

## Brief communication

## CLL1 expression distinguishes chronic lymphocytic leukemia from other mature B-cell neoplasms

Elisabeth Oppliger Leibundgut<sup>a,b,\*</sup>, Daniela Rogenmoser-Dissler<sup>a</sup>, Dirk de Beer<sup>b</sup>, Naomi A. Porret<sup>a</sup>, Silvia Zibellini<sup>c</sup>, Jan Dürig<sup>d</sup>, Alexander Röth<sup>d</sup>, Dirk Klingbiel<sup>e</sup>, Gabriela M. Baerlocher<sup>a,b</sup>

<sup>a</sup> Department of Hematology, University Hospital and University of Bern, Bern, Switzerland

<sup>b</sup> Experimental Hematology, Department of Clinical Research, University of Bern, Bern, Switzerland

<sup>c</sup> Department of Hematology, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy

<sup>d</sup> Department of Hematology, University Hospital Essen, Essen, Germany

<sup>e</sup> Swiss Group for Clinical Cancer Research (SAKK), Coordinating Center, Bern, Switzerland

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## ABSTRACT

The distinction of CLL from other mature B-cell neoplasms, especially from leukemic forms of mantle cell lymphoma or splenic marginal zone lymphoma, can be difficult but has important prognostic and therapeutic implications. We measured *CLL1* (CLL upregulated gene1) mRNA by qPCR and found a highly significant difference between CLL and other lymphoid neoplasms (AUC 0.96, 95%CI 0.93–0.99). Based on our cut-off values we can predict CLL and other mature B-cell neoplasms with high probability (PPV 99% and 94%). Analysis of *CLL1* expression is a rapid and reliable tool that may facilitate the diagnosis of mature B-cell neoplasms especially in inconclusive cases.

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### 1. Introduction

The accurate diagnosis of B-cell lymphocytosis has important prognostic and therapeutic implications. The distinction of chronic lymphocytic leukemia (CLL) from other B-cell neoplasms, however, can be difficult since leukemic manifestations of mantle cell lymphoma (MCL) and splenic marginal zone lymphoma (SMZL) can be morphologically indistinguishable and can immunophenotypically overlap with CLL. The diagnostic scoring system for the specific immunophenotype of CLL proposed by Matutes et al. comprises a set of five markers which have proven to be useful in many cases [1]. In cases with 2 or 3 of five markers, additional parameters are needed to establish the correct diagnosis. A number of surface and molecular markers have been investigated and proposed to be diagnostically useful and/or to have prognostic significance in B-cell lymphocytosis, five of which have recently been combined into a new molecular scoring system for risk stratification for CLL, including ZAP70, LPL (lipoprotein lipase), CLL1, microRNA-29c and microRNA-223 [2].

The chronic lymphocytic leukemia upregulated gene-1 (*CLL1*) has been shown to be the first highly specific CLL gene [3]. Six transcripts have been identified, two of which contain a putative coding sequence with low similarity to human interleukin-4. Overexpression of the transcript cDNA1 appears to be specific for CLL, is of prognostic significance and can be used for MRD detection [4–6]. High levels of *CLL1* expression in patients with CLL have been correlated with unfavorable prognostic markers, i.e. unmutated IgVH, high CD38 expression, high ZAP70 expression and unfavorable cytogenetics (17p, 11q). Single cases of patients with B-cell neoplasms other than CLL showed low levels of *CLL1* expression [3].

The goal of this study was to assess the specificity and sensitivity of the *CLL1* mRNA expression in B-cell lymphoproliferative disorders (B-LPD) and to validate its utility to distinguish CLL from other B-cell neoplasms, especially MCL.

### 2. Materials and methods

Our retrospective study was performed on 167 blood or bone marrow samples taken at diagnosis from patients with lymphoid neoplasms including CLL ( $n=83$ ), MCL ( $n=18$ ), SMZL ( $n=26$ ), follicular lymphoma ( $n=12$ ), diffuse large B-cell lymphoma ( $n=9$ ), B lymphoblastic leukemia/lymphoma ( $n=11$ ) and T lymphoblastic leukemia/lymphoma ( $n=8$ ). All patients were diagnosed according to the World Health Organization criteria. Diagnostic characterization included PCR and/or FISH analysis for translocations  $t(11;14)$  or  $t(14;18)$  in patients with MCL and follicular lymphoma (FL), and IgVH mutational status, CD38 ( $n=63$ ) and ZAP70 ( $n=43$ )

\* Corresponding author at: Department of Hematology, University Hospital Bern, Freiburgstrasse 4, 3010 Bern, Switzerland. Tel.: +41 31 632 9807; fax: +41 31 632 5038.

E-mail address: [elisabeth.oppliger@insel.ch](mailto:elisabeth.oppliger@insel.ch) (E. Oppliger Leibundgut).

**Table 1**  
CLL1 mRNA expression in patients with lymphoid neoplasms.

Diagnosis	Number of samples	PBL/BM	CLL1 median <sup>a</sup>	CLL1 range <sup>a</sup>
Mature B-cell neoplasms				
CLL with unmutated IgVH	43	42/1	655.15	0.28–51,324
CLL with mutated IgVH	40	39/1	55.87	0.08–2871
Mantle cell lymphoma	18	13/5	0.06	<0.01–4.05
Splenic marginal zone lymphoma	26	9/17	0.45	<0.01–12.29
Follicular lymphoma	12	6/6	0.08	<0.01–0.56
Diffuse large B-cell lymphoma	9	7/2	<0.01	<0.01–8.74
Precursor lymphoid neoplasms				
B lymphoblastic leukemia/lymphoma	11	7/4	0.12	<0.01–4.38
T lymphoblastic leukemia/lymphoma	8	6/2	0.12	<0.01–1.69
Controls				
Healthy volunteers	15	15/0	0.52	0.18–3.03

PBL, peripheral blood; BM, bone marrow.

<sup>a</sup> Expression levels relative to B-cells of healthy donors.

in patients with CLL as previously described [7]. 15 blood samples from healthy volunteers were included as normal controls.

Normal CD19+ lymphocytes were isolated from Ficoll separated peripheral blood mononuclear cells from healthy donors by magnetic cell sorting using MACS CD19 Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of CD19+ cells was 93% on average (range 82.5–97.4%).

Total RNA was extracted from Ficoll separated mononuclear cells using the QIAmp RNA Blood Mini Kit (Qiagen, Hombrechtikon, Switzerland) or TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed by the Superscript system (Invitrogen). CLL1 expression was determined by quantitative real-time PCR using published primers and probes for the cDNA1 transcript [3]. The endogenous reference gene ABL was measured according to the EAC protocol [8]. CLL1 expression was normalized for ABL ( $\Delta Ct = Ct_{\text{target}} - Ct_{\text{ABL}}$ ) and expressed relative to B-lymphocytes from healthy donors ( $2^{-\Delta\Delta Ct}$ ). A normalized quantity greater than 1.00 was defined as overexpression of CLL1 mRNA.

Statistical analyses were performed using the R statistical software package (<http://www.r-project.org>).

### 3. Results and discussion

CLL1 mRNA was detected in all samples from CLL patients, ranging from 0.08 to 51,300-fold compared to B-cells from healthy individuals, with medians of 655-fold in patients with unmutated IgVH and 56-fold in patients with mutated IgVH (Table 1). 76 of 83 (92%) CLL samples demonstrated overexpression of CLL1 mRNA. In contrast, the great majority of cases with lymphoid neoplasms other than CLL showed undetectable or very low CLL1 mRNA expression (median 0.19, range <0.01–12.3). The difference between CLL and other mature B-cell neoplasms including MCL, SMZL and FL was highly discriminant (area under the curve (AUC) 0.96, 95% CI 0.93–0.99). Taking into account the overlap in CLL1 expression between IgHV-mutated CLL and other B-LPD, we chose, based on the receiver operating characteristic (ROC) analysis and the AUC, to determine two cut-off levels for CLL and other B-LPD: A CLL1 value of 6.54 and higher predicts CLL with a positive predictive value (PPV) of 99% and a negative predictive value (NPV) of 81%, and a value of 0.35 and lower predicts a mature B-cell neoplasm other than CLL with a PPV of 94% and a NPV of 78% (Fig. 1).

Furthermore, for patients with CLL, we correlated the CLL1 expression with IgVH mutational status, CD38 and ZAP70 using the two-sided Wilcoxon rank sum test with *p*-values adjusted for multiple testing. Our results confirmed the known significant association of high CLL1 expression with the poor prognostic markers of unmutated IgVH (*p* < 0.001), high expression of CD38 (*p* = 0.002) and ZAP70 (*p* = 0.002).

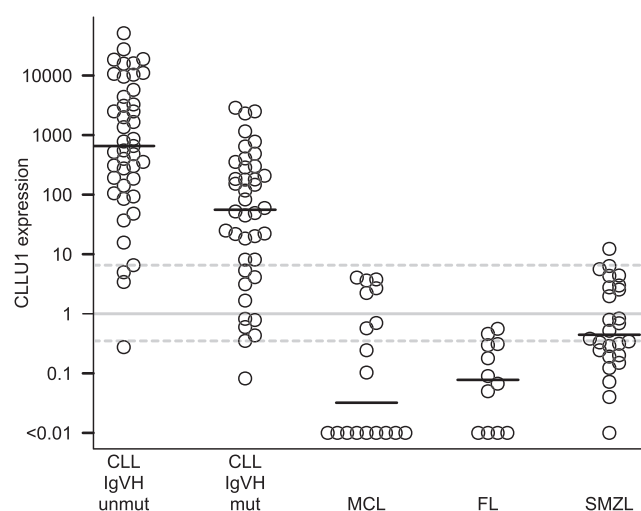
In order to test the utility of CLL1 mRNA expression as a diagnostic biomarker, we analyzed an independent validation set of 9 samples from patients with inconclusive morphologic and immunophenotypic results (Table 2). Six cases with CLL1 values above or below the cut-off levels allowed a correct allocation to the diagnostic entities of CLL and other mature B-cell neoplasms, as defined by subsequent analysis of additional diagnostic markers

or bone marrow biopsies and/or follow-up data where available. Three patients had intermediate CLL1 values.

In the following two cases are presented in detail:

**Patient 1:** A 61 year-old male patient presented with a lymphadenopathy and a lymphocytosis of 5.4 G/l. The lymphocytes were immunophenotypically characterized as monoclonal B-cells with lambda light chain restriction expressing CD19 (dim), CD20, CD79b, CD38, CD25 and surface IgG, with a partial expression of FMC-7 (dim), CD23 (dim), CD11c and a coexpression of CD5 and CD43. CD10 was negative. Based on the CD5 positivity and a Matutes's CLL score of 2 of 5 points, a differential diagnosis of MCL or atypical CLL was made. The CLL1 mRNA expression level was 123. This result is highly suggestive for the diagnosis of a CLL. In line with this, PCR was negative for *t*(11;14).

**Patient 7:** A 64 year-old male patient presented with splenomegaly and lymphadenopathy without other symptoms and a lymphocytosis of 11 G/l. The immunophenotype showed monoclonal CD5 negative B-cells expressing CD19, CD20, CD23, CD25, IgM and kappa light-chains, with a partial expression of CD79b, CD38, CD43 and FMC7. Despite CD5-negativity, the morphology and the CLL score of 3 of 5 points suggested the diagnosis of an atypical CLL. A SMZL or a lymphoplasmacytic lymphoma was discussed as differential diagnoses. CLL1 mRNA expression was 0.04,



**Fig. 1.** Comparison of relative CLL1 mRNA expression levels in CLL and mature B-cell neoplasms. CLL1 expression indicates fold expression compared to normal B-cells (continuous line). MCL, mantle cell lymphoma; FL, follicular lymphoma; SMZL, splenic B-cell marginal zone lymphoma. A value of 6.54 fold or greater predicts a CLL with a probability of 99% (upper dashed line) and a value below 0.35 fold predicts a mature B-cell neoplasm with a probability of 94% (lower dashed line). The horizontal black bars indicate the median expression level for each sample type.

**Table 2**  
 CLLU1 mRNA expression in patients with inconclusive diagnosis.

Patient No.	Age at Diagnosis, y	Sex	Reason for assignment	Material	Tumor Cells %	Immuno-phenotype: Matutes score	Differential diagnosis	CLLU1 value	Allocation based on CLLU1 value	Confirmatory or excluding findings	Additional findings	Most probable diagnosis
1	61	m	Lymphadenopathy, lymphocytosis	PBL	55	2/5	MCL or atypical CLL	122.9	CLL	t(11;14) negative by PCR.	IgVH mutated	CLL
2	50	m	Lymphocytosis, splenomegaly	PBL	85	2/5	Atypical CLL or MCL	3.09	Not conclusive	t(11;14) negative by PCR, Cyclin D1 negative, ZAP70 positive, del13q14. 7 years later: progressive splenomegaly and stable lymphocytosis.	IgVH mutated	Not conclusive
3	75	m	Thrombocytopenia and leukopenia	BM	17	2/5	Mature B-cell neoplasm, SMZL	1.11	Not conclusive	Normal cytogenetics 4 ys later: splenectomy. Histological diagnosis of SZLM.		Not conclusive
4	43	m	Lymphocytosis	PBL	50	3/5	Atypical CLL or MCL	0.64	Not conclusive	Trisomy 12, del(14)(q24q32).	IgVH mutated	Not conclusive
5	42	m	Leukocytosis, asymptomatic, familial CLL	PBL	60	1/5	SMZL, MCL or CLL	0.33	Mature B-cell neoplasm excluding CLL	t(11;14) negative by PCR, bone marrow biopsy typical for SMZL, hepatomegaly and splenomegaly.	IgVH mutated	SMZL
6	71	m	Isolated lymphocytosis, splenomegaly	PBL	85	1/5	SMZL or atypical CD5-MCL	0.15	Mature B-cell neoplasm excluding CLL	Cyclin D1 negative, 2 M-gradients, bone marrow biopsy typical for SMZL, splenomegaly.		SMZL
7	64	m	Lymphocytosis	PBL	73	3/5	Atypical CLL, SLL or SMZL	0.04	Mature B-cell neoplasm excluding CLL	bone marrow biopsy consistent with SMZL, splenomegaly.	IgVH mutated	SMZL
8	65	m	Lymphocytosis	PBL	93	1.5/5	CLL, B-PLL or SMZL	<0.01	Mature B-cell neoplasm excluding CLL	del17p13, del13q14, t(11;14) and t(14;18) negative by PCR; CD23 negativity.		B-PLL
9	63	m	Lymphadenopathy	PBL	58	3/5	MCL, CLL or SMZL	<0.01	Mature B-cell neoplasm excluding CLL	t(11;14) negative by PCR. Diagnosis of SMZL in lymph node biopsy.		SMZL

CLLU1 values of 6.54 and higher were assigned to the diagnosis of CLL, values of less than 0.35 were assigned to the diagnostic group of mature B-cell neoplasms excluding CLL and values of 0.35–6.53 were considered as not conclusive. PBL, peripheral blood; BM, bone marrow; MCL, mantle cell lymphoma; CLL, chronic lymphocytic leukemia; IgVH, variable gene of immunoglobulin heavy chain; SMZL, splenic B-cell marginal zone lymphoma; SLL, small lymphocytic lymphoma; B-PLL, B-cell prolymphocytic leukemia.

thereby excluding a CLL with high probability. *t*(11;14) was negative by molecular analysis. The results of the bone marrow biopsy were typical for the diagnosis of SMZL.

In summary, we defined two cut-off values and demonstrated the usefulness of the *CLLU1* expression as an additional biomarker to compile the diagnosis of patients with mature B-cell neoplasms. Furthermore, we validated our cut-off values on a set of inconclusive cases and could assign a diagnosis based on *CLLU1* values in 67% of these cases. A larger prospective study is ongoing. We conclude that *CLLU1* analysis by qPCR is a simple, rapid and accurate diagnostic tool that could potentially facilitate the differential diagnosis of B-LPD, especially in cases where known diagnostic parameters such as the Matutes's score for CLL and immunohistochemistry for other mature B-cell neoplasms according to the diagnostic work-up of the WHO classification fail to establish the diagnosis.

#### Conflict of interest statement

The authors declare no competing financial interests.

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**Contributors.** EOL and GMB designed the study, coordinated the research and wrote the paper; DR, DB and NP performed the experiments and interpreted the data, SZ, AR and JD collected the clinical data and were involved in the management of patients and selection of cases, DK provided the statistical analysis.

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