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The *CLLU1* expression level is a stable and inherent feature of the chronic lymphocytic leukemia clone

AM Buhl[1,2], **GW Novotny**[3], **P Josefsson**[1], **JE Nielsen**[3], **LB Pedersen**[1], **C Geisler**[1], **LZ Rassenti**[2,4], **TJ Kipps**[2,4], **J Jurlander**[1], and **H Leffers**[3]

[1]The Leukemia Laboratory, Department of Hematology, Rigshospitalet, Copenhagen, Denmark

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

[3]University Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark

[4]The CLL Research Consortium, La Jolla, CA, USA

Although most leukemias harbor a common genetic aberration, useful for the classification and subgrouping of the disease, no common molecular event leading to chronic lymphocytic leukemia (CLL) has been described. *CLLU1* was identified in a comparative screen of patients with and without somatic hypermutations in the variable region of their immunoglobulin receptor heavy chain genes (IgV_H). The expression of *CLLU1* was shown to be highly upregulated in patients without somatic hypermutations. Subsequent analysis showed that *CLLU1* upregulation can be detected only in CLL cells, but not in any other tissue, cell line or in samples from patients with other hematological malignancies.[1,2] In earlier studies we found *CLLU1* to have independent prognostic significance in CLL.[3,4] When *CLLU1* was analyzed as a continuous variable, a doubling of the *CLLU1* expression level was shown to be associated with an increase of 7% in the relative risk of early death.[4] Others have since then confirmed the prognostic significance of *CLLU1* in CLL.[5,6] The function of the *CLLU1* gene is unknown. The gene is not conserved in other species and even though several of the *CLLU1* splice variants contain a putative open reading frame of 121 amino acids, we have not yet been able to convincingly detect expression of the putative CLLU1 protein in CLL patient samples. Thus, we do not know whether *CLLU1* plays a role in the pathogenesis of CLL, or whether its expression is a reflection of other events in the CLL cells.

The goal of this study was to further investigate the biological properties of the *CLLU1* mRNAs. We first developed specific QRT-PCR primer sets to measure the relative expression levels of the different *CLLU1* splice variants in samples from 30 CLL patients (Primer and probe sequences are in Supplementary Table 1.) (Figure 1a). The expression pattern of cDNA4/cDNA5 was earlier shown to mirror that of cDNA1,[3] and as the cDNA4/cDNA5 Coding Sequence (CDS) PCR reaction does not amplify across an exon–intron boundary, it was not included here. For 25 of the 30 samples we obtained readings in all three QRT-PCR reactions, but in 5 cases we were only able to detect expression of the

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cDNA1 splice variant. Pair-wise linear regression analysis showed a significant linear relation between the expression levels of all the splice variants ($P < 0.0001$), suggesting that all the splice variants are derived from one primary transcript and regulated by a common promoter upstream from Exon 1 (Figure 1b). As cDNA1 is the most abundantly expressed splice variant (cDNA1 > cDNA3 > cDNA2/4), we used the cDNA1 QRT-PCR for further analyses. Our results suggest that high-risk CLL patients express all *CLLU1* splice variants, whereas low-risk CLL patients may only have detectable expression of the cDNA1 splice variant. The poor prognostic status associated with high *CLLU1* expression levels may be a consequence of selective expression of certain *CLLU1* splice variants, such as cDNA2/4. Alternatively, the spectrum of outcomes could be the result of a dosage effect incurring a function of the *CLLU1* cDNA1 splice variant that is expressed in all CLL samples.

To determine whether *CLLU1* upregulation is a specific feature of CLL cells only, we isolated CD3 + cells (T cells) and CD19 + cells (B cells and CLL cells) from two CLL patient samples, and determined their *CLLU1* expression level. In the first sample (UPN100), CLL cells constituted 96% of the CD19 + -purified cells and the *CLLU1* expression level was 218. T cells constituted 90% of the CD3 + -selected sample and this sample had a *CLLU1* expression value of 10.3. For UPN 600 the CD19-selected sample had 97% CLL cells and the *CLLU1* expression level was 5239. In contrast, the T-cell population constituted 87% of the CD3-selected sample, which had a *CLLU1* expression value of 74. These results suggest that expression of *CLLU1* is restricted to the CLL cells. We subsequently identified 15 CLL patients, from whom blood and marrow samples were collected on the same date. The samples were Ficoll separated, and the *CLLU1* expression levels were determined. Linear regression analysis showed that *CLLU1* expression levels were similar in the paired blood and marrow samples (Figure 1c).

The *CLLU1* expression level in a sample may be a result of all CLL cells expressing a similar level of *CLLU1* or a fraction of cells expressing very high levels of *CLLU1*. To investigate this we performed *in situ* hybridization (ISH) on cytospins of Ficoll separated CLL patient samples ($n = 3$). RNA probes were derived by *in vitro* transcription of a 240-bp fragment of Exon 3 and a 278-bp fragment from the CDS region (Figure 2a). The probes were hybridized to cells from CLL patients with high *CLLU1* levels (UPN73 RQ(cDNA1) = 1034 (shown in Figures 2b–g), UPN34 RQ(cDNA1) = 305 and UPN120 RQ(cDNA1) = 324) or to purified normal B-cells. This revealed that the CLL cells had uniform expression of *CLLU1*, as assessed with either probe (Figures 2b and d). In contrast, we did not see any distinct staining resulting from hybridization of the probes to the control normal B-cells (Figures 2c and e). These results suggest that the measured *CLLU1* expression level reflects a common *CLLU1* expression level in all the CLL cells within the clone. The *in situ* hybridization also allowed us to study the cellular localization of the *CLLU1* mRNAs within the CLL cells. The cytoplasm of the CLL cells is very small and localized on one side of the nucleus, as illustrated by the Giemsa-stained CLL cells with distinct dark blue nuclear staining (Figure 2g). We detected a distinct cytoplasmic localization of the *CLLU1* mRNAs, suggesting that the *CLLU1* mRNAs are both spliced and polyadenylated, similar to functional mRNAs (Figure 2f).

To address whether the level of *CLLU1* changes during the course of the disease, we collected blood samples from untreated CLL patients from at least three consecutive time points. The samples were Ficoll separated and *CLLU1* expression levels were determined. Despite the relatively minor fluctuations seen in some samples, all of the patients had low, intermediate or high *CLLU1* levels at all the time points investigated. There were some notable exceptions. One patient presented with one clone expressing mutated IgV_H 3–23 (89.6% homology to germ line), another rearrangement being unmutated IgV_H 1–3 (100% homology to germ line) (closed circles and the dotted line in Figure 3a). At the first time point, at which the *CLLU1* expression level was 204, the IgV_H 3–23 clone was the dominant band in IgV_H analysis. At the later time points the *CLLU1* expression level increased to 1397, and this coincided with the V_H 1–3 band becoming the strongest band in IgV_H analysis, which may be evidence for the presence of two CLL clones in this patient. These results indicate that the *CLLU1* expression level of any given CLL clone is stable over time.

To investigate whether the *CLLU1* expression level is affected by treatment, we identified patients who obtained at least a partial response after treatment, with a decrease in the white blood cell (WBC) count to $\leq 11 \times 10^9 l^{-1}$, but then later had relapsed with a considerable increase in the WBC count (Figure 3b). Patients had received fludarabine, fludarabine and rituximab, or steroids and rituximab, and in some cases Campath maintenance. FACS analysis showed that in all the samples CLL cells constituted at least 84% of the cell population (results not shown). Samples from before treatment and after relapse were analyzed for *CLLU1* expression, and we found that the *CLLU1* expression levels in these samples were similar (Figure 3c). These results show that the *CLLU1* level is an inherent marker of the particular CLL clone, and it is not affected by treatment.

The *CLLU1* assay was initially developed as an RNA-based one-step QRT-PCR analysis. However, a comparative study of 12 CLL patient samples showed that cDNA synthesis followed by QRT-PCR works equally well (Supplementary Figure 1A). We next collected five tubes of blood from 11 CLL patients, and purified and analyzed the samples successively on days 0–4. The *CLLU1* levels were found to be essentially constant over the 5-day period (Supplementary Figure 1B). This suggests that *CLLU1* analysis by QRT-PCR is a robust technique that can be successfully applied under circumstances in which transportation or storage of samples over several days is required.

In this report, we show that the expression level of *CLLU1* is stable in any given CLL clone. First, we find similar levels of *CLLU1* in CLL samples from blood and marrow samples from the same patient. Second, the *in situ* hybridization experiments show that all the CLL cells within the clone express *CLLU1*. Third, in our longitudinal study of *CLLU1* expression in untreated patients we find that *CLLU1* expression is stable over time. Fourth, *CLLU1* expression levels are similar in patient samples before treatment and after relapse; hence a high *CLLU1* level does not appear to be a feature that is acquired by the CLL clone during the course of the disease. These results show that the level of *CLLU1* is a constant and inherent parameter for the CLL clone. As of today, only two molecular CLL clone-specific features are known: the IgV_H structure and the *CLLU1* expression level. These two parameters may be considered the fingerprint of a particular CLL clone. The biological properties associated with different IgV_H structures are currently being unraveled.[7,8] Future

research should help elucidate the potential biological implications of varying *CLLU1* expression levels.

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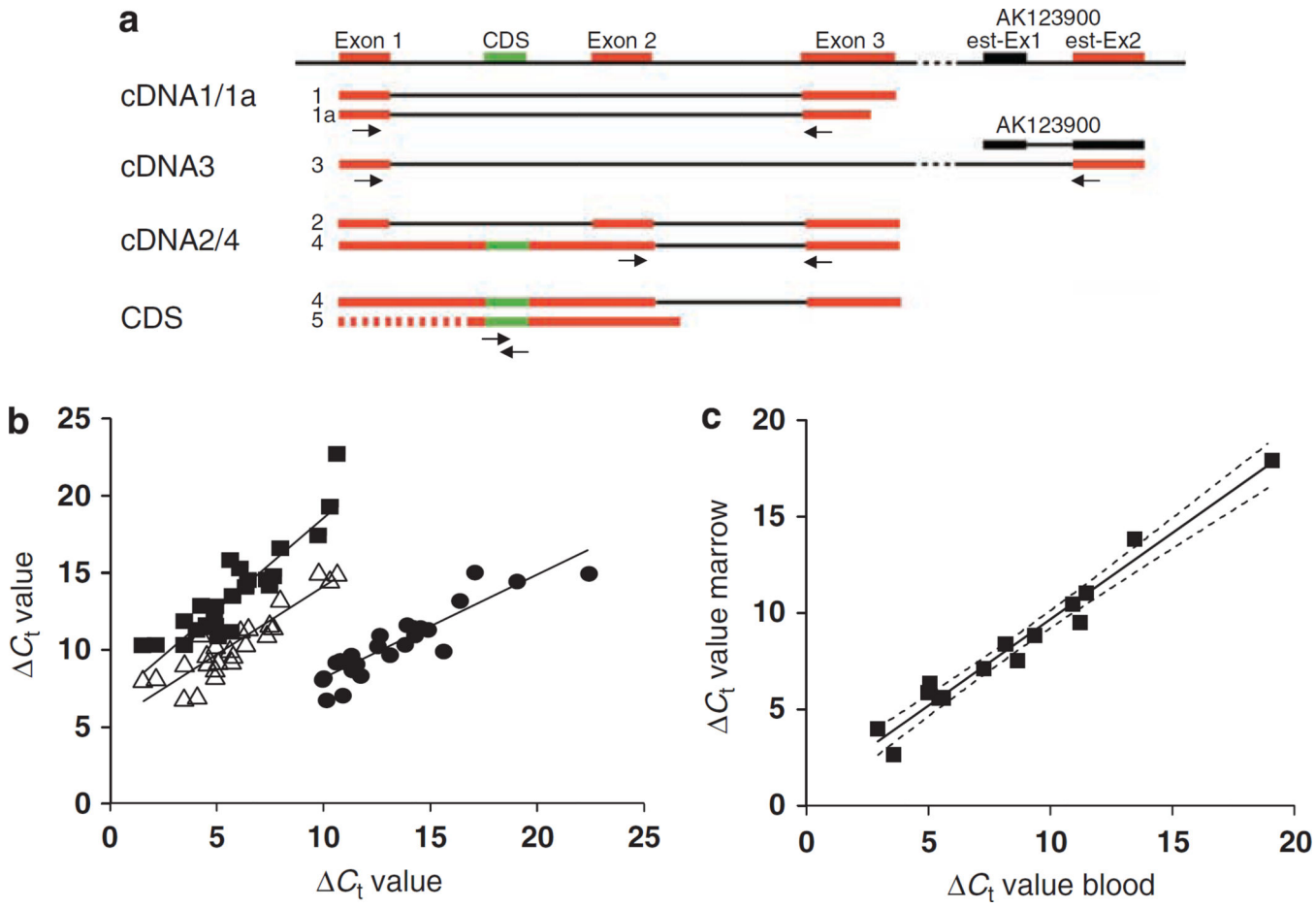

Figure 1.

Expression of the *CLLU1* splice variants. (**a**) Overview of the location of primers used for the *CLLU1* splice variant-specific QRT-PCR reactions. (**b**) The expression patterns of the *CLLU1* splice variants were investigated in 25 chronic lymphocytic leukemia (CLL) patients. ΔC_t is the number of PCR cycles required to reach the threshold level for *CLLU1* after normalization to the β*2-M* internal standard. ■ — X: cDNA1, Y: cDNA2/4, $r = 0.90$, $P < 0.0001$, Δ — X: cDNA1, Y: cDNA3, $r = 0.90$, $P < 0.0001$, ● — X: cDNA2/4, Y: cDNA3, $r = 0.91$, $P < 0.0001$. (**c**) Blood or marrow samples were collected from the same untreated patient at the same time point ($n = 15$). *CLLU1* expression was determined by QRT-PCR (cDNA1 splice variant). Using linear regression analysis we found $\beta = 0.89 \pm 0.05$, $r = 0.98$, $P < 0.0001$. The dotted lines show the 95% confidence intervals.

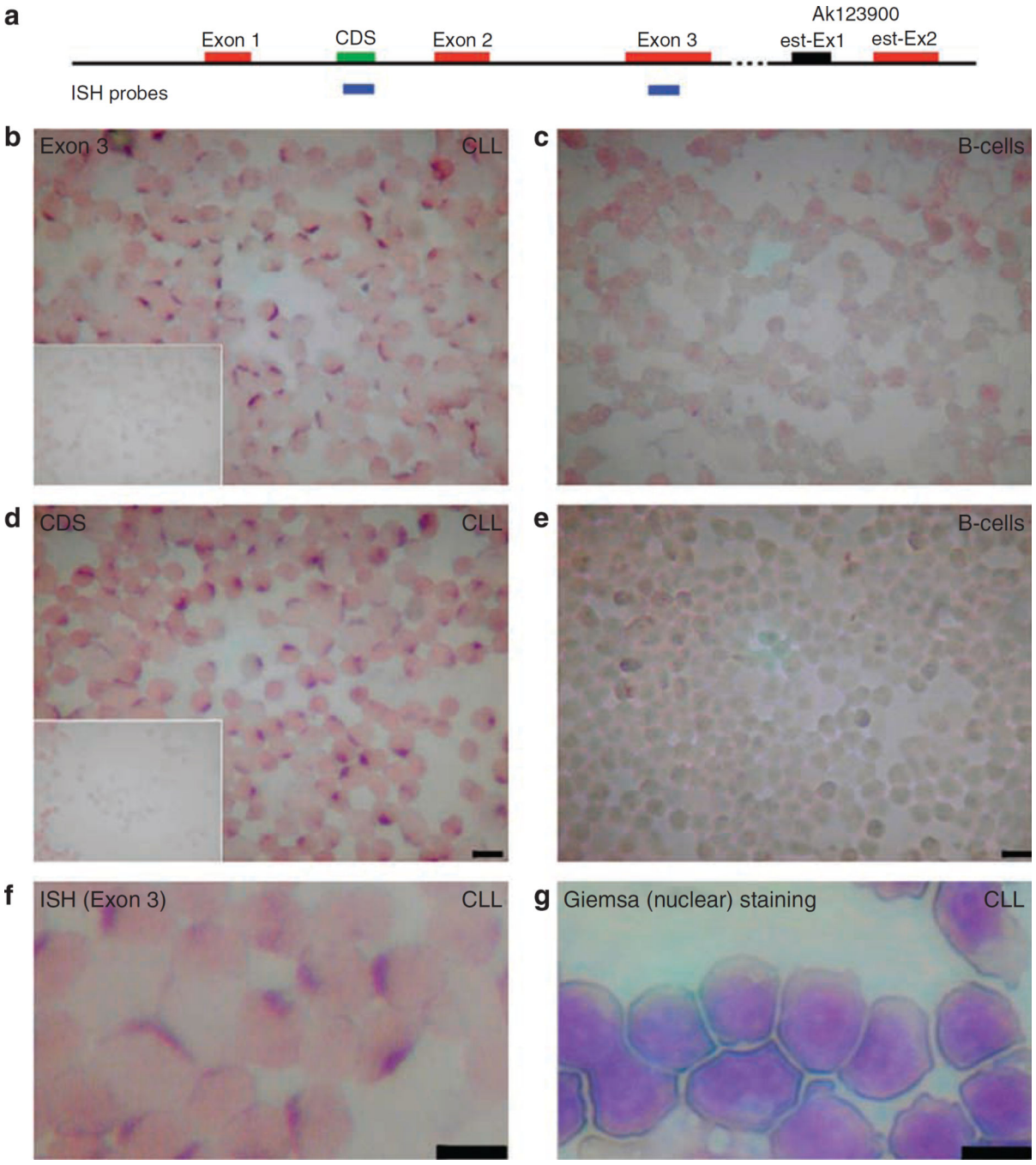

Figure 2.

In situ hybridization (ISH) using probes complementary to Exon 3 or CDS sequences of *CLLU1*. (**a**) Overview of the *CLLU1* gene on 12q22. The two ISH probes are shown in blue. Left panels (**b** and **d**) show ISH to sample UPN73, a CLL patient with a *CLLU1* expression level of 1034-fold above B-cell levels. The right panels (**c** and **e**) show ISH to purified normal B-cell controls. The insets show hybridization with sense probe controls. The bars correspond to 10 μM. (**f**) Enlargement of ISH-stained chronic lymphocytic leukemia (CLL) cells from UPN73 (exon 3 probe). (**g**) Giemsa-stained CLL cells.

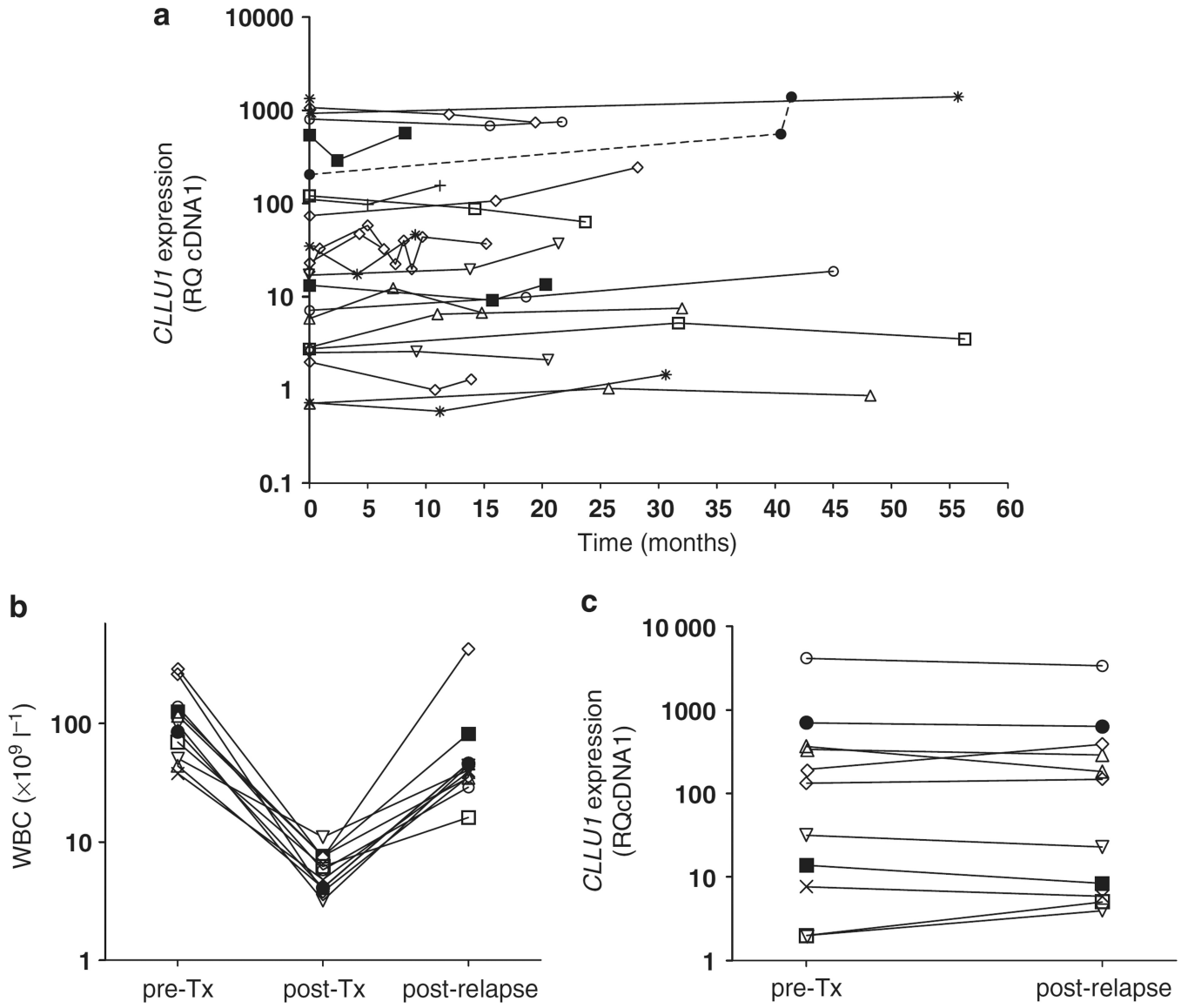

Figure 3.

CLLU1 expression is a stable feature of the particular chronic lymphocytic leukemia (CLL) clone. (**a**) In all, 21 untreated CLL patients were followed longitudinally to determine whether *CLLU1* expression changed over time. RQ(cDNA1) indicates the *CLLU1* expression level (cDNA1 splice variant) relative to the normal B-pool calibrator. The biclonal patient is marked with closed circles and a dotted line. (**b**) Cell samples were obtained from 11 CLL patients before treatment and after relapse. White blood cell (WBC) counts in the patient before treatment (pre-Tx), after treatment (post-Tx) and after relapse (post-relapse). (**c**) *CLLU1* expression RQ(cDNA1) measured in CLL samples pre-Tx and post-relapse.