Published in final edited form as:
Eur J Haematol. 2011 May ; 86(5): 405–411. doi:10.1111/j.1600-0609.2011.01588.x.

Analysis of *CLLU1* expression levels before and after therapy in patients with chronic lymphocytic leukemia

Anne Mette Buhl[#1,2], **Danelle F. James**[#2,3], **Donna Neuberg**[3,4], **Sonia Jain**[5], **Laura Z. Rassenti**[2,3], and **Thomas J. Kipps**[2,3]

[1]Department of Hematology, Rigshospitalet, Copenhagen, Denmark

[2]Division of Hematology/Oncology, Department of Medicine, Moores UCSD Cancer Center, University of California San Diego, La Jolla, CA

[3]The CLL Research Consortium, Dana-Farber Cancer Institute, Boston, MA

[4]Department of Biostatics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA

[5]Division of Biostatistics and Bioinformatics, UCSD, La Jolla, CA, USA

[#] These authors contributed equally to this work.

Abstract

Objective—Chronic lymphocytic leukemia (CLL) is incurable, but therapy leading to eradication of minimal residual disease (MRD) in CLL is associated with improved clinical outcomes. CLL upregulated gene 1 (*CLLU1*) is solely upregulated in CLL patient samples. We hypothesized that *CLLU1* could be used to monitor for residual disease in CLL patient samples after therapy.

Methods—We examined whether the *CLLU1* real-time quantitative PCR (RQ-PCR) could detect small numbers of CLL cells in mixtures of normal peripheral blood mononuclear (PBMC) cells. We then performed a retrospective analysis on time-matched cryopreserved specimens from patients who achieved MRD-negative remissions that underwent serial marrow biopsies for evaluation of residual disease by 4-color flow cytometry. RNA from PBMC samples collected at the time of the marrow assessments was analyzed for *CLLU1*. Nine patients underwent a total of 46 paired blood and marrow evaluations (median 5 assessments per patient).

Results—*CLLU1* RQ-PCR on PBMCs of healthy donors reconstituted with varying amounts of CLL cells demonstrated leukemia cells could be reliably detected with high sensitivities depending on the *CLLU1* expression level. Analysis of time-matched samples assessed for *CLLU1*

Correspondence Dr. Anne Mette Buhl, Department of Hematology, L4041, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. Tel: +45 35454045; Fax: +45 35454295; annemette.buhl@gmail.com.

Author contributions

A.M. Buhl designed the study, performed the experiments, and wrote the manuscript, D. James designed the study, interpreted the clinical data, and wrote the manuscript, D. Neuberg interpreted data and assisted with writing the manuscript, S. Jain performed statistical analysis, L. Rassenti identified samples and provided clinical data for the study, T. Kipps designed the study, interpreted data, and wrote the manuscript.

Disclosures

All authors declare no competing financial interest.

levels in the blood by RQ-PCR and residual disease of the marrow determined by 4-color flow cytometry revealed a correlation coefficient of 0.96 ($P < 0.0001$).

Conclusion—The *CLLU1* RQ-PCR is a sensitive and specific assay for detecting residual CLL cells after therapy. Assessment of blood *CLLU1* levels can be used as a reliable marker of tumor burden and has the potential to complement currently used techniques for MRD monitoring in patients with CLL.

Keywords

chronic lymphocytic leukemia; minimal residual disease; real-time quantitative PCR; chronic lymphocytic leukemia upregulated gene 1

The chronic lymphocytic leukemia upregulated gene 1 (*CLLU1*) gene on chromosome 12q22 was identified in the search of genes differentially expressed between chronic lymphocytic leukemia (CLL) cases with mutated immunoglobulin heavy chain variable region genes (IGHV) and unmutated IGHV. The expression pattern of the *CLLU1* gene is highly restricted, because only CLL cells express elevated levels of *CLLU1* (1–3). *CLLU1* levels in the CLL patient population range from low levels, comparable to the levels found in normal B-cells, to several thousandfold higher (4, 5). *CLLU1* is a prognostic marker in CLL, as high expression levels are associated with shorter time to treatment and poor overall survival in patients (4–6), and correlates with poor-risk prognostic markers such as leukemia-cell use of unmutated IGHV and high-level expression of ZAP-70 (3, 4, 6, 7).

Advances in the therapy of patients with CLL have led to higher complete response (CR) rates, prolonged response duration, and in some cases eradication of detectable minimal residual disease (MRD). Irrespective of treatment regimen, patients who achieve a CR with elimination of MRD, as assessed by quantitative PCR (RQ-PCR) for leukemia-cell IGHV (8), or multiparametric flow cytometry (9) have significantly better progression free and overall survival than patients who have achieved a CR, but have persistent MRD (10–16). Hence, achievement of a MRD-negative CR has become a desirable treatment endpoint (17, 18), and ways to maintain MRD-negativity at the end of induction therapy are being investigated, with monoclonal antibodies or immune modulating compounds being tested in ongoing trials. Moreover, some have advocated for studies investigating the value of early therapy for progressive MRD (10, 12, 17) prior to development of clinical manifestations that define the current basis for recommending treatment (18, 19).

Previously, we demonstrated that *CLLU1* levels in serial CLL samples of untreated patients were stable over time and that the blood and marrow samples from the same untreated patient collected on the same date expressed similar levels of *CLLU1* (20). Moreover, the CLL cells of any one patient had uniform expression of *CLLU1* as assessed by *in situ* hybridization (20). Furthermore, CLL cells collected from patients who had relapsed from therapy expressed the same level of *CLLU1* as that noted for CLL cells collected from the same patient prior to therapy (20). However, it was not known whether the blood mononuclear cells collected during and following therapy expressed *CLLU1* and whether detection of *CLLU1* could be used to monitor tumor burden and assess for MRD.

Patients and methods

Sample collection and flow cytometric analysis

This research was approved by the UCSD Human Research Protections program and conducted in accordance with the Declaration of Helsinki. For evaluation of the relation between *CLLU1* expression levels in blood and marrow of treated patients with CLL, 17 sets of paired samples were collected from 13 different patients. For one patient, three sets were collected from different dates, from two additional patients, two sets of samples from separate dates were used, and the remaining ten samples were from independent patients. To determine the *CLLU1* background level and establish a cutoff, we purified peripheral blood mononuclear cells (PBMC) from 15 healthy donors. For our retrospective analysis on time-matched blood specimens from patients with CLL who had marrow aspirate samples evaluated for disease by 4-color flow cytometry, we collected 46 paired samples from nine different patients (median 5 samples per patient). Blood samples were considered appropriate for analysis if drawn within 3 months of the marrow assessment without intervening therapy. Flow cytometry was performed at time of marrow evaluation. Four-color flow cytometry on aspirated marrow specimens was performed by evaluating for CD5, CD19, CD20, and CD79b expression as previously described (12). Patients with <0.1% residual CLL cells in the marrow who also satisfied criteria for CR by NCI-WG criteria (18) were designated as having achieved a MRD-negative CR. IGHV mutational status, ZAP-70 levels, and CD38 levels were determined as previously described (21). A patient sample was considered ZAP-70 positive if the percentage of CLL cells expressing ZAP-70 was >20%, and a patient sample was classified as CD38 positive, when 30% or more of the CLL cells had fluorescence above the background-fluorescence threshold.

RQ-PCR analysis for *CLLU1* expression

CLLU1 RQ-PCR was performed as previously described (4). RNA was extracted from cryopreserved PBMCs or marrow mononuclear cells using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA) with on-column DNAse treatment. Samples (100 ng RNA per reaction if possible, at least 10 ng RNA per reaction) were analyzed for *CLLU1* expression (cDNA1 splice variant) in triplicates using one-step real-time RQ-PCR reagent (#4309169; Applied Biosystems, Foster City, CA, USA) by the comparative Ct method of relative quantification with *β2-microglobulin* (*β2M*) as endogenous control. A pool of purified normal B-lymphocytes was used as calibrator to express the level of *CLLU1* in the samples relative to the level found in normal B-lymphocytes, which was assigned the value of *CLLU1* RQ = 1. Analysis was performed using an ABI 7000 system (Applied Biosystems Inc., Carlsbad, CA, USA) with the threshold line set at 0.1. Samples with poor RNA quality, identified by a *β2M* Ct value above 24, were excluded from the analysis. For *CLLU1* Ct values below 35, a single reaction deviating more than one Ct value form the remaining two reactions of the triplicate was excluded from the mean. Because reactions with a low RNA copy number are prone to a higher variation within the triplicate, for Ct values above 35, all three reactions were included in the mean value determination, irrespective of Ct value differences. This influenced the evaluation of two samples, in which one and two, respectively, of the triplicate *CLLU1* Ct values were not detectable. The not detectable values were set as Ct 40, and the mean of the detected and not detected Ct values was used

as the mean *CLLU1* Ct value. For sequential analysis within patients, subsequent samples were normalized to the pre-treatment *CLLU1* level.

Statistical analysis

Between-subjects correlation analysis (22) was performed to evaluate *CLLU1* levels in paired marrow and blood samples following therapy. For the between-subjects correlation analysis, the number of observations was used as weight. To determine whether a change in the quantity of CLL cells in the marrow was associated with a change in blood *CLLU1* levels within individual subjects monitored sequentially, we studied within-subject correlation via multiple linear regression and analysis of variance methods (23). For the purpose of graphing and statistical analysis, MRD results by 4-color flow cytometry below 0.1% residual cells were assigned a value of 0.05%, reflecting half the detection limit for the assay (24).

Results

We first examined 17 paired blood and marrow samples from treated patients with CLL. Time-matched blood and marrow CLL cells in each pair expressed virtually the same levels of *CLLU1* ($r = 0.997$, $P < 0.0001$; Fig. 1A). Therefore, as blood is a more easily accessible compartment, we focused subsequent analyses on peripheral blood samples.

To determine the background level of *CLLU1*, we next evaluated PBMCs of fifteen healthy donors and found these cells had very low to negligible expression of *CLLU1* (Fig. 1B), the mean *CLLU1* expression level ± standard deviation being 0.14 ± 0.09. Based on these results, we estimated that a *CLLU1* value of *CLLU1* RQ = 0.32, corresponding to two standard deviations above the mean *CLLU1* value found in PBMCs of healthy donors, would be suitable as cutoff value to distinguish CLL cells in a sample from normal hematopoietic cells. Hence, the expected sensitivities of the *CLLU1* assay, based on the patients *CLLU1* pretreatment level, could be predicted as outlined in Fig. 1B (dotted lines in Fig. 1B).

We then performed reconstitution experiments to determine whether the *CLLU1* assay could be used to detect small numbers of CLL cells admixed with PBMCs from healthy donors. CLL cells isolated from various patients were mixed with PBMCs from healthy donors in proportions ranging from 0.01% to 100%. As shown in Fig. 1C–E, these dilution experiments revealed that we could detect CLL cells in normal leucocytes at ratios down to 10^{-4}, dependent on the *CLLU1* expression level in the given patient sample.

To evaluate whether the *CLLU1* level remains stable in the context of therapy, we examined the blood samples of nine patients who received treatment for progressive CLL and achieved a MRD-negative CR as assessed by 4-color flow cytometry analyses on marrow mononuclear cells. Six patients were treated with the combination of high-dose methylprednisolone and rituximab, and five of these patients had consolidation therapy with alemtuzumab all achieving MRD-negative CRs (12). Three patients received fludarabine-based regimens followed by alemtuzumab consolidation therapy, similarly attaining MRD-negative CRs (patient characteristics are provided in Table 1). All patients had serial marrow

biopsies to monitor MRD by 4-color flow cytometry on the aspirated samples. Forty-six time-matched blood samples were assessed for *CLLU1*, and *CLLU1* values were normalized to pretreatment levels. Figure 2 shows the profiles of the reduction in disease burden after treatment as monitored by 4-color flow cytometry of marrow mononuclear cells (right axes, solid lines) and the relative *CLLU1* expression level in the blood (left axes, dotted lines). Detection of increasing *CLLU1* levels in the blood over time after therapy was associated with detection of increasing levels of residual disease as assessed by 4-color flow cytometry on the marrow, often in the absence of clinically detectable disease. Evaluation of the association between the change in blood *CLLU1* levels and the change in residual disease as determined by 4-color flow cytometry of the marrow within each subject (median 5 assessments per patient) provided a correlation coefficient of 0.96 ($P < 0.0001$).

Discussion

High expression of the *CLLU1* gene is restricted to CLL, and we have previously demonstrated that the *CLLU1* level within the leukemic clone is constant in untreated CLL samples (20). We therefore hypothesized that the *CLLU1* expression level would be constant during treatment and following the completion of therapy.

First, we performed dilution experiments that confirmed the *CLLU1* QRT-PCR could detect few CLL cells in a mixture of normal PBMCs, with the assay sensitivity being dependent on the *CLLU1* expression level of the given sample.

Next, we performed longitudinal analysis on time-matched blood and marrow samples from nine different patients with CLL, who received antibody or purine-analog-based therapy and achieved MRD-negative remissions. We found that the relative *CLLU1* expression level on blood samples mirrored the residual level of CLL cells as determined by 4-color flow cytometry on cells from the aspirated marrow. This strong correlation (correlation coefficient 0.96, $P < 0.0001$) confirms that the *CLLU1* expression level within CLL cells does not change in the context of different therapies and that normal cells do not aberrantly express *CLLU1* at any point in time during hematopoietic reconstitution following therapy. Hence, the *CLLU1* expression level is an intrinsic and constant parameter of the CLL clone. This observation suggests that monitoring for expression of *CLLU1* might provide a reliable means with which to evaluate residual disease in the context of therapy.

The *CLLU1* assay will be most useful for patients with CLL that express high levels of *CLLU1*. As demonstrated in Fig. 2, the *CLLU1* test is informative for patients with CLL with high and intermediate *CLLU1* values (patients CLL1–7, *CLLU1* RQ range 23–1734), whereas, for patients with low *CLLU1* values (patients CLL 8–9, *CLLU1* RQ range 1.3–3.5), the *CLLU1* test has limited use. Reflecting on the *CLLU1* distribution in a previously characterized cohort of untreated CLL patients (5), we predict the *CLLU1* assay to be very useful for about half of all patients with CLL. Prior studies indicate that patients with CLL with high *CLLU1* expression have shorter time intervals from diagnosis to initial therapy and decreased overall survival (4–6). Furthermore, high *CLLU1* expression has been clearly linked to other poor prognostic markers in CLL such as unmutated IGHV and ZAP-70 positivity (3, 4, 7). Several groups have demonstrated that patients with leukemia cells that

express unmutated IGHV genes experience early relapse following chemoimmunotherapy (15, 25, 26). Therefore, the patients with CLL most likely to require early and repetitive treatment will more often have high *CLLU1* expression levels. These high-risk patients are most likely to benefit from improved monitoring of leukemia following treatment that will allow for identification of early relapse and easier incorporation of post remission treatment strategies such as consolidation or maintenance therapies. Because of the simplicity of the *CLLU1* test and the fact that it can be performed on a blood sample, we envision this test could complement current techniques used for disease monitoring in CLL. In analogy with acute myeloid leukemia, where the WT1 gene is used as a disease marker following therapy (27), patients with CLL with high *CLLU1* expression can be monitored by the *CLLU1* RQ-PCR following treatment. A RQ-PCR assay to monitor residual disease could be particularly useful in institutions that have access to general clinical biochemistry labs, but not to specialized hematological flow facilities.

Conceivably, the *CLLU1* blood assay could be incorporated into future studies designed to monitor the molecular response to treatment including consolidation or maintenance strategies that may ultimately lead to a better understanding of the relationship between the kinetics of the treatment response, disease relapse, and the long-term outcome.

Acknowledgments

The authors would like to thank the CRC Tissue Core, E. Avery for technical assistance, F. He for data analysis, M. Cook for assistance with database searches, L. B. Pedersen for helpful discussions, and C. Geisler for comments to the manuscript. This work was supported by grants from The Alfred Benzon Foundation, the CLL Research Consortium (CRC) and NIH PO1-CA081534. DFJ is supported by a Lymphoma Research Foundation Fellowship and a TACF-ASP Young Investigator Award.

References

1. Buhl AM, Jurlander J, Jorgensen FS, Ottesen AM, Cowland JB, Gjerdrum LM, Hansen BV, Leffers H. Identification of a gene on chromosome 12q22 uniquely overexpressed in chronic lymphocytic leukemia. Blood. 2006; 107:2904–11. [PubMed: 16339396]
2. Oppliger Leibundgut E, Dissler D, de Beer D, Marti D, Schuller JC, Duerig J, Roeth A, Baerlocher GM. CLLU1 expression distinguishes B-cell chronic lymphocytic leukemia from other B-cell lymphoproliferative disorders. Haematologica. 2007; 92(Suppl 1):0522.
3. Roumelioti M, Palaiologou D, Stravodimos G, et al. Overexpression of CLLU1 gene: a unique characteristic of unmutated CLL lymphocytes. Haematologica. 2009; 93(s3):S35.
4. Buhl AM, Jurlander J, Geisler CH, Pedersen LB, Andersen MK, Josefsson P, Petersen JH, Leffers H. CLLU1 expression levels predict time to initiation of therapy and overall survival in chronic lymphocytic leukemia. Eur J Haematol. 2006; 76:455–64. [PubMed: 16529606]
5. Josefsson P, Geisler CH, Leffers H, Petersen JH, Andersen MK, Jurlander J, Buhl AM. CLLU1 expression analysis adds prognostic information to risk prediction in chronic lymphocytic leukemia. Blood. 2007; 109:4973–9. [PubMed: 17284524]
6. Kaderi MA, Kanduri M, Mansouri M, et al. LPL is the strongest prognostic factor in a comparative study of RNA-based markers in chronic lymphocytic leukemia. Blood (ASH Annual Meeting Abstracts). 2009; 114:1254.
7. Chen L, Li J, Zheng W, Zhang Y, Wu Y, Li L, Qian S, Xu W. The prognostic evaluation of CLLU1 expression levels in 50 Chinese patients with chronic lymphocytic leukemia. Leuk Lymphoma. 2007; 48:1785–92. [PubMed: 17786715]

8. Bottcher S, Stilgenbauer S, Busch R, et al. Standardized MRD flow and ASO IGH RQ-PCR for MRD quantification in CLL patients after rituximab-containing immunochemotherapy: a comparative analysis. Leukemia. 2009; 23:2007–17. [PubMed: 19641522]
9. Rawstron AC, Villamor N, Ritgen M, et al. International standardized approach for flow cytometric residual disease monitoring in chronic lymphocytic leukaemia. Leukemia. 2007; 21:956–64. [PubMed: 17361231]
10. Varghese AM, Rawstron AC, Hillmen P. Eradicating minimal residual disease in chronic lymphocytic leukemia: should this be the goal of treatment? Curr Hematol Malig Rep. 2010; 5:35–44. [PubMed: 20425395]
11. Tam CS, O'Brien S, Wierda W, Kantarjian H, Wen S, Do KA, Thomas DA, Cortes J, Lerner S, Keating MJ. Long-term results of the fludarabine, cyclophosphamide, and rituximab regimen as initial therapy of chronic lymphocytic leukemia. Blood. 2008; 112:975–80. [PubMed: 18411418]
12. Castro JE, James DF, Sandoval-Sus JD, Jain S, Bole J, Rassenti L, Kipps TJ. Rituximab in combination with high-dose methylprednisolone for the treatment of chronic lymphocytic leukemia. Leukemia. 2009; 23:1779–89. [PubMed: 19693094]
13. Moreton P, Kennedy B, Lucas G, et al. Eradication of minimal residual disease in B-cell chronic lymphocytic leukemia after alemtuzumab therapy is associated with prolonged survival. J Clin Oncol. 2005; 23:2971–9. [PubMed: 15738539]
14. Schweighofer CD, Ritgen M, Eichhorst BF, Busch R, Abenhardt W, Kneba M, Hallek M, Wendtner CM. Consolidation with alemtuzumab improves progression-free survival in patients with chronic lymphocytic leukaemia (CLL) in first remission: long-term follow-up of a randomized phase III trial of the German CLL Study Group (GCLLSG). Br J Haematol. 2009; 144:95–8. [PubMed: 19016732]
15. Hallek M, Fischer K, Fingerle-Rowson G, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. Lancet. 2010; 376:1164–74. [PubMed: 20888994]
16. Kwok M, Rawstron AC, Varghese A, Hillmen P. Minimal residual disease is a predictor for progression-free and overall survival in chronic lymphocytic leukemia (CLL) that is independent of the type or line of therapy. Blood (ASH Annual Meeting Abstracts). 2009; 114:540.
17. Montserrat E. Treatment of chronic lymphocytic leukemia: achieving minimal residual disease-negative status as a goal. J Clin Oncol. 2005; 23:2884–5. [PubMed: 15738532]
18. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood. 2008; 111:5446–56. [PubMed: 18216293]
19. Binet JL, Caligaris-Cappio F, Catovsky D, et al. Perspectives on the use of new diagnostic tools in the treatment of chronic lymphocytic leukemia. Blood. 2006; 107:859–61. [PubMed: 16223776]
20. Buhl AM, Novotny GW, Josefsson P, Nielsen JE, Pedersen LB, Geisler C, Rassenti LZ, Kipps TJ, Jurlander J, Leffers H. The CLLU1 expression level is a stable and inherent feature of the chronic lymphocytic leukemia clone. Leukemia. 2009; 23:1182–6. [PubMed: 19212335]
21. Rassenti LZ, Jain S, Keating MJ, et al. Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia. Blood. 2008; 112:1923–30. [PubMed: 18577710]
22. Bland JM, Altman DG. Calculating correlation coefficients with repeated observations: part 2 - correlation between subjects. BMJ. 1995; 310:633. [PubMed: 7703752]
23. Bland JM, Altman DG. Calculating correlation coefficients with repeated observations: part 1 - correlation within subjects. BMJ. 1995; 310:446. [PubMed: 7873953]
24. Baccarelli A, Pfeiffer R, Consonni D, Pesatori AC, Bonzini M, Patterson DG Jr, Bertazzi PA, Landi MT. Handling of dioxin measurement data in the presence of non-detectable values: overview of available methods and their application in the Seveso chloracne study. Chemosphere. 2005; 60:898–906. [PubMed: 15992596]
25. Byrd JC, Gribben JG, Peterson BL, Grever MR, Lozanski G, Lucas DM, Lampson B, Larson RA, Caligiuri MA, Heerema NA. Select high-risk genetic features predict earlier progression following

chemoimmunotherapy with fludarabine and rituximab in chronic lymphocytic leukemia: justification for risk-adapted therapy. J Clin Oncol. 2006; 24:437–43. [PubMed: 16344317]

26. Lin KI, Tam CS, Keating MJ, et al. Relevance of the immunoglobulin VH somatic mutation status in patients with chronic lymphocytic leukemia treated with fludarabine, cyclophosphamide, and rituximab (FCR) or related chemoimmunotherapy regimens. Blood. 2009; 113:3168–71. [PubMed: 19050308]
27. Cilloni D, Renneville A, Hermitte F, et al. Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute myeloid leukemia: a European LeukemiaNet study. J Clin Oncol. 2009; 27:5195–201. [PubMed: 19752335]

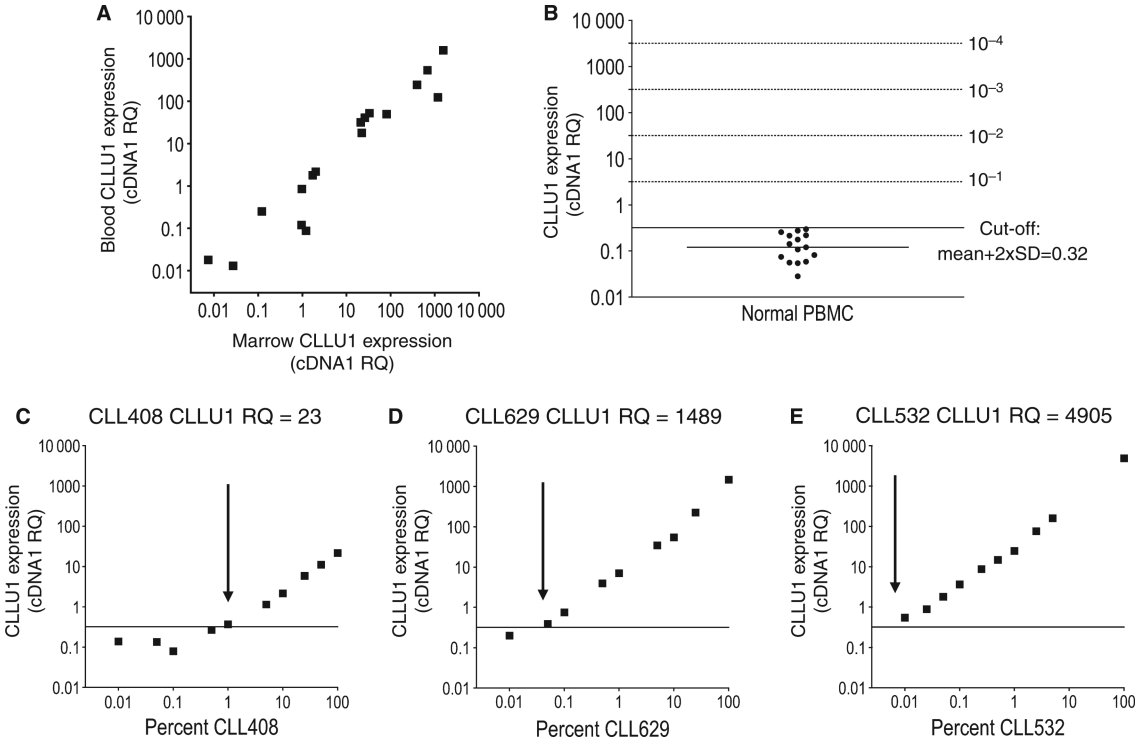

Figure 1.

(A) Paired blood and marrow samples were collected from patients with chronic lymphocytic leukemia (CLL) after treatment (n = 17). CLL upregulated gene 1 (*CLLU1*) expression was determined using RQ-PCR (cDNA1 splice variant). Bland and Altman between samples correlation analysis demonstrated a strong correlation between the *CLLU1* levels in the two tissues (correlation coefficient = 0.997, $P < 0.0001$). (B) Determination of the background *CLLU1* expression level in normal peripheral blood mononuclear cells (PBMCs). Shown is the *CLLU1* expression level of 15 healthy donor samples with a line at the mean, *CLLU1* RQ = 0.14, and the cutoff value (line across graph) representing the mean *CLLU1* of normal PBMCs + two SD = 0.32. The dotted lines and the associated numbers delineate the predicted *CLLU1* assay sensitivity for a patient with a pretreatment *CLLU1* expression level at or above the level marked by the line. (C–E) Dilution curves of purified CLL cells diluted into normal PBMCs. The straight line reflects the *CLLU1* cutoff value (see above) that allows for distinguishing CLL cells from normal PBMCs. Arrows mark the level of sensitivity reached using the *CLLU1* MRD assay for the individual patient samples.

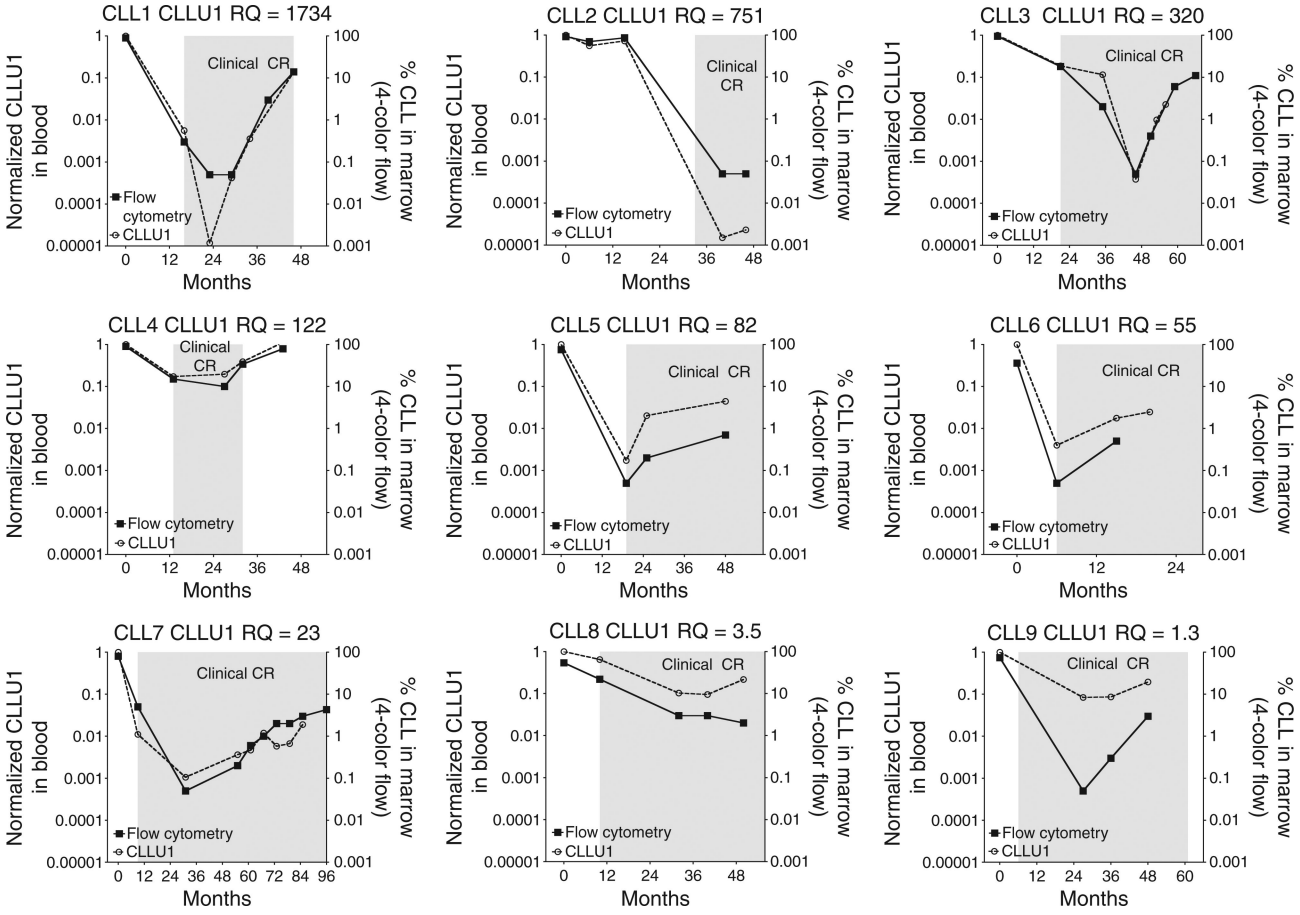

Figure 2.

Nine patients with chronic lymphocytic leukemia (CLL) were evaluated for minimal residual disease (MRD) by 4-color flow cytometry on aspirated marrow samples (right axes, black squares, solid lines) and relative CLL upregulated gene 1 (*CLLU1*) expression on blood samples (left axes, open circles, dotted lines). For two patients (CLL4 and CLL8), samples from the time of MRD-negative complete response were not available for *CLLU1* analysis. The gray shaded area denotes the time during follow-up when the patients remain in clinical complete remission (no clinically detectable leukemia by physical exam, in conjunction with a normal absolute lymphocyte count, hemoglobin, and platelet count).

Table 1

Overview of clinical characteristics for the nine patients with CLL monitored for residual disease by 4-color flow cytometry of the marrow and blood *CLLU1* expression analysis

ID	Age	*CLLU1*	IGHV	ZAP70	CD38	*β*2M	Treatment	Response	Consolidation	Final response
CLL1	48	1734	Unmut	Pos	Pos	2	FCR	MRD+ CR	Alem	MRD– CR
CLL2	62	751	Unmut	Pos	Pos	2.2	HDMP-R, later FR	PR	Alem	MRD– CR
CLL3	56	320	Unmut	Neg	Pos	4.7	HDMP-R	PR	Alem	MRD– CR
CLL4	62	122	Unmut	Pos	Neg	2.5	HDMP-R	MRD+ CR	Alem	MRD– CR
CLL5	54	82	Mut	Neg	Neg	2.2	HDMP-R	MRD+ CR	Alem	MRD– CR
CLL6	61	55	Mut	Neg	Neg	1.4	HDMP-R	MRD– CR	None	MRD– CR
CLL7	62	23	Mut	Neg	Neg	2.8	F	MRD+ CR	Alem	MRD– CR
CLL8	70	3.5	Mut	Neg	Neg	2.7	HDMP-R	PR	Alem	MRD– CR
CLL9	53	1.3	Mut	Neg	Neg	1.4	HDMP-R	PR	Alem	MRD– CR

Unmut, unmutated; Mut, mutated; Pos, positive; Neg, negative; *β*2M, soluble β2-microglobulin (mg/L); HDMP-R, high-dose methylprednisolone and rituximab; F; Fludarabine; FR, fludarabine and rituximab; FCR, fludarabine, cyclophosphamide, and rituximab; Alem, Alemtuzumab; MRD, minimal residual disease; MRD+ CR, MRD positive complete remission; MRD– CR, MRD negative complete remission; PR, partial remission; CLL, chronic lymphocytic leukemia; *CLLU1*, CLL upregulated gene 1; IGHV, immunoglobulin heavy chain variable region genes.