Cryptochrome-1 expression: a new prognostic marker in B-cell chronic lymphocytic leukemia

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ABSTRACT

Chronic lymphocytic leukemia is an adult-onset leukemia with a heterogeneous clinical behavior. When chronic lymphocytic leukemia cases were divided on the basis of IgV$_H$ mutational status, widely differing clinical courses were revealed. Since IgV$_H$ sequencing is difficult to perform in a routine diagnostic laboratory, finding a surrogate for IgV$_H$ mutational status seems an important priority. In the present study, we proposed the use of Cryptochrome-1 as a new prognostic marker in early-stage chronic lymphocytic leukemia. Seventy patients (Binet stage A, without treatment) were included in the study. We correlated Cryptochrome-1 mRNA with well established prognostic markers such as IgV$_H$ mutations, ZAP70, LPL or CD38 expression and chromosomal abnormalities. High Cryptochrome-1 expression correlated with IgV$_H$ unmutated samples. In addition, Cryptochrome-1 was a valuable predictor of disease progression in early-stage chronic lymphocytic leukemia, therefore it can be introduced in clinical practice with the advantage of a simplified method of quantification.

Key words: Cryptochrome-1, prognostic marker, chronic lymphocytic leukemia, ZAP70, LPL, IgV$_H$.

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Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with a variable clinical course and overall survival times ranging from months to decades.[1] The staging systems developed by Rai[2] and Binet[3] are the standard methods of assessing survival and treatment requirements in CLL patients, however, they are insufficient for the identification of patients at risk of progression, especially in the early-stages of CLL. There is a need to investigate reliable prognostic factors for predicting stable or progressive forms of CLL that facilitate risk-adapted treatment strategies.[4] Mutational status of immunoglobulin heavy chain variable region (IgV$_H$) correlates with clinical behavior and is a powerful prognostic factor in CLL. Unmutated IgV$_H$ patients have a reduced survival and responsiveness to chemotherapy.[5,6]

However, IgV$_H$ sequencing is difficult to perform in a routine laboratory, thus, finding a surrogate for IgV$_H$ mutations seems to be an important priority. One of the proposed markers identified through transcriptional studies is the quantification of ZAP70 expression,[7,8] but there are still problems related to the technical validation and standardization of the flow cytometric (FC) method used for its determination, as well as around 20% of discordant results between ZAP70 and IgV$_H$ mutations.[9] Another prognostic marker in CLL is lipoprotein lipase (LPL), which shows itself to be as effective as IgV$_H$ mutational status and more reliable than ZAP70 when tested in unpurified CLL samples.[10,11]

In a previous gene profiling study, we identified a limited set of genes which were expressed differentially between unmutated and mutated CLLs.[12] Cryptochrome-1 (CRY1) emerged as one of the genes that provides a good segregation of samples according to IgV$_H$ mutational status, in concordance with previous reports.[13,14] CRY1 is one of the key components involved in circadian clock control but its role in CLL is still little understood.

The objective of this study was to evaluate the prognostic significance of CRY1 expression in early-stage CLL, and its relationship with other well established prognostic markers in CLL.

Design and Methods

Chronic lymphocytic leukemia patients and samples

Seventy samples from previously untreated CLL patients (Binet stage A) were included in this study after informed consent was obtained. Clinical diagnosis was based on standard morphological and immunophenotypic criteria. Definition of progressive disease was based on the NCI guidelines.[15]

Mononuclear cells (PBMC) were isolated from peripheral blood by Ficoll-Hypaque (Lymphoprep™) density centrifugation. Samples were then processed for CD19+ selection, FC analysis and DNA/RNA isolation.

Analysis of IgV$_H$ somatic mutation status

DNA was prepared using a standard protocol and IgV$_H$ gene rearrangements were performed using BIO-MED-2 primers.[16] We considered unmutated those samples with ≥98% homology with the closest germinal line.

RTqPCR assays

cDNA was synthesized from 1.5 µg of total RNA using random hexanucleotides and SuperScriptII (Invitrogen) following the manufacturer's instructions. RTqPCR assays were carried out using "Hs00172734-m1 (CRY1), Hs00277148-m1 (ZAP70) and Hs00173425-m1 (LPL) primers and probe sets (TaqMan® Gene Expression Assays, Applied Biosystems). Amplification of GUS gene was performed in all cases to normalize gene expression.

Interphase fluorescence in situ hybridization (FISH)

A panel of commercially available probes (Vysis) was used to target chromosomes 11q22.3 (ATM), 13q14 (D13S25 and D13S319), 17p13 (TP53) deletions and trisomy 12 on blood smears according to a standard protocol.

ZAP70 flow cytometry assay

Intracellular ZAP-70 expression was analyzed in 69 samples by FC according to Crespo *et al*.[17] with some modifications. We use an isotype control as negative control. Results ≥20% were considered positive.

Statistical analysis

Significant differences between groups were assessed by the Mann-Whitney test and considered significant when *p* values <0.05. All statistical calculations were performed using the SPSS 13.0 software. Progression free survival (PFS) was calculated from the date of diagnosis to disease progression or last follow-up. For more detailed information see the *Online Supplementary Appendix*.

Results and Discussion

CRY1 expression and IgV$_H$ mutational status

Out of the 70 Binet's stage A patients tested for IgV$_H$ mutational status, 50 were mutated in IgV$_H$ genes (71.4%). These percentages are slightly different from those reported in previous studies[6,18,19] because in most of them, patients with different stages of the disease were included.

To assess the usefulness of CRY1 expression as surrogate marker for IgV$_H$ mutational status, we determined CRY1 by RTqPCR in CD19+ cell samples. The mean ± 2 SEM relative concentration in unmutated and mutated samples were: 0.311±0.081 and 0.034±0.023, respectively, *p*<0.0001 (*Online Supplementary Figure S1*).

The optimal predictive model based on CRY1 expression in relation to IgV$_H$ mutational status yielded an area under ROC curve of 0.963. A cut-off value that best segregates IgV$_H$ unmutated from mutated cases for CRY1 relative concentration of 0.090 was selected (95% sensitivity and 92% specificity). Our data show 92.8% of concordant results between CRY1 expression and IgV$_H$ mutational status (*Online Supplementary Table S1*).

We have also analyzed CRY1 expression in 18 samples without B-cell selection. The results showed that CRY1 was able to distinguish between IgV$_H$ unmutated and mutated samples (mean values: 0.380 and 0.051, for unmutated and mutated groups respectively, *p*=0.007). Therefore, our results reveal that CRY1 mRNA expression can be analyzed in PBMC as well as CD19+ selected B cells. CRY1 expression was higher in unmutated CLLs than in mutated ones, but the reasons for this differential expression are still unknown.

CRY1 expression in relation to ZAP70, CD38 or LPL expression and cytogenetic abnormalities

ZAP70 expression was measured by FC and RTqPCR in leukemic B cells, CD38 was determined by FC and LPL by RTqPCR. ROC curves were used to evaluate the performance of each assay. The areas under the curves were 0.835 and 0.880 for ZAP70 measured by FC and RTqPCR respectively, 0.963 for LPL and 0.666 for CD38. Taking into account these results, CRY1 and LPL are the markers that better assess IgV$_H$ mutational status.

Next, we analyzed correlations between continuous variables using Spearman's test, and strong significance was found for CRY1 and LPL or ZAP70 (*p*<0.001).

Other independent prognostic markers in CLL are cytogenetic abnormalities. We performed FISH analysis on 61 CLL samples. Abnormalities were seen in 54.1% (33/61) of patients. The correlation of CRY1 expression and the presence of the different chromosomal aberrations is shown in Table 1. Remarkably, loss of 13q14, which as a single abnormality is associated with a favourable prognosis,[20] was associated with low expression of CRY1, except in one case. Deletion of 17p13 which is associated with bad prognosis was detected in

Table 1. Cryptochrome-1 gene expression in relation to cytogenetic abnormalities.

Cytogenetic abnormalities	CRY1+ (≥0.090) n=18	CRY1- (<0.090) n=43	Total n=61
normal	10	18	28
del 13q14	1	22	23
trisomy 12	5	2	7
del 11q22.3 (ATM)	1	1	2
del 17p13 (TP53)*	1	0	1

*There were 61 cases with FISH results available. *This case shows a complex karyotype, combining del 17p13 and trisomy 12.*

Table 2. Relation between CRY1 and CD38, ZAP70 or LPL expression.

	Status (cut-off value)	CRY1+ (≥0.090) n		CRY1- (<0.090) %		Concordance n	%	p (χ²) %
ZAP70 by FC	Positive (≥20%)	18	26.1	6	8.7	84.1		<0.0001
	Negative (<20%)	5	7.2	40	58.0			
ZAP70 by RTqPCR	Positive (≥0.1050)	22	31.4	9	12.9	85.7		<0.0001
	Negative (<0.1050)	1	1.4	38	54.3			
LPL by RTqPCR	Positive (≥82.45)	21	30.0	5	7.1	90.0		<0.0001
	Negative (<82.45)	2	2.9	42	60.0			
CD38 by FC	Positive (≥20%)	12	19.0	9	14.3	69.8		0.012
	Negative (< 20%)	10	15.9	32	50.8			

CRY1 and LPL were determined by RTqPCR. CD38 was determined by FC. ZAP70 was measured by both methods: FC and RTqPCR. Cut-off values for RTqPCR were selected using Youden index.

Figure 1. Kaplan–Meier plots. (A) PFS according to IgV$_H$ mutational status. (B) PFS according to CRY1 gene expression.

one case in combination with trisomy 12, this case was IgV$_H$ unmutated, with high expression of CRY1 and positive values of CD38 and ZAP70.

In order to compare variables, we chose a cut-off value that best segregates IgV$_H$ unmutated from mutated cases for each variable, therefore cases were scored as *positive* (+) or *negative* (–) according to this criteria. Table 2 shows concordance percentages between expression of CRY1 and ZAP70, CD38 or LPL. Both methods for ZAP70 estimation displayed strong concordance with CRY1 expression, with slightly better results in the case of RTqPCR. A better correlation was obtained between CRY1 and LPL expression, with a concordance ratio of 90.0% whereas CD38 show the lowest percentage of concordant results (69.8%).

Given the strong prognostic value shown by CRY1, ZAP70 and IgV$_H$ mutational status, we studied the correlation between all of them, as well as the discordant results. A perfect correlation was obtained with all double-negative patients, ZAP70- by RTqPCR and CRY1-,
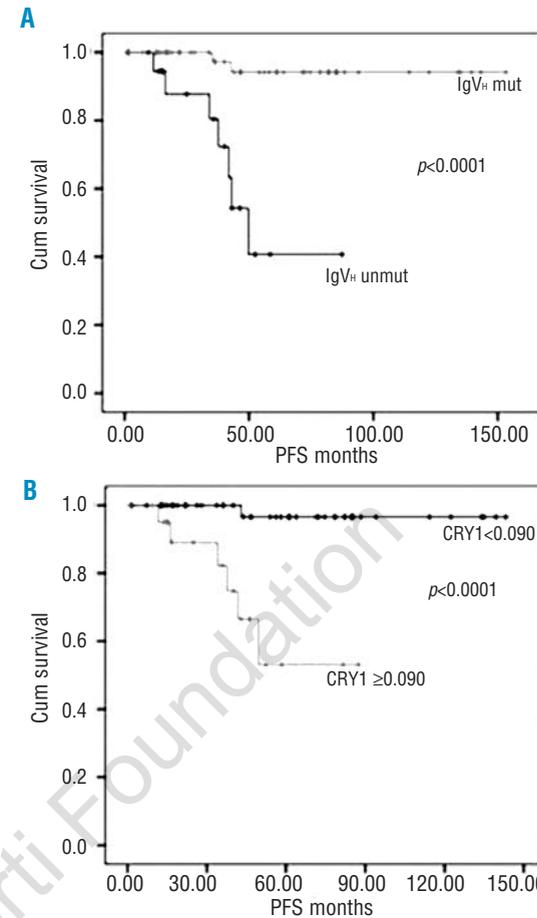
(n=38) showing in all cases mutated IgV$_H$ genes; whereas, in the case of all double-positive patients, ZAP70+ by RTqPCR and CRY1+ (n=22), 3 samples expressed mutated IgV$_H$ genes, in which one of these samples was ZAP70- by FC as well as CD38<20% and the other 2 samples were ZAP70+ and CD38>20% by FC in both cases. The strong correlation accounted for these markers is remarkable, but still discordant profiles represent a 14.3% of CLL patients in our study. They included 9 patients expressing ZAP70+ by RTqPCR and CRY1-, and one patient expressing ZAP70- and CRY1+. Among these discordant patients with ZAP70+/CRY1- profiles, 8 had mutated IgV$_H$ gene, whereas one was unmutated (this sample was ZAP70- by FC). On the other hand, the discordant patient with ZAP70-/CRY1+ displayed mutated IgV$_H$ gene, but this patient had high CD38 expression. Notably, from the 10 CRY1/ZAP70 discordant patients, in 8 cases CRY1 was useful to assign IgV$_H$ mutational status correctly (*Online Supplementary Table S2*).

Interestingly, with IgV$_H$ mutational status as the comparison method, CRY1 expression showed 92.8% agreement in terms of results, a much better performance than ZAP70 measured by FC and RTqPCR (82.6% and 84.3% of agreement respectively) which is until now the most used surrogate marker for IgV$_H$ mutations.

We have also compared the performance of CRY1 against LPL which is one of the newest prognostic markers proposed in CLL.[10,11,13,21] The concordance between CRY1 and LPL expression was high, with only 7 discordant results. All double-positive samples, except 2, were unmutated for IgV$_H$. Among the discordant results, there were 2 cases CRY1$^+$/ LPL$^-$, and both were IgV$_H$ mutated, thus LPL would assign mutational status correctly in those cases. In the case of the other 5 discordant samples (CRY1-/LPL$^+$), 4 of them were IgV$_H$ mutated, therefore CRY1 behaves as a better surrogate marker than LPL in this group of samples (*Online Supplementary Table S2*).

CRY1 expression and survival

We evaluated CRY1 as a new marker of disease progression in our group of early-stage CLL patients in comparison with other prognostic markers. The median PFS was 49.67 months (95% CI, 38.79-69.55 months) in CLLs displaying unmutated IgV$_H$ genes whereas the median PFS was not achieved in mutated patients, $p<0.0001$ (Figure 1A). The estimated mean survival for patients with CRY1 values ≥0.090 was 63.20 months (95% CI, 48.17–78.23), and 139 months (95% CI, 133.12–146.43) for patients with CRY1 values below 0.090, $p<0.0001$ (Figure 1B). In PFS curves for ZAP70 measured by FC and RTqPCR there was a better distinction between groups in the case of RTqPCR determinations ($p=0.001$ and $p=0.054$ for RTqPCR and FC respectively). LPL was also a strong predictor for PFS in our group of patients. Thus, LPL positive cases have a significantly shorter median PFS than negative cases, $p<0.0001$ (*Online Supplementary Figure S2*). Among the parameters studied, CRY1, LPL and IgV$_H$ mutational status were those which better exhibited association with PFS in early-stage CLL.

In summary, it is well known that IgV$_H$ mutational status is a reliable prognostic marker in CLL,[5,6] but its determination is laborious and time-consuming, It is tempting, therefore, to search for other markers. Here, we showed that CRY1 quantification by RTqPCR may constitute a new prognostic marker in CLL. CRY1 is a key component of the circadian clock. Recent epidemiological studies have raised the possibility that disruption of the circadian clock may increase the risk of developing cancer in humans and adversely affect prognosis in cancer patients.[22] A cyclic and significant expression of circadian clock genes in peripheral mononuclear blood cells of healthy individuals[23] and an anomalous expression of CRY1 (among other components of the biological clock) has been documented during chronic and blastic phases of chronic myeloid leukemia.[24] In our study, CRY1 show a similar or better behavior than other well established biomarkers in CLL, such as ZAP70, CD38, LPL or cytogenetic abnormalities, and an excellent correlation with IgV$_H$ mutational status. Our observation, along with the easiness of CRY1 determination by RTqPCR on B lymphocytes or unselected PBMC, indicate that CRY1 may be used as a new marker of disease progression for the initial prognostic assessment of early-stage CLL. In addition, it would be interesting to introduce this marker in clinical trials for its evaluation in larger cohorts of patients.

Authorship and Disclosures

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