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Cryptochrome-1 Gene Expression is a Reliable Prognostic Indicator in Egyptian Patients with Chronic Lymphocytic Leukemia: A Cohort Prospective Study

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List of abbreviations:
CLL: Chronic lymphocytic leukemia.
PB: Peripheral blood.

BM: Bone marrow.

IgHV: Immunoglobulin heavy-chain variable-region.

CRY-1: Cryptochrome-1.

TFT: Time to first treatment.

K-EDTA: Potassium ethylene diamine tetra-acetic acid.

FISH: Fluorescence in situ hybridization.

qRT-PCR: Real-time reverse-transcription polymerase chain reaction.

RNA: Ribonucleic acid.

cDNA: Complementary deoxyribonucleic acid.

CT: Cycle threshold.

SD: Standard deviation.

IQR: Interquartile range.

HR: Hazard ratio.

CI: Confidence interval.

Abstract

Objectives Traditional prognostic factors have proven insufficient to account for the observed heterogeneity in the clinical behavior of chronic lymphocytic leukemia (CLL). *Cryptochrome-1 (CRY-1)* is a circadian clock gene essential in maintaining circadian rhythm and regulating cell proliferation. We evaluated *CRY-1* gene expression in CLL patients, and assessed its putative role as a prognostic indicator for the clinical course of CLL. **Materials and Methods** Hundred CLL patients at diagnosis were studied for *CRY-1* gene expression by qRT-PCR, and were followed up for assessment of time to first treatment (TFT). **Results** *CRY-1* was expressed in 94% CLL patients at diagnosis. The median *CRY-1* relative gene expression level (0.006) stratified patients into high and low expression groups. Forty of 100(40%) CLL patients showed high *CRY-1*, 54/100 (54%) showed low *CRY-1*, while 6/100 (6%) had undetectable *CRY-1* gene expression. High *CRY-1* gene expression was significantly associated with $CD38^+$, $Zap\text{-}70^+$, double $CD38^+Zap\text{-}70^+$, unfavorable/intermediate cytogenetics, unmutated *IgHV* gene, and diffuse marrow infiltration. Patients with high*CRY-1* gene expression had shorter TFT than the low *CRY-1* gene expression group. Cox-proportional hazard regression model identified *CRY-1* gene expression to be independently predictive for TFT. **Conclusions** *CRY-1* is differentially expressed among CLL patients stratifying them into low-risk and high-risk groups. *CRY-1* gene expression could constitute a reliable prognostic indicator for CLL progression, complementing the role of standard well-established prognostic factors. *CRY-1* gene expression could be employed as a prognostic indicator for disease progression during the initial prognostic work-up and follow-up for CLL patients.

Keywords: Chronic lymphocytic leukemia; *Cryptochrome-1*; Circadian genes; Time to first treatment; Prognosis; Real-time polymerase chain reaction; CD38; Zap-70.

Introduction

Chronic lymphocytic leukemia (CLL) is a chronic lymphoproliferative disorder characterized by progressive proliferation and accumulation of morphologically mature, immunologically dysfunctional monoclonal B cells in the peripheral blood (PB), bone marrow (BM), and lymphoid tissue (1).

The clinical course of CLL is heterogeneous and difficult to predict, with some patients experiencing rapid disease progression and others living for decades without requiring treatment (2). Early treatment of the latter group could place patients at risk for therapy-related complications that might compromise their quality of life and/or survival. Defining markers that can reliably stratify patients into groups with good-risk or poor-risk disease could facilitate clinical trials evaluating the potential benefit of early treatment and assist risk-adapted treatment strategies (3). Traditional prognostic factors have proven insufficient to account for the observed heterogeneity in the clinical behavior of CLL necessitating a search for further prognostic indicators that can better correlate with patients' clinical outcome and survival (4,5).

Mutational status of the *immunoglobulin heavy-chain variable-region (IgHV)* gene correlates with the clinical behavior and is a powerful prognostic factor in CLL. However, *IgHV* gene sequencing is laborious, time-consuming, and difficult to perform in routine laboratory, thus, finding a surrogate for*IgHV* gene mutations seems an important priority (6).

Several genes constituting the circadian clock machinery have been found to establish functional interplays with regulators of the cell cycle. Thus, the aberrant expression of circadian clock genes can result in the aberrant expression of downstream target genes associated with cell proliferation and apoptosis, leading to the emergence of different types of cancers, including CLL (6-9).

Cryptochrome-1 (CRY-1) gene (12q23-q24.1); a key component of the circadian clock, has been shown to be essential to the maintenance of circadian rhythm because of its role in the negative arm of the circadian feedback loop. However, apart from its circadian function it has an additional role as a transcriptional regulator for several genes involved in cell metabolism and proliferation (10).

Accordingly, the objective of this study was to evaluate the expression of *CRY-1* gene in CLL patients, and to assess its putative role as a prognostic indicator for the clinical course of CLL.

Materials and Methods

This cohort prospective study was conducted on 100 newly-diagnosed untreated CLL patients attending the Hematology/Oncology Clinic of Ain Shams University Hospitals. The patients were selected for the study on the basis of standard clinical, hematologic, and immunophenotypic criteria for the diagnosis of CLL (11). CLL patients were 64 males and 36 females with a male to female ratio of 1.8:1, and a median age of 61 years (IQR, 55-68). All patients were followed up after diagnosis for assessment of time to first treatment (TFT) (median, 20 months; range, 10-24 months).

TFT was defined as the time from diagnosis to any initial treatment or, alternatively, as time from diagnosis to last follow-up. Briefly, criteria for initiating primary treatment were the following: (i) the presence of constitutional symptoms; (ii) evidence of progressive marrow failure as manifested by the development of, or worsening of, anemia and/or thrombocythemia; (iii) autoimmune anemia and/or thrombocytopenia poorly responsive to corticosteroid therapy; (iv) massive (>6 cm below the left costal margin) or progressive splenomegaly; (v) massive nodes or clusters (>10 cm in longest diameter) or progressive lymphadenopathy; (vi) progressive

lymphocytosis with an increase of >50% over a 2-month period or an anticipated doubling time of <6 months (12). Informed consents were obtained from all patients prior to enrollment. The study was approved by the Ethical Committee for Human Experimentation of Ain Shams University and was in accordance with the Helsinki Declaration of 1975, as revised in 2002. The demographic and clinicopathologic characteristics of CLL patients at diagnosis are presented in Table 1.

Sampling

PB and BM samples were collected on potassium ethylene diamine tetra-acetic acid (K-EDTA) (1.5 mg/mL) for morphologic, immunophenotypic and molecular analyses, and on lithium heparin for cytogenetic analysis. PB samples were used for flow cytometric immunophenotyping and real-time reverse-transcription polymerase chain reaction (qRT-PCR) for quantification of *CRY-1* gene expression, while PB or BM samples, when available, were used for the cytogenetic analysis.

Flow cytometric immunophenotyping

Immunophenotyping using the standard panel for chronic lymphoproliferative disease (CD5, CD19, CD23, FMC7, CD20, CD38, CD79b, CD10, CD25, CD103, CD123, kappa, lambda, surface IgM) (Beckman Coulter, Miami, USA), along with ZAP-70 (BioLegend, CA, USA) were performed on EPICS XL Flow Cytometer (Coulter Electronics, Hielach, FL, USA). Positivity threshold was defined as the expression of the marker by ≥30% of the B-lymphocytes (13), however, for CD38 and Zap-70 a cut-off value of ≥20% and ≥10% of the B-lymphocytes were considered positive, respectively (14).

Fluorescence in situ hybridization (FISH)

Probes for del(13q), del(17p), del(11q) and trisomy 12 (Vysis, Downers Grove, USA) were used. The patients were stratified into cytogenetic-based risk groups: *favorable*; del(13q), or normal karyotype, *intermediate*; trisomy 12, and *unfavorable*; del(17p), del(11q), or complex karyotype (≥ 3 chromosomal aberrations) (13).

Real-time reverse-transcription polymerase chain reaction (qRT-PCR) for quantification of *CRY-1* gene expression

qRT-PCR amplification was done using gene expression sets for the *CRY-1* gene (Homosapines; Hs00172734-m1 TaqMan® Gene Expression Assays; Applied Biosystems, Foster City, CA, USA), TaqMan *β-actin* control reagents (Applied Biosystems) for the *β-actin* reference gene, andTaqMan Universal PCR Master Mix (Applied Biosystems). The total ribonucleic acid (RNA) was extracted from PB samples using a QIAamp® RNA blood kit (Applied Biosystem, Qiagen, Valencia, CA, USA), and the complementary deoxyribonucleic acid (cDNA) was synthesized using QuantiTectReverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. The cDNA was stored at -20°C until used.

PCR products were synthesized from cDNA samples using sequence-specific primers, and TaqMan oligonucleotide fluorescence-labeled probes; FAM for *CRY-1* gene (Hs_*CRY-1*_1_FAM Quantifast Probe Assay) and MAX for *β-actin* gene (Hs_*β-actin*_1_MAX Quantifast Probe Assay). Both probes were labeled with Iowa Black Fluorescent Quencher (IBFQ). Each PCR contained all the necessary reagents and 50 ng of cDNA in a final volume of 25 µL. A negative control (cDNA replaced by nuclease-free water) was included in each assay. The reaction protocol comprised 40 cycles of heating at 95°C for 5 min (hot start PCR), followed by heating at 95°C for 30 s (denaturation), and finally heating at 60°C for 30 s (annealing/extension). PCR and data analysis were carried out on Startagene Mx3000P

(Startagene Inc., CA, USA). Undetectable *CRY-1* expression was applied to cases where cycle threshold (CT) values exceeded the 40th cycle.

CRY-1 expression levels in unknown samples were calculated by relative quantification using the ΔΔCT method which relies on comparison of CT values of *CRY-1* (target gene) to *β-actin* (reference gene) in unknown and normal calibrator samples. The results were presented as the fold change in gene expression normalized to the endogenous reference gene and relative to the normal calibrator (15).

Statistical analysis

Data were analyzed using SPSS (version 17) statistical software package under Windows 7 operating system (SPSS Inc., Chicago, Illinois, USA).Categorical data were presented in the form of frequency and percentage, and compared using Chi-square test (χ^2).Continuous data were presented in the form of mean ± standard deviation (SD) or median and interquartile range (IQR) for parametric and non-parametric variables, respectively. Student's *t*-test and Mann-Whitney test were used for comparing continuous parametric and non-parametric variables between two groups, respectively. Curves for TFT were plotted according to the Kaplan-Meier method, with log-rank test used to compare the TFT distributions between the high and low *CRY-1*gene expression groups. Multivariate Cox-proportional hazard regression model (hazard ratio; HR) was done to identify the independent association of*CRY-1* gene expression with TFT. A *P* value of <0.05 was considered significant and of <0.01 was considered highly-significant in all analyses.

Results

***CRY-1* gene expression in CLL patients at diagnosis**

CRY-1 gene was expressed in 94 of 100 (94%) CLL patients at diagnosis (median, 0.006; IQR, 0.000008-0.32) (Table 1). The median *CRY-1*relative gene expression level (0.006) was employed as the cut-off value for stratifying high and low *CRY-1* gene expression groups. Accordingly, 40 of 100 (40%) CLL patients showed high *CRY-1* gene expression (≥0.006) (median, 0.295; IQR, 0.034-3.618) and 54 of 100 (54%) CLL patients showed low *CRY-1* gene expression (<0.006) (median, 0.000008; IQR, 0.0000005-0.0002). Six of 100 (6%) CLL patients had undetectable *CRY-1* gene expression (Table 1, Figure 1).

High and low *CRY-1* gene expression in relation to the studied parameters of CLL patients at diagnosis

High *CRY-1* gene expression was significantly associated with $CD38^+$, $Zap\text{-}70^+$, double $CD38^+Zap\text{-}70^+$ expression, unfavorable/intermediate cytogenetics, unmutated *IgHV* gene, and diffuse BM infiltration in trephine biopsy ($p<0.05$). On the other hand, low *CRY-1* gene expression was significantly associated with $CD38^-$, $Zap\text{-}70^-$, double $CD38^-Zap\text{-}70^-$ expression, and favorable cytogenetics ($p<0.05$). On the other hand, no further significant difference was found between high and low *CRY-1* gene expression groups regarding other studied demographic or clinicopathologic parameters ($p>0.05$) (Table 2).

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Figure Legends

Figure 1 *CRY-1* gene expression in CLL patients at diagnosis. (A) All CLL patients, (B) Low and High *CRY-1* gene expression groups.

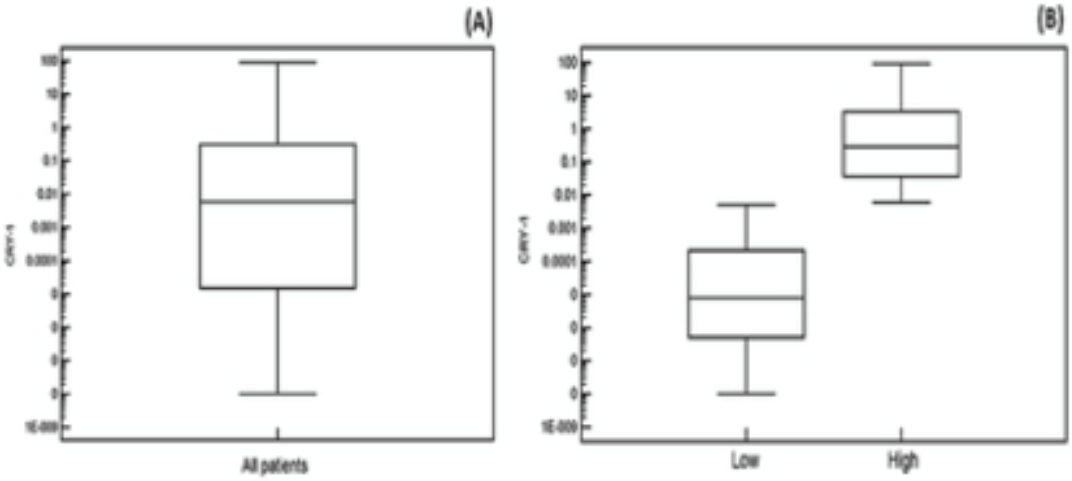

Figure 2 Kaplan-Meier curve showing the time to first treatment for CLL patients based on *CRY-1* gene expression levels.

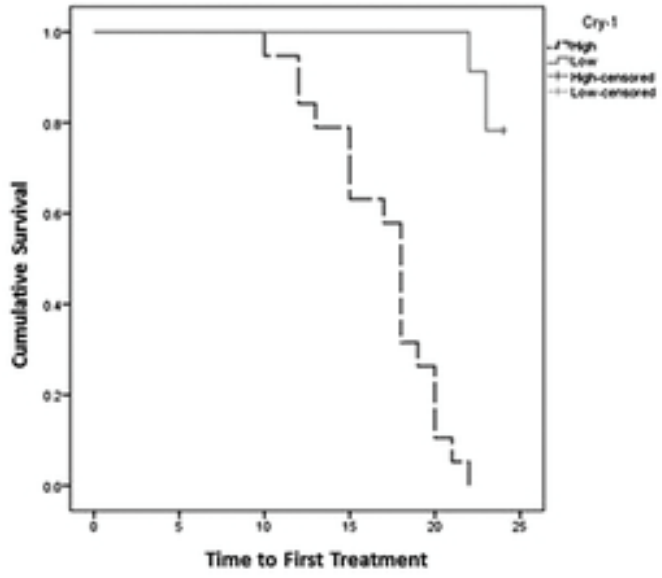

Table 1: Demographic and clinicopathologic characteristics of CLL patients at diagnosis

Parameters	CLL patients (n = 100)
Age (years), median (IQR)	61 (55 - 68)
Gender (Male:Female)	1.8:1
Lymphadenopathy, n (%)	76 (76)
Splenomegaly, n (%)	38 (38)

Hepatomegaly, n (%)	22 (22)
Binet Stage, n (%) A (Low-risk) B (Intermediate-risk) C (High-risk)	 36 (36) 12 (12) 52 (52)
TLC ($\times 10^9$/L), median (IQR)	38.5 (19.8 - 90)
Hemoglobin (g/dL), mean ± SD	10.73 ± 2.5
Platelets ($\times 10^9$/L), mean ± SD	157 ± 82
PB Lymphocytes ($\times 10^9$/L), median (IQR)	34 (15 - 72)
BM Lymphocytes (%), median (IQR)	85 (78 - 90)
CD38 positive, n (%)	44 (44)
Zap-70 positive, n (%)	22 (22)
Cytogenetic abnormalities, n (%)* Favorable Intermediate Unfavorable Not available	 42 (42) 20 (20) 22 (22) 16 (16)
IgHV gene, n (%) Mutated Unmutated Not available	 37 (37) 32 (32) 31 (31)
Pattern of BM infiltration, n (%)** Diffuse Non-diffuse Not available	 40 (40) 50 (50) 10 (10)
CRY-1 gene expression, median (IQR) All CLL patients High *CRY-1* group, n = 40 (40%) Low *CRY-1* group, n = 54 (54%) Undetectable *CRY-1*, n = 6 (6%)	 0.006 (0.000008 - 0.32) 0.295 (0.034 - 3.618) 0.000008 (0.0000005 - 0.0002) -
Time to first treatment (months), median (range)	20 (10 - 24)

BM, Bone marrow; CLL, Chronic lymphocytic leukemia; *CRY-1, Cryptochrome-1*; *IgHV, Immunoglobulin heavy-chain variable-region*; IQR, Interquartile range; PB, Peripheral blood; SD, Standard deviation; TLC, Total leucocytic count.

*Favorable: del(13q), normal karyotype; Intermediate: trisomy 12; Unfavorable: del(17p), del(11q), complex karyotype (≥ 3 chromosomal aberrations) (13).

**Non-diffuse infiltration included nodular, interstitial, or mixed nodular/interstitial infiltrations.

Table 2: High and low *CRY-1* gene expression in relation to the studied parameters of CLL patients at diagnosis

Parameters	High *CRY-1* (n = 40)	Low *CRY-1* (n = 54)	P value
Age (years), median (IQR)	58.5 (54.3 - 64.8)	62 (55 - 67)	0.892
Gender, n (%) Male Female	 22 (55) 18 (45)	 40 (74.1) 14 (25.9)	0.172
Lymphadenopathy, n (%) Present Absent	 30 (75) 10 (25)	 42 (77.8) 12 (22.2)	1.000
Splenomegaly, n (%) Present Absent	 16 (40) 24 (60)	 22 (40.7) 32 (59.3)	0.959
Hepatomegaly, n (%) Present Absent	 12 (30) 28 (70)	 10 (18.5) 44 (81.5)	0.489
Binet Stage, n (%) A (Low-risk) B (Intermediate-risk) C (High-risk)	 12 (30) 8 (20) 20 (50)	 20 (37) 4 (7.4) 30 (55.6)	0.436
TLC ($\times 10^9$/L), median (IQR)	21.9 (10.8 - 66)	46 (25 - 125)	0.100
Hemoglobin (g/dL), mean ± SD	10.7 ± 2.9	10.8 ± 2.3	0.856
Platelets ($\times 10^9$/L), mean ± SD	145 ± 69	158 ± 89	0.572
PB Lymphocytes ($\times 10^9$/L), median (IQR)	19.8 (7.6 - 52)	38.5 (21 - 95)	0.114
BM Lymphocytes (%), median (IQR)	84.5 (78 - 90)	85 (78 - 90)	0.367
CD38 expression, n (%) Positive Negative	 24 (60) 16 (40)	 16 (29.6) 38 (70.4)	0.031
Zap-70 expression, n (%) Positive Negative	 16 (40) 24 (60)	 6 (11.1) 48 (88.9)	0.021
CD38 and Zap-70 expression, n (%) Double positive Double negative Discordant	 12 (30) 12 (30) 16 (40)	 0 (0) 32 (59.3) 22 (40.7)	0.002
Cytogenetic abnormalities, n (%)* Favorable Intermediate Unfavorable	 6 (15) 14 (35) 18 (45)	 32 (59.3) 10 (18.5) 4 (7.4)	0.005
IgHV gene, n (%) Mutated Unmutated	 14 (35) 19 (47.5)	 23 (42.6) 13 (24.1)	0.037
Pattern of BM infiltration, n (%)** Diffuse	23 (57.5) 19 (47.5)	15 (27.8) 27 (50)	0.041

Non-diffuse			
Time to first treatment (months), Median 95% confidence interval	 16.89 15.38 - 18.41	 23.69 23.44 - 23.95	 <0.001

BM, Bone marrow; CLL, Chronic lymphocytic leukemia; *CRY-1, Cryptochrome-1; IgHV, Immunoglobulin heavy-chain variable-region*; IQR, Interquartile range; PB, Peripheral blood; SD, Standard deviation; TLC, Total leucocytic count.

*Favorable: del(13q), normal karyotype; Intermediate: trisomy 12; Unfavorable: del(17p), del(11q), complex karyotype (≥ 3 chromosomal aberrations) (13).

**Non-diffuse infiltration included nodular, interstitial, or mixed nodular/interstitial infiltrations.

Table 3: Predictors for earlier time to first treatment according to Cox-proportional hazard regression model

Predictors	Hazard Ratio (95% Confidence Interval)	P value
Binet stage	2.80 (1.32 - 4.96)	0.036
CD38 expression	0.38 (0.08 - 1.84)	0.232
Zap-70 expression	2.41 (0.26 - 7.65)	0.003
Cytogenetic-based risk groups	1.26 (0.90 - 2.79)	0.017
IgHV gene mutational status	4.23 (2.52 - 6.98)	<0.001
Pattern of BM infiltration	0.98 (0.17 - 1.78)	0.360
CRY-1 gene expression	3.99 (2.12 - 6.19)	0.001

BM, Bone marrow; *CRY-1: Cryptochrome-1; IgHV, Immunoglobulin heavy-chain variable-region.*