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***IGHV* gene mutational status and *LPL/ADAM29* gene expression as clinical outcome predictors in CLL patients in remission following treatment with oral fludarabine plus cyclophosphamide**

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Abstract Several prognostic factors can predict the rapid progression in chronic lymphocytic leukaemia (CLL), including *IGHV* mutational status, cytogenetic abnormalities and, more recently, *LPL/ADAM29* expression. In contrast, few

studies have been devoted to the influence of these factors on clinical outcome in responding patients after therapy. We here propose to analyse the impact of *IGHV* gene status, *LPL* and *ADAM29* gene expression on disease-free survival (DFS) and overall survival (OS) in 41 stage B or C CLL patients in remission after oral fludarabine plus cyclophosphamide. The median follow-up was of 64 (16–74) months. Sequencing of *IGHV* showed mutated (M) *VH* genes in 16 of 41 cases and unmutated (UM) in 25 cases. Analysis of *LPL* and *ADAM29* expression in 35 of 41 cases showed overexpression of *ADAM29* in 17 cases (14 M and three UM) and *LPL* in 18 cases (all UM). Patients expressing UM *IGHV* and *LPL* had shorter DFS and OS when compared to patients expressing M *IGHV* and/or *ADAM29*. Furthermore, blood minimal residual disease (MRD) evaluation using four-colour flow cytometry was performed in 33 out of the 41 patients. We showed that patients who achieved phenotypic remission displayed longer DFS than those with MRD⁺. Our results support the use of *LPL* and *ADAM29* gene expression associated to *IGHV* mutational status for predicting the clinical outcome of patients treated by oral fludarabine + cyclophosphamide and could be considered for treatment strategies.

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Keywords CLL · Oral fludarabine · Cyclophosphamide · *LPL* · *ADAM29* · *IGHV* · Mutational status

Introduction

Chronic lymphocytic leukaemia (CLL) is recognised as a heterogeneous disease [7]. Although clinical staging sys-

tems described by Rai et al. [24] and Binet et al. [1] are useful methods for assessing patient prognosis, they do not permit to understand the basis of this heterogeneity. In the last decade, it has been demonstrated that the immunoglobulin (Ig) *VH* gene mutational status predicts the natural history of the disease. Indeed, the presence of unmutated (UM) *VH* genes was highly associated with a much poorer prognosis. Median survival in patients whose CLL cells express UM *IGHV* genes ranges between 60 and 119 months. In contrast, patients whose CLL cells express mutated (M) *IGHV* genes have a distinctly longer median survival and many may never require treatment. It is now admitted that an UM germ line configuration of the *IGHV* genes constitutes a crucial adverse prognostic factor in CLL under conventional chemotherapy treatment as well as after autologous stem cell transplantation [6, 11, 16, 22]. Several other prognostic factors have been described in CLL, including cytogenetic abnormalities [8], ZAP-70 expression [5], CD38 expression [6], serum markers such as soluble CD23 [26], thymidine kinase [10] and β_2 -microglobulin [13]. Our group has suggested that expression of *lipoprotein lipase (LPL)* and *a disintegrin and metalloproteinase domain 29 (ADAM29)* genes are closely correlated to *IGHV* mutational status with a concordance rate of 82% for *ADAM29* gene and M status and 76% for *LPL* gene and UM status. Interestingly, the value of the *LPL/ADAM29* ratio as well as *LPL/ADAM29* expression assessed by a qualitative multiplex polymerase chain reaction (PCR) improves this correlation with a concordance rate of 90%. Both have been described to be very good prognostic factors associated with event-free survival and overall survival [21]. Until now, few studies were devoted to the impact of prognostic factors on the treatment response and disease-free survival (DFS). Byrd et al. [2] showed that although no correlation was observed between *IGHV* mutational status and cytogenetic aberrations with treatment response, these prognostic factors were both highly correlated to DFS in CLL patients in complete remission (CR) after treatment with rituximab, fludarabine and cyclophosphamide combination. More recently, the same group showed that interphase cytogenetic analysis, but not *IGHV*, CD38 expression or ZAP-70 status, can predict the outcome of fludarabine-based chemotherapy and that patients with high-risk cytogenetic features should be considered for alternative therapies [9]. Very recently, Laurenti et al. [14] reported that oral fludarabine + cyclophosphamide could be an appropriate treatment in CLL patients with low-risk biological parameters. They showed that a shorter PFS and a shorter time to retreatment (TTR) were observed in patients with “high-risk” cytogenetic abnormalities. They also showed that patients with UM *IGHV* displayed shorter TTR when compared to M cases. No study has analysed yet the impact of *LPL* and

ADAM29 gene expression on the clinical outcome for patients in CR. Minimal residual disease (MRD) status has been shown to be a good clinical outcome predictor, since patients who achieved complete phenotypic remission as assessed by four-colour flow cytometry in the blood and/or in the bone marrow had a superior response duration and survival when compared with patients who were MRD⁺ [15, 18, 25, 29]. Our results confirm the importance of MRD assessment as a good clinical outcome predictor and demonstrate the impact of *IGHV* mutational status and *LPL/ADAM29* gene expression on DFS and OS in CLL patients in response following oral fludarabine + cyclophosphamide.

Materials and methods

Patients

Among the 75 progressive CLL patients treated by first-line oral fludarabine + cyclophosphamide recently reported by Cazin et al. [3], 41 were included in this study, of whom 38 were in CR and three in nodular partial remission (nPR; $n=3$) according to the NCI criteria [4].

IGHV mutational status

The *IGHV* gene sequences were determined as previously described [23]. Briefly, amplification of immunoglobulin heavy chain variable regions by PCR was performed on DNA from leukaemic cells with consensus primers for the *VH* framework region 1 and *JH* genes or following the BIOMED-2 protocols. Purified PCR products were sequenced either directly or after a cloning procedure using an automated DNA sequencer. Sequence data were analysed using IgBLAST (<http://www.ncbi.nlm.nih.gov/igblast>) and the ImMunoGeneTics database (IMGT; <http://imgt.cines.fr>). *IGHV* sequences were considered as M if their homology with the closest germ line counterpart was less than 98%.

LPL and *ADAM29* gene expression

We have previously demonstrated that the qualitative multiplex-PCR method is simple and as reliable as the quantitative RQ-PCR for the *LPL/ADAM29* gene expression in CLL patients. In the present study, we evaluated the relative expression of *LPL* and *ADAM29* gene by multiplex reverse transcriptase PCR, by using primers designed with the Oligo 6 software (Table 1). These primers were validated by performing this PCR on 33 known samples previously quantified by RQ-PCR [21]. Total cellular RNA was extracted and its integrity was assessed by visualisation of the 18S and 28S RNA species on electrophoresis in

Table 1 Sequences of primers used in multiplex PCR

Primer	Sequence (5'→3')
<i>LPL</i> forward	cagatgccctacaaagtctcc
<i>LPL</i> reverse	tttctctgtatggcaccgtggc
<i>ADAM29</i> forward	ggcaaccaccaataactaaat
<i>ADAM29</i> reverse	tttaatgtgaagcgtgtggga

agarose gel after ethidium bromide staining. First-strand complementary DNA (cDNA) was synthesised from 2 µg total RNA using Superscript II reverse transcriptase (Invitrogen, Cergy-Pontoise, France) and oligodT or random hexamer primers. Optimised PCR conditions were obtained with primers at the final concentrations of 0.5 µM, 1.5 mM MgCl₂ and 200 µM dNTP. Amplifications were performed on 60 ng cDNA and included an initial denaturation step at 94°C for 4 min, followed by 29 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C. Finally, the reaction was completed with a final elongation step at 72°C for 5 min. PCR products were analysed on ethidium-bromide-stained 11% acrylamide gel electrophoresis where they appeared as a 130-bp band for *ADAM29* and a 110-bp band for *LPL* (Fig. 1). Amplification of ABL was performed in parallel to ensure cDNA integrity.

MRD assessment

Two months after the treatment ended, blood flow cytometry (FC) analysis was carried out in order to detect residual leukaemic cells in the blood as previously described [17]. Briefly, we used a four-colour FC with a panel of antibodies including anti-CD19, anti-CD20, anti-CD5, anti-CD79b antibodies and a Becton Dickinson FACScalibur device (San Jose, CA, USA). The sensitivity of this FC technique was of 10⁻⁴.

Statistical analysis

Survival analyses were performed using the Kaplan–Meier method. Statistical significance of associations between

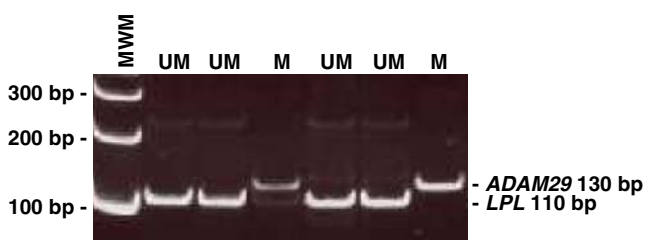


Fig. 1 *MWM* molecular weight marker, *M* mutated, *UM* unmutated. *LPL* and *ADAM29* transcripts were amplified simultaneously; the PCR products were then separated by electrophoresis on acrylamide gel and visualised under UV illumination after ethidium bromide staining

individual variables and survival was calculated by the log-rank test. These analyses were performed using MedCalc® Version 9.3.0.0 (<http://www.medcalc.be>).

Results

Clinical characteristics

Forty-one advanced CLL (Binet stage B or C) patients achieving remission after treatment with oral fludarabine plus cyclophosphamide were enrolled in this study. Thirty-eight were in CR and three in nPR according to NCI criteria [4]. Median follow-up was 64 (16–74) months.

IGHV mutational status and clinical outcome

IGHV sequencing was performed in all patients. Sixteen expressed M *VH* genes (ten VH3, of which three VH3-21, four VH4, one VH2 and one VH1). Twenty-five cases expressed UM *VH* genes (14 VH1, of which 11 VH1-69, ten VH3, none of them was VH3-21, and one VH4). In the M group, only five out of 16 patients relapsed with a median time to progression (TTP) of 28 (23–60) months, whilst in the UM group, 16 out of 25 experienced relapse with a median TTP of 29 (3–52) months ($p=0.01$; Fig. 2a). Of note if that two out of the VH3-21 cases did not experience relapse and the latter relapsed 28 months after the treatment evaluation. No death was observed in the M group of patients, whilst ten out of 25 UM cases died at a median time of 41 (16–64) months ($p=0.01$; Fig. 2b).

LPL and *ADAM29* gene expression and clinical outcome

LPL and *ADAM29* gene expression were assessed in 35 out of the 41 responder patients. Seventeen expressed *ADAM29*, of whom 14 had M *IGHV* genes and three UM *IGHV* genes. *LPL* expression was observed in 18 out of 35 cases, all of them expressing UM *IGHV* genes. Regarding TTP according to *LPL* and *ADAM29* gene expression, relapse rate was higher in the *LPL*-expressing group, with 14 of 18 patients who relapsed with a median TTP of 32 (3–52) months, whilst only six out of 17 expressing *ADAM29* patients relapsed with a median TTP of 28 (21–60) months ($p=0.01$; Fig. 2c). Although the median survival was not reached, six out of 18 *LPL* patients died at 16, 25, 41, 45, 53 and 57 months, whilst no death was observed in the group of *ADAM29*-expressing patients ($p=0.01$; Fig. 2d) [21]. Interestingly, three discordant cases regarding *LPL* and *ADAM29* expression vs mutational status were observed with *ADAM29* overexpression and UM *IGHV*. Among them, two patients achieved phenotypic

remission and are still alive in CR at 58 and 70 months; the remaining patient relapsed at 21 months and is still alive with a follow-up of 66 months.

MRD and clinical outcome

MRD evaluation in the blood was performed 2 months after the end of the treatment in 33 out of the 41 patients. Among these 33 patients, 21 achieved phenotypic remission at the time of the treatment evaluation, of whom 13 were still in remission with a median time follow-up of 66 (53–68) months, and the remaining eight patients relapsed at a median time of 44 (22–56) months. In the group of the 12 MRD⁺ patients, ten relapsed at a median time of 28 (3–60) months. As shown in Fig. 2e, patients achieving phenotypic remission displayed longer DFS ($p=0.0009$). However, no significant difference was observed regarding OS (Fig. 2f). Interestingly, among the 13 patients expressing M *VH*

genes, ten achieved phenotypic remission (77%). In contrast, 11 out of 20 patients with UM *VH* genes achieved phenotypic remission (55%). Although there is a tendency of a higher rate of phenotypic remission in the group of M patients when compared with UM patients, the difference is not statistically significant ($p=0.2$). When regarding *LPL* and *ADAM29* gene expression and MRD, the results are similar to those observed with the mutational status and showed that 11 of 18 MRD⁻ patients expressed *ADAM29*, whilst eight of 11 MRD⁺ patients expressed *LPL* ($p=0.07$). Data are visualised in Table 2.

Discussion

In this work, we have described a series of CLL patients in clinical remission after oral fludarabine plus cyclophosphamide, for whom we have assessed the recently described

Fig. 2 Disease-free survival according to **a** *IGHV* mutational status showing that patients with unmutated (UM) profile displayed shorter response duration than those with mutated (M) genes; **c** *LPL*-expressing patients demonstrated shorter response duration than *ADAM29*-expressing patients; **e** Minimal residual disease (MRD) status with longer duration response for MRD-negative patients. Overall survival according to **b** *IGHV* status with longer survival for M patients; **d** *LPL* and *ADAM29* expression with longer survival for *ADAM29* group of patients and **f** MRD status which did not show significant different OS for MRD⁺ vs MRD⁻ patients

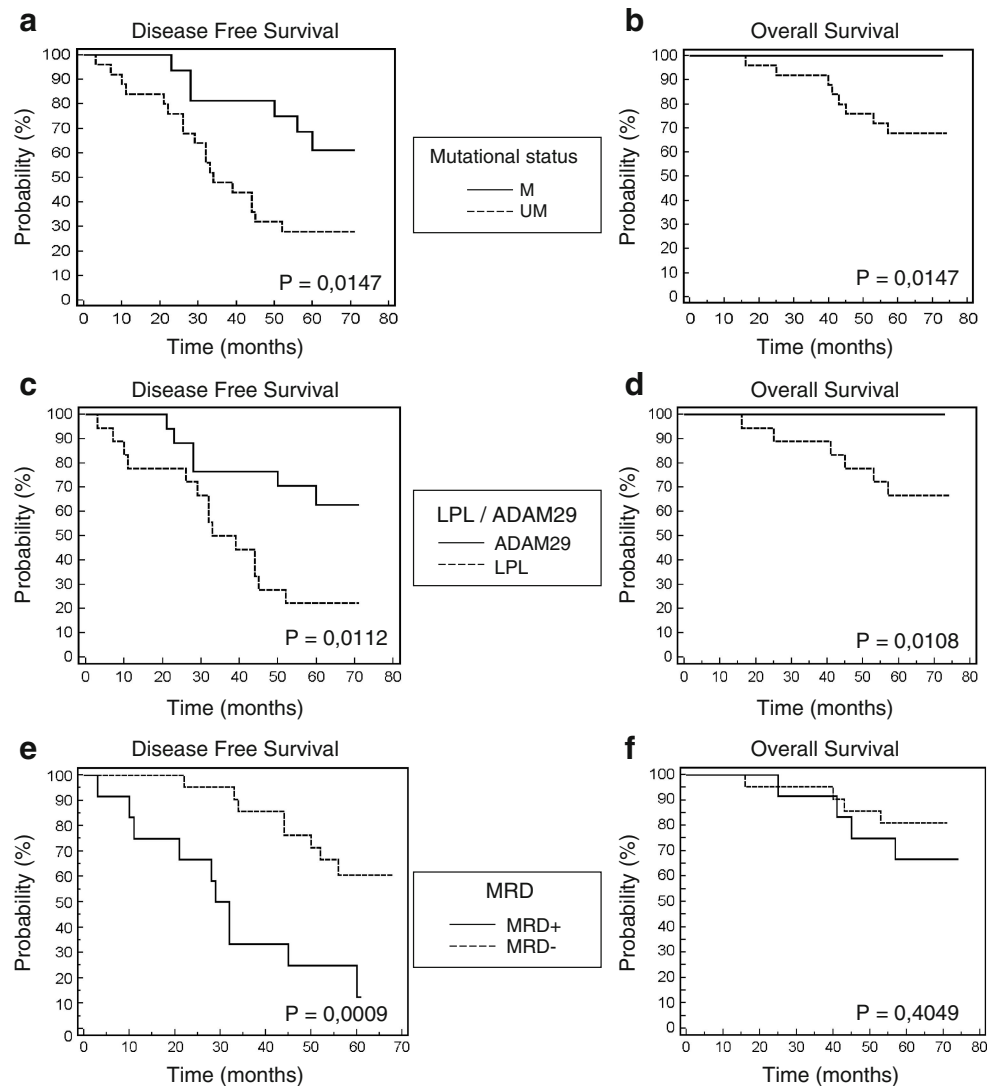


Table 2 MRD status according to *IGHV* mutational status and *LPL/ADAM29* expression

	MRD ⁻	MRD ⁺	Total
M	10	3	13
UM	11	9	20
<i>ADAM29</i>	11	3	14
<i>LPL</i>	7	8	15

Patients expressing *ADAM29* and M *IGHV* achieved more frequently phenotypic remission than those expressing *LPL* and UM *IGHV*

M mutated, UM unmutated

prognostic factors, including *IGHV* mutational status and *LPL/ADAM29* gene expression and, for some of them, interphase cytogenetics. Furthermore, we looked at the predictive value of MRD status at the time of the treatment evaluation for relapse and OS. The influence of *IGHV* mutational status and cytogenetic abnormalities on time to treatment from initial CLL diagnosis and OS has been demonstrated by previous studies [6, 11]. Our group has shown that *LPL* and *ADAM29* gene expression is closely correlated to *IGHV* gene mutational status. Indeed, in a series of 119 CLL patients, *LPL* gene showed a concordance rate of 76% with UM *IGHV* status and for *ADAM29* gene a concordance rate of 82% with M profile. Interestingly, the correlation was higher when using *LPL/ADAM29* ratio with a concordance rate of 90%. Furthermore, *LPL* and *ADAM29* gene expression appeared to be a strong prognostic factor for event-free survival and OS [21]. Other reports have confirmed the prognostic value of *LPL* expression gene in CLL [12, 19, 20, 27, 28]. In contrast, very few studies have looked at the impact of these prognostic factors on the clinical outcome of the CLL patients in remission after treatment. In a recent report by Byrd et al. [2], *IGHV* UM status and high-risk interphase cytogenetics, including del(17p) or del(11q), predict statistically inferior DFS and OS in patients in CR after chemoimmunotherapy. Herein, we have shown that patients with M *IGHV* genes had longer DFS and OS when compared to UM patients. The expression of *LPL* and *ADAM29* genes is correlated to the *VH* mutational status, since all *LPL*-expressing patients have UM *VH* genes and the majority of *ADAM29*-expressing patients have M *VH* genes. Furthermore, patients with MRD-negative displayed significant longer DFS than those with persistent leukaemic cells in the blood. However no significant difference in term of OS was observed when comparing MRD⁺ patients with MRD⁻ patients. More recently, Laurenti et al. [14] showed that the oral combination of fludarabine and cyclophosphamide was an effective and well-tolerated regimen and that a shorter TTR was observed in *IGHV*

UM cases when compared to M ones ($p=0.035$). We recently showed the high efficacy of this combination in 75 pre-untreated CLL patients with a very high overall response (80%) and CR (53%) [3]. Herein, we analysed the *IGHV* mutational status and *LPL/ADAM29* expression in 41 remitters among the 75 patients of this series. We showed that the UM cases represented 61% and M cases 39% as expected in progressive CLL. In addition, the *IGHV* usage corresponded to classical distribution displaying an overrepresentation of VH3 and VH1 genes, particularly VH1-69 gene [22]. Regarding OS according to mutational status, the M group had longer OS since all of them were still alive at the endpoint time with a median follow-up of 64 months. This is in agreement with previous reports which looked at the influence of this parameter on survival from initial CLL diagnosis [6, 11, 16, 22]. Concerning DFS, among the 21 cases who relapsed, 16 expressed UM *VH* genes and only five expressed M *VH* genes ($p=0.01$). These results are in agreement with those reported by Byrd et al. [2] who showed that M patients displayed longer DFS and OS and those of Laurenti et al. [14] showing a shorter TTR in UM patients. Similarly, we looked at the impact of *LPL* and *ADAM29* expression on the clinical outcome of these responder patients, showing that *LPL*-expressing patients display shorter DFS and OS since 14 of the 18 cases relapsed with a median TTP of 32 months, whilst only six out of the 17 *ADAM29* cases relapsed with a median TTP of 28 months. We have also confirmed that *LPL* and *ADAM29* expression could be a surrogate of *IGHV* mutational status, as previously described [21]. Of note is that the three discordant cases with overexpression of *ADAM29* and UM *IGHV* were still alive at 58, 66 and 70 months, and two of them did not relapse. Altogether, these results suggest that mutational status as well as *LPL* and *ADAM29* gene expression represent important outcome predictive factors not only from initial diagnosis as previously described [21] but also in CLL patients in remission after chemotherapy. In addition, our work confirms the high interest of MRD evaluation in CLL patients since we found a good correlation between MRD status and DFS as previously described [15, 18, 25, 29]. In this series, the achievement of phenotypic remission was slightly higher in the group of patients with M *VH* genes and *ADAM29* gene expression when compared with UM and *LPL*-expressing patients. However, the difference was not statistically significant, and it will be interesting to verify this result on a larger series.

These data strongly suggest that *LPL* and *ADAM29* gene expression associated to *IGHV* mutational status could be helpful in adapting treatment modalities. Further studies are needed to confirm the prognostic interest of these markers used in combination for outcome prediction after different treatment strategies.

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