CLLU1 expression has prognostic value in chronic lymphocytic leukemia after first-line therapy in younger patients and in those with mutated *IGHV* genes

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ABSTRACT

CLLU1, located at chromosome 12q22, encodes a transcript specific to chronic lymphocytic leukemia and has potential prognostic value. We assessed the value of *CLLU1* expression in the LRF CLL4 randomized trial. Samples from 515 patients with chronic lymphocytic leukemia were collected immediately before the start of treatment. After RNA extraction and cDNA synthesis, *CLLU1* expression was assessed by quantitative polymerase chain reaction. In total, 247 and 268 samples were identified as having low and high *CLLU1* expression, respectively. The median follow-up was 88 months. High *CLLU1* expression was significantly correlated with unmutated *IGHV* genes, ZAP-70 and CD38 positivity, and absence of 13q deletion (all r>0.2, $P<0.0001$). At 6 years, patients with high *CLLU1* expression had significantly worse progression-free survival (9% *versus* 17%; $P=0.03$) and overall survival (42% *versus* 57%; $P=0.0003$) than patients with low *CLLU1* expression. Among patients with mutated *IGHV* genes, overall survival at 6 years was 50% in those with high *CLLU1* expression and 76% in those with low *CLLU1* expression ($P=0.005$). However, *CLLU1* expression was not an independent predictor of overall survival in a multivariate model including *TP53* aberrations, beta-2 microglobulin level, age and *IGHV* mutation status. Nor did it predict response to treatment. *CLLU1* expression analysis helps to refine the prognosis of patients with chronic lymphocytic leukemia who have mutated *IGHV* genes. *(controlled-trials.com identifier: ISRCTN58585610)*

Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with a highly variable outcome depending on clinical and biological characteristics. Clinical staging is a relatively simple method of assessing the outcome of CLL patients based on clinical parameters.[1,2] In the last two decades, multiple biological markers have been shown to have prognostic relevance in CLL, most notably chromosomal aberrations, *IGHV* mutations and expression of surface markers such as ZAP-70 and CD38.[3-8] Gene expression analyses have also revealed the heterogeneity of CLL, with distinct gene expression signatures being associated with particular genetic subgroups of CLL.[9-11] In particular, the expression of genes such as *CLLU1*, *ADAM29* and *LPL* have recently been associated with different outcomes in CLL.[12-17]

CLLU1 located at chromosome 12q22, encodes a novel transcript, which has been identified as a marker specific to CLL.[18] In particular, high *CLLU1* expression (*CLLU1*-H) is significantly more frequent in unmutated *IGHV* and CD38$^+$ CLL.[19,20] *CLLU1*-H has been shown to be associated with shorter overall survival and shorter time to first treatment in patients with early stage CLL,[20] especially those younger than 70 years.[13] In addition, *CLLU1* expression has been shown to be a specific and stable marker in CLL cells,[21] making it a promising marker not only for prognosis but also for minimal residual disease analysis.[22] However, the effect of *CLLU1* expression on response to therapy is currently unknown and, to date, there are no data on the significance of *CLLU1* expression with regards to progression-free survival and overall survival in patients receiving first-line therapy. We assessed the value of *CLLU1* expression as a prognostic marker in the LRF CLL4 randomized controlled trial.

Design and Methods

Patients and samples

Pre-treatment blood samples were collected at the time of entry into the trial and were available for *CLLU1* expression analysis from 515 of the 777 patients. The patients were previously untreated, 25% having Binet stage A-progressive disease, 45% stage B and 30% stage C. The male:female ratio was 3:1 and the median age was 65 years (range, 35–86 years). Patients were randomized to receive chlorambucil, fludarabine, or fludarabine with cyclophosphamide. The trial was approved by a multicenter research ethics committee and all patients gave informed consent.

RNA extraction and CLLU1 expression analysis

RNA was extracted using the RNeasy Mini Kit (Qiagen) after which

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cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (LifeTechnologies). *CLLU1* mRNA expression levels were assessed by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using *B2M* as the endogenous control, as described previously.[20] In brief, *CLLU1* and *B2M* were amplified in duplicate in a 7500 Fast Real-Time PCR system (LifeTechnologies) using 5 μL of cDNA in a total volume of 25 μL containing TaqMan Fast Universal PCR Master Mix (LifeTechnologies), and 200 μM of primers/probes. The ΔΔCt method was used, with RNA extracted from a normal B-cell pool as a calibrator, to calculate the relative expression of *CLLU1*. The assay was validated in a preliminary set of 15 samples from the original series[13] and a real-time relative quantification (RQ) of >40 was used to define cases with high *CLLU1* expression, as in the original series.

Statistical methods

Correlations between *CLLU1* expression values (high or low; *CLLU1*-H or *CLLU1*-L, respectively) and other relevant clinical and biological variables were assessed using the Pearson's product-moment correlation coefficient and multiple regression analysis. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Overall survival was calculated from randomization to death while progression-free survival was calculated from randomization to relapse or death, censored at the date of latest follow-up for patients remaining event-free. The follow-up was to 31st October 2010, with the median follow-up of survivors being 88 months (range, 48 - 141 months). Six years was the time-point chosen for comparing survival between groups, because follow-up data were available for all survivors up to this point, except for two patients lost to follow-up at 48 and 50 months. Cox regression was used for analysis of variables associated with survival. *P* values ≤0.05 were considered statistically significant. All analyses were performed using the Statistica software package (StatSoft, Tulsa, OK, USA).

Results

CLLU1 expression in patients in the LRF CLL4 trial

The demographic, clinical and biological characteristics of the 515 trial patients with *CLLU1* mRNA expression data were not significantly different from those of the 262 patients without available data (*Online Supplementary Table S1*), demonstrating that the series of 515 cases was representative of the whole trial population. The RQ values of *CLLU1* expression ranged from 0.02 to 46,757 (median 47). Various cut-offs to differentiate between high and low expression were investigated. However, no important differences were seen (*data not shown*) and the value used by Josefsson *et al.*[13] (RQ=40) was, therefore, selected for this analysis. A total of 247 and 268 samples were identified as *CLLU1*-H (RQ>40) and *CLLU1*-L (RQ<40), respectively.

Table 1. The degree of correlation between *CLLU1* expression and other prognostic variables.

Patient/disease characteristic	Low *CLLU1* expression (n=247*) %	High *CLLU1* expression (n=268)† %	Correlation coefficient (r)	*P*
Unmutated *IGHV* genes	45	77	0.33	<0.0001
Zap-70 positivity	33	64	0.31	<0.0001
CD38 positivity	52	74	0.23	<0.0001
Absence of 13q deletion	30	51	0.22	<0.0001
Raised β_2 microglobulin (≥4 mg/L)	38	55	0.17	0.001
Trisomy 12	11	18	0.11	0.02
TP53 deletion and/or mutation	6	10	0.08	0.09
Older age (≥70 years)	28	32	0.05	0.3
11q deletion	20	21	0.02	0.7

**Minimum n. of cases for any variable: 173; †Minimum n. of cases for any variable: 185.*

Correlations between CLLU1 expression, biological and clinical variables

The variables most closely correlated with high *CLLU1* mRNA expression were unmutated *IGHV* genes, ZAP-70 positivity (defined as >10% of cells[8]), CD38 positivity (defined as ≥7% of cells[8]), and absence of 13q deletion (all r>0.2, *P*<0.0001, Table 1). Higher *CLLU1* expression was also moderately correlated with serum β_2 microglobulin ≥4 mg/L and trisomy of chromosome 12, the location of *CLLU1* (both r>0.1, *P*<0.05, Table 1). There was no correlation between *CLLU1* expression and age, gender, disease stage, white blood count, hemoglobin concentration or platelet count (*data not shown*).

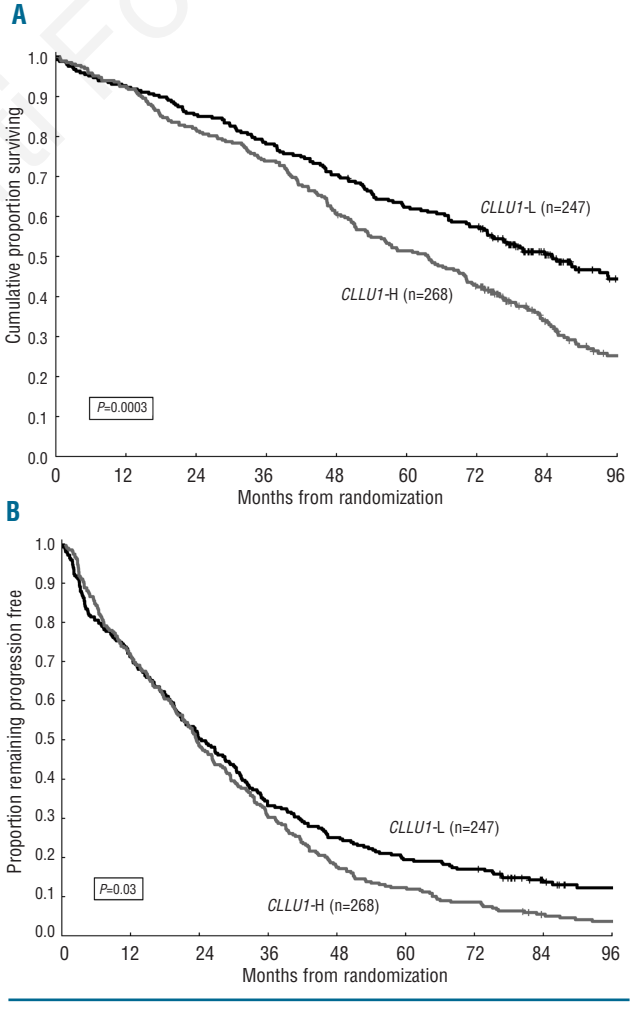

Figure 1. Survival from randomization according to *CLLU1* expression (high – *CLLU1*-H *vs.* low – *CLLU1*-L): (A) overall survival; (B) progression-free survival.

Table 2. Response to treatment in patients divided according to *CLLU1* expression and treatment group.

Treatment group	Assessable patients n.	Overall response rate *CLLU1*-H %	*CLLU1*-L %	*P*	Complete response rate *CLLU1*-H %	*CLLU1*-L %	*P*
All	485	79	78	0.7	16	16	0.9
Chlorambucil	236	70	72	0.8	6	7	0.8
Fludarabine	119	82	74	0.3	16	6	0.1
Fludarabine +cyclophosphamide	130	94	93	0.9	33	43	0.3

CLLU1-*L* – *low* CLLU1 *expression;* CLLU1-*H* – *high* CLLU1 *expression.*

Association between CLLU1 *expression and clinical outcomes*

The main results of the LRF CLL4 trial, as well as the prognostic factors affecting outcomes, have already been published.[8,23-24] Patients with *CLLU1*-H had significantly shorter overall survival from randomization than patients with *CLLU1*-L. The 6-year overall survival rate was 42% [95% confidence interval (95% CI) 36-48%] in patients with *CLLU1*-H *versus* 57% (95% CI: 51-63%) in patients with *CLLU1*-L [hazard ratio (HR) 1.48 (95% CI: 1.20-1.84); *P*=0.0003; Figure 1A]. *CLLU1* mRNA expression was also significantly associated with progression-free survival. At 6 years the progression-free survival rate was 9% (95% CI: 6-12%) for patients with *CLLU1*-H *versus* 17% (95% CI: 13-22%) for patients with *CLLU1*-L [HR 1.23 (95% CI: 1.03-1.47); *P*=0.03; Figure 1B]. However, *CLLU1* expression did not predict response to treatment. The overall response rate was 79% in patients with *CLLU1*-H and 78% in those with *CLLU1*-L. The rate of complete responses was 16% in both groups. Moreover, there were no differences in response rates between patients with *CLLU1*-H and *CLLU1*-L expression within any of the treatment groups (Table 2).

CLLU1 mRNA expression was significantly associated with overall survival in patients with mutated *IGHV* genes. Within this group, the overall survival rate at 6 years was 50% (95% CI: 36-62%) for patients with *CLLU1*-H *versus* 76% (95% CI: 67-83%) for patients with *CLLU1*-L [HR 1.93 (95% CI: 1.24-3.00); *P*=0.005; Figure 2A]. On the other hand, in patients with unmutated *IGHV* genes there was no difference. In this group the overall survival rate at 6 years was 40% (95% CI: 33-47%) for patients with *CLLU1*-H *versus* 35% (95% CI: 26-45%) for those with *CLLU1*-L [HR 0.91 (95% CI: 0.69-1.20); *P*=0.5]. Altogether, for patients with unmutated *IGHV* genes, the overall survival rate at 6 years was significantly worse than for patients with mutated *IGHV* who had *CLLU1*-H [39% *versus* 50%, HR 1.52 (95% CI: 1.06-2.18); *P*=0.02].

Similarly, the difference in overall survival according to *CLLU1* expression appeared more marked in younger than in older patients. In patients aged <70 years, the 6-year survival rate was 51% (95% CI: 43-58) in those with *CLLU1*-H *versus* 68% (95% CI: 60-74%) in those with *CLLU1*-L [HR 1.62 (95% CI: 1.23-2.13); *P*=0.0005; Figure 2B]. In patients ≥70 years there was no difference: 25% (95% CI:17-35%) in patients with *CLLU1*-H *versus* 30% (95% CI: 19-41%) in patients with *CLLU1*-L [HR 1.11 (95% CI:0.78-1.58); *P*=0.6]. In contrast, β_2-microglobulin level predicted overall survival at 6 years not only within the group of patients with mutated *IGHV* genes, the rate being 46% (95% CI: 31-60%) in those with high β_2-microglobulin (≥ 4mg/L) *versus* 83% (95% CI: 73-89%) in those with low β_2-microglobulin (<4 mg/L) [HR 4.00 (95% CI: 2.34-6.83); *P*<0.0001], but also within the group with unmutated *IGHV* genes, in which it was 25% (95% CI: 17-32%) among the patients with high β_2-microglobulin *versus* 54% (95% CI: 44-63%) among those with low β_2-microglobulin [HR 2.04 (95% CI: 1.51-2.75); *P*<0.0001].

Table 3. Multivariate analysis of prognostic factors associated with shorter overall survival.

Variable	Hazard Ratio	95% CI	*P*
IGHV unmutated (>98% homology)	2.53	1.78-3.61	<0.0001
Raised β_2 microglobulin (≥4 mg/L)	2.03	1.51-2.73	<0.0001
Older age (years)	1.04	1.02-1.06	<0.0001
TP53 aberrations (deletion and/or mutation)	1.88	1.17-3.04	0.01
Treatment group	0.88	0.75-1.04	0.1
High *CLLU1* expression	1.10	0.82-1.48	0.5

CLLU1 mRNA expression was not an independent predictor of overall survival in a multivariate model including the variables previously shown to be independent predictors of overall survival in this trial: *TP53* aberrations (deletion and/or mutation), β_2-microglobulin level, age and *IGHV* mutational status.[8,24] Treatment group did not predict survival but was also included in the model (Table 3).

Discussion

The prognostic value of *CLLU1* mRNA expression has previously been investigated in four retrospective studies,[13,15,16,20] including between 59 and 252 patients each and at a median follow-up from diagnosis of between 43 and 102 months. The end-points in all four studies were time to first treatment and overall survival from diagnosis. Our study, reported here, is the first investigation of the prognostic value of *CLLU1* mRNA expression in a controlled randomized trial. In this trial *CLLU1* expression was analyzed in samples collected at baseline from 515 previously untreated patients and the median follow-up from randomization was 88 months. We analyzed the effect of *CLLU1* expression across three different therapies, including an alkylating agent (chlorambucil) and a purine analog (fludarabine) given alone or in combination with cyclophosphamide. In addition to overall survival, we included response to treatment and progression-free survival as end-points. Time to first treatment was not an end-point as all patients required treatment from the outset of the study. The cut-off used to distinguish between low and high *CLLU1* expression remained the same as in the study by Josefsson *et al.*,[13] making it possible to com-

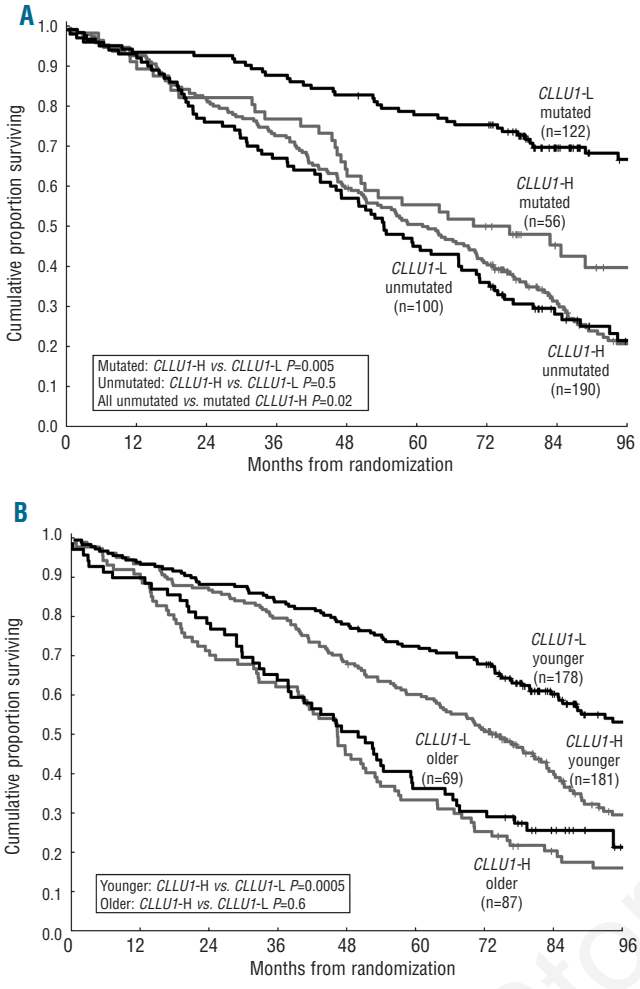

Figure 2. Overall survival from randomization: (A) by *CLLU1* expression (high – *CLLU1*-H *vs.* low – *CLLU1*-L) and *IGHV* mutation status (B) by *CLLU1* expression (high – *CLLU1*-H *vs.* low – *CLLU1*-L) and age group (<70 *versus* ≥70 years of age).

pare our results directly with theirs and to validate their findings prospectively.

We were able to confirm that patients who had high *CLLU1* mRNA expression had significantly shorter overall survival than those with low expression. They also had significantly shorter progression-free survival. The prognostic effect of *CLLU1* expression on overall survival has been previously suggested to be greatest in patients under the age of 70 years and in *IGHV* mutated cases[13]. We confirmed these findings in this randomized trial with respect to both younger patients and the *IGHV* mutated group. It was previously shown that younger age and mutated *IGHV* genes were indicators of good prognosis in the LRF CLL4 trial.[8] As suggested by Josefsson *et al.*, a possible explanation for the greater adverse effect of high *CLLU1* expression in these groups with good prognosis, compared with its effect in the corresponding groups with poorer prognosis,[13,15] may be that high *CLLU1* expression exerts its clinical effect in the longer term and does not, therefore, affect patients who have shorter survival because of older age or unmutated *IGHV* genes. This could also explain why *CLLU1* expression did not predict overall survival in the study by Stamatopoulos *et al.* in 170 patients with a median follow-up of only 64 months from diagnosis.[15] In this context, it is interesting that in our trial *CLLU1* expression did not affect response to treatment, in any treatment group. Unlike overall and progression-free survival, the rates of these shorter-term end-points (overall response rate and complete response rate) were no different between patients with high or low *CLLU1* expression.

Correlations between higher *CLLU1* mRNA expression and other variables indicative of more aggressive disease were previously established.[18-20] We were able to confirm these findings, particularly with regards to unmutated *IGHV* status, ZAP-70 and CD38 positivity, absence of 13q deletion and raised levels of β_2-microglobulin.

Our findings suggest that *CLLU1* mRNA expression may be useful as a prognostic indicator in patients requiring treatment, specifically within the groups with longer life expectancy defined by younger age or mutated *IGHV* genes. Within these groups *CLLU1* expression was able to identify patients with particularly good prognosis. However, we cannot recommend *CLLU1* expression as a prognostic test in all patients. β_2-microglobulin level, which can be measured by a simple laboratory test, retained independent significance in a multivariate analysis of factors predicting overall survival, while *CLLU1* expression did not. β_2-microglobulin level distinguished between subgroups with relatively good and poor prognosis not only in patients with mutated *IGHV* genes but also in those whose *IGHV* genes were unmutated. It was perhaps to be expected that *CLLU1* expression would not be an independent predictor of overall survival in a multivariate model which included *IGHV* mutation status, because of the correlation between these two variables. However, Kaderi *et al.*[16] also found that *CLLU1* expression did not independently predict overall survival, in 252 newly diagnosed patients (median follow-up of 102 months), in a multivariate model including other RNA-based markers. They found that *LPL* expression was the most significant of the markers included and, like *CLLU1* expression, it was particularly able to discriminate between subgroups within the groups with good prognosis, such as those with mutated *IGHV* genes. β_2-microglobulin level was not used as a comparator in their analysis.

We have confirmed that the level of *CLLU1* mRNA expression provided prognostic information in patients with CLL in the LRF CLL4 trial. We recommend the inclusion of *CLLU1* expression in the risk stratification of patients with CLL, in particular in those defined by other markers as having a good prognosis.

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Authorship and Disclosures

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