), and the third was CD38 negative but was mutated in BCL-6 gene. In the whole group, FC and RTqPCR results were concordant in 82.3% of the patients. In the discordant group, RTqPCR was better in assessing IGHV unmutated cases. Follw-up for the cohort was a median of 42.07 months (range: 1.00–141.33) in order to record patient treatment requirements due to disease progression. As expected, IGHV mutations defined two subsets of early-stage CLL; IGHV mutated cases had longer TTP than those unmutated (p<0.0001) (Figure 1A). In the case of RTqPCR, the TTP curve showed that ZAP-70 negative patients had a longer TTP than the ZAP-70 positive group (p=0.001) (Figure 1B). Our data show that 100% of IGHV mutated and 100% of ZAP-70 negative by RTqPCR patients have the longest TTP. On the contrary, 7 IGHV unmutated patients and 7 ZAP-70 positive patients required treatment. In the case of ZAP-70 by FC, again patients were divided into two groups but the statistical significance was lower (p=0.041) (Figure 1C).

Our results support the proposal of Catherwood *et al.*[9] about the usefulness of a RTqPCR assay to analyze ZAP-70 expression assigning a high percentage of CLL samples to the correct IGHV mutational status. We show that RTqPCR is a good, sensitive and accurate method to determine ZAP-70 expression having some advantages over FC, which is actually the technique most widely used. It is known that FC has some drawbacks, such as: requirement of cytoplasmic permeabilization, different performance depending on the monoclonal antibodies and fluorochromes used, and different cut-off values reported.[6,10-12] RTqPCR is easier to standardize and the requirement of B cell selection could be automatized.

ZAP-70 expression as a prognostic marker was analyzed by TTP curves. In agreement with previous results, RTqPCR methodology performed well, whereas FC showed a poorer performance and less statistical significance. Rassenti *et al.*[10] reports that measurement of ZAP-70 expression is even more significant than IGHV mutational status. In our cohort, IGHV mutational status and ZAP-70 by RTqPCR were strong predictors of TTP.

In conclusion, we have demonstrated that determination of *ZAP-70* in B cells by RTqPCR has an excellent correlation with IGHV mutational status and also with TTP in early-stage CLL. These observations suggest that ZAP-70 by RTqPCR would be a useful clinical test that might be used at the time of diagnosis to identify patients who

Table 1. Relation between ZAP-70 expression and IGHV mutational status.

	IGHV Unmut	*IGHV Mut*	*p (ψ^2 test)*
ZAP-70 by RTqPCR			
≥0.1050 (n=31)	20 (64.5%)	11 (35.5%)	<0.0001
<0.1050 (n=39)	0 (0%)	39 (100%)	
ZAP-70 by FC			
≥20% (n=23)	16 (69.6%)	7 (30.4%)	<0.0001
<20% (n=46)	4 (8.7%)	42 (91.3%)	

Optimal cut-off values were calculated by Youden index.

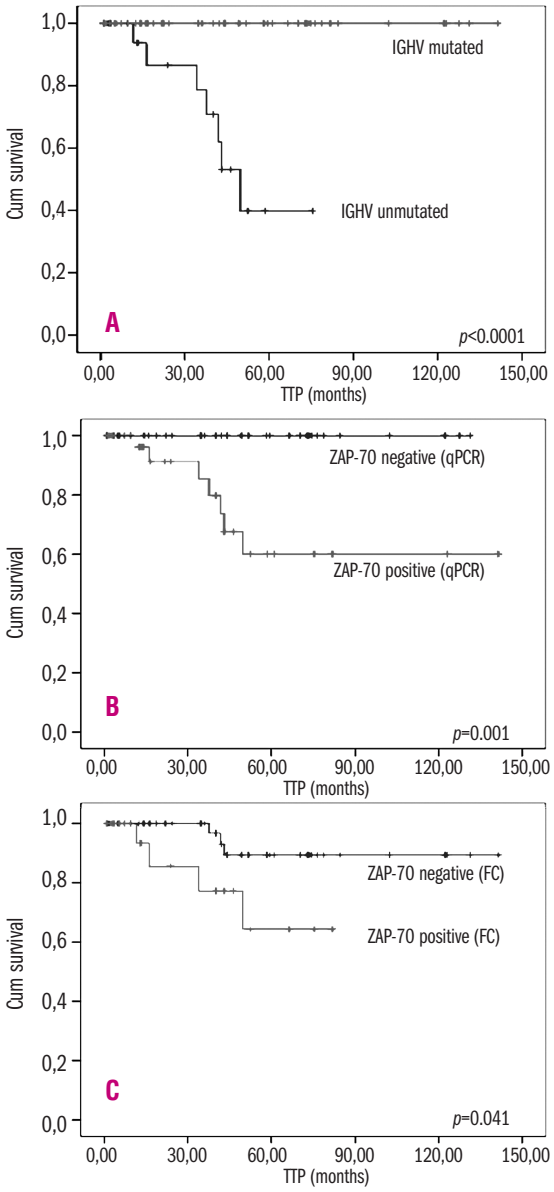

Figure 1. TTP survival curves according to IGHV mutational status and ZAP-70 expression. (A) IGHV mutational status, (B) ZAP-70 mRNA expression by RTqPCR and (C) ZAP-70 expression by FC. Assessment of mutational status was based on a 98% cut-off value, ZAP-70 expression was determined by FC analysis of whole blood (n=69) or by RTqPCR analysis of CD-19 selected cells (n=70).

have an increased risk of early disease progression and to guide treatment decision, since this technique is more amenable to application in clinical laboratories than IGHV sequencing.

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Epstein-Barr virus reactivation is a potentially severe complication in chronic lymphocytic leukemia patients with poor prognostic biological markers and fludarabine refractory disease

Chronic lymphocytic leukemia (CLL) patients who are refractory to, or have early relapse after, fludarabine and cyclophosphamide (FC) based therapy have a poor prognosis.[1] A major clinical problem in FC-refractory disease is a profound immunodeficiency, resulting in a high incidence of severe opportunistic infections.[2] The immunodeficiency may be caused either by the natural history of high-risk fludarabine-refractory CLL disease in itself and/or immunosuppressive side effects of the CLL treatment used for this group of patients. Over a two-year period, we identified 11 CLL patients with EBV-reactivation, defined as measurable EBV-DNA copies by quantitative PCR.

All 11 patients had IgG antibodies (VCA and/or EBNA) against EBV. Ten patients had negative CMV qPCR at the time of EBV-reactivation, while one (UPN 6) had concomitant CMV-reactivation. All 11 patients had biochemical signs of hypogammaglobulinemia, while 8 (UPN 1, 2, 5-6 and 8-11) patients had neutropenia. The median age at diagnosis of CLL was 58 years (range 43-75 years). The median time from CLL diagnosis to EBV-reactivation was 4.5 years (range 1-13 years). With a follow-up time of 824 days, the median overall survival from date of EBV-reactivation was 264 days, despite aggressive rituximab based chemo-immunotherapy in symptomatic cases. Eight deaths were observed, 3 as a direct result of EBV-reactivation.

All 11 patients had high-risk disease as defined by either IGVH mutational status and/or FISH analysis of recurrent cytogenetic aberrations associated with CLL (Table 1). Ten patients (UPN 1-2 and 4-11) had received fludarabine based treatment prior to EBV-reactivation, and 9 patients (UPN 1-2, 4-6 and 8-11) had fludarabine refractory disease at the time of EBV-reactivation. Whether these observations suggest that EBV-reactivation is associated with the immunosuppressive side-effects caused by treatment of advanced CLL cannot be determined from our data set. However, we do note that EBV-reactivation occurred before exposure to alemtuzumab in 4 patients (UPN 1, 7-8 and 11), and in one patient (UPN 3) prior to any CLL treatment. This last patient turned out to be fludarabine-resistant, did not receive rituximab, but responded well to standard alemtuzumab treatment on which EBV-copies in sequential blood samples disappeared. Of the 4 patients not treated with alemtuzumab, one developed proven EBV-driven CLL-related BLPD, one hemophagocytic syndrome, one a possible BLPD and one did not have any symptoms.

The clinical presentation of the patients was related to the level of EBV copies/mL plasma.

i) Low-grade EBV-reactivation. Seven patients (UPN 1-7) had low EBV-levels of up to 6,600 copies/mL. The patients were either asymptomatic (UPN 1 and 4) or presented with fever, fatigue, night sweats and/or enlarged lymph nodes, that is, symptoms identical to the development of active CLL. In retrospect, UPN 5-7 were considered to have pos-