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LETTER TO THE EDITOR

Lipoprotein lipase expression in unmutated CLL patients is the consequence of a demethylation process induced by the microenvironment

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Chronic lymphocytic leukaemia (CLL) can be defined as a low-grade B-cell tumor with antigen-experienced monoclonal CD5$^+$ B cells that, having escaped programmed cell death and undergone cell cycle arrest in the G0/G1 phase, relentlessly accumulate in lymphoid organs and circulate into the peripheral blood.[1] This leukemic B-cell accumulation results from a complex balance between activation of cell proliferation and inhibition of apoptotic death.[2] During the past few years, several new prognostic markers have emerged in CLL. Among them, the mutational status of the immunoglobulin heavy-chain variable (*IGHV*) genes is considered one of the strongest.[3] Results from gene expression profile in CLL led us to propose that expression of the lipoprotein lipase (*LPL*) gene could constitute a suitable surrogate marker of the mutational status of IGHV.[4] Despite the usefulness of LPL for CLL prognosis,[5–8] its functional role and the molecular mechanism regulating its expression remain elusive as yet.

LPL has a central role in lipid metabolism by catalyzing the hydrolysis of chylomicrons and very-low-density lipoproteins. In addition to its catalytic function, LPL acts as a bridging protein between cell surface proteins and lipoproteins, by increasing the contact between monocytes and endothelial cell surface through its interaction with heparan sulfate proteoglycans.[9] In CLL B cells, LPL expression has been related to functional pathways involved in fatty acid degradation and signaling, which may influence CLL biology and clinical outcome.[10]

There is increasing evidence that regulation of gene expression during normal lymphocyte development is mediated through changes in chromatin structure and/or through the methylated patterns of CpG islands. Tissue-specific patterns of methylated cytosine residues can be altered by environmental factors, and are often abnormal in tumor disorders.[11,12]

To gain insight into the molecular mechanisms responsible for the high LPL expression in Unmutated (Um) CLL B cells, we investigated: (a) the methylation status of the CpG island from this gene in 26 CLL cases and (b) the possibility that LPL expression could be related to specific signals delivered from an activated CLL microenvironment.

In a first step, we analyzed the CpG sites in the *LPL* gene. This analysis revealed CpG-rich sequences encompassing a CpG island of 1163 bp with 112 CpG dinucleotides. This area includes a region within the first exon and the first intron of the *LPL* gene. To better characterize this CpG island, we focused on methylation status of CpG dinucleotides in two different regions (R1 = 248 bp, from +87 bp to +335 bp and R2 = 261 bp, from +446 bp to +707 bp). Our results comparing methylation changes between R1 and R2 region in preliminary six CLL samples showed that the main differences appeared to be restricted to exon 1 (CpG dinucleotides number 1–18) and to the first region of intron 1 (CpG dinucleotides number 19–23) (Supplementary Figures 1A–C). Importance of the DNA methylation in the first exon has been recently linked to transcripcional gene expression.[11] To confirm these results, we performed methylation analysis on the R1 region of LPL-CpG island in 26 CLL patients, 14 Um/LPL-positive CLLs and 12 mutated (Mut)/LPL-negative patients (clinical and molecular CLL characterization is shown in Supplementary Table 1). All samples were analyzed following bisulphite DNA conversion, methylation-specific primer-PCR and confirmed by PCR amplification, cloning and sequencing of bisulphite DNA corresponding to R1-LPL region (Material and Methods available as Supplementary Material online). Results have shown that Mut CLL samples and Daudi Human Burkitt's lymphoma cell line (negative control) did not express, or expressed minimal levels of LPL mRNA. In contrast, Um CLL cases expressed high levels of LPL mRNA, though lower than adipocyte cells (AT) (Figure 1a). Interestingly, a different methylation pattern between Um and Mut CLL samples has been found (Figures 1b and c), suggesting that differential methylation status is responsible for *LPL* gene expression in Mut and Um CLL patients. To confirm these results, we studied LPL mRNA expression of these 26 CLL patients by quantitative reverse transcription PCR and correlated LPL expression to the analysis of methylation status by bisulphite sequencing. Results showed a significant correlation ($P < 0.0001$) between LPL expression and demethylated status in Um CLL patients and absence of LPL expression and methylated status in Mut CLL patients (Figure 1d). To further characterize this observation, *in vitro* treatment with DNA methyltransferase inhibitor 5-Aza-dC on Daudi cell line was performed. Results showed that exposure to this drug triggered LPL mRNA expression at significant levels compared with untreated cells and that 5-Aza-dC was capable to induce a clear demethylation of R1-LPL region (Supplementary Figure 2). Overall, these data confirm that demethylation in Exon 1/Intron 1 of *LPL* gene correlates with LPL expression in leukemic CLL B cells.

Previous work suggests that lipid metabolism activation is associated with high LPL expression in Um and progressive CLL patients.[13] Therefore, we investigated whether this anomalous expression could be related with proliferative microenvironment signals delivered to the leukemic clone. For this, we stimulated PBMC from six LPL-negative patients with CD40 ligand plus IL-4, anti-IgM, CpG-ODN or Pam3CSK4, (see Material and Methods in Supplementary Data). Activation through CD40/IL-4 was able to induce high expression of *LPL* gene at mRNA and protein levels (Figures 2b and c). Accordingly, this expression was associated with both, DNA demethylation of R1-LPL region and with proliferation of CLL B cells as evidenced by Ki-67 protein expression (Figures 2a and d). Stimulation through the BCR also increased LPL expression and demethylation of R1-LPL region in four out of six CLL samples, as well as Ki-67 protein expression in three of them. In contrast, stimulation through TLR receptors did not result in DNA demethylation and Ki-67 protein expression, nor induced LPL expression in any of the six samples evaluated (Supplementary Figures 3A–C). Results from one representative LPL-negative CLL patient before and following these different stimulations are shown in Figure 2. To better characterize these results, we evaluated whether LPL methylation status and LPL

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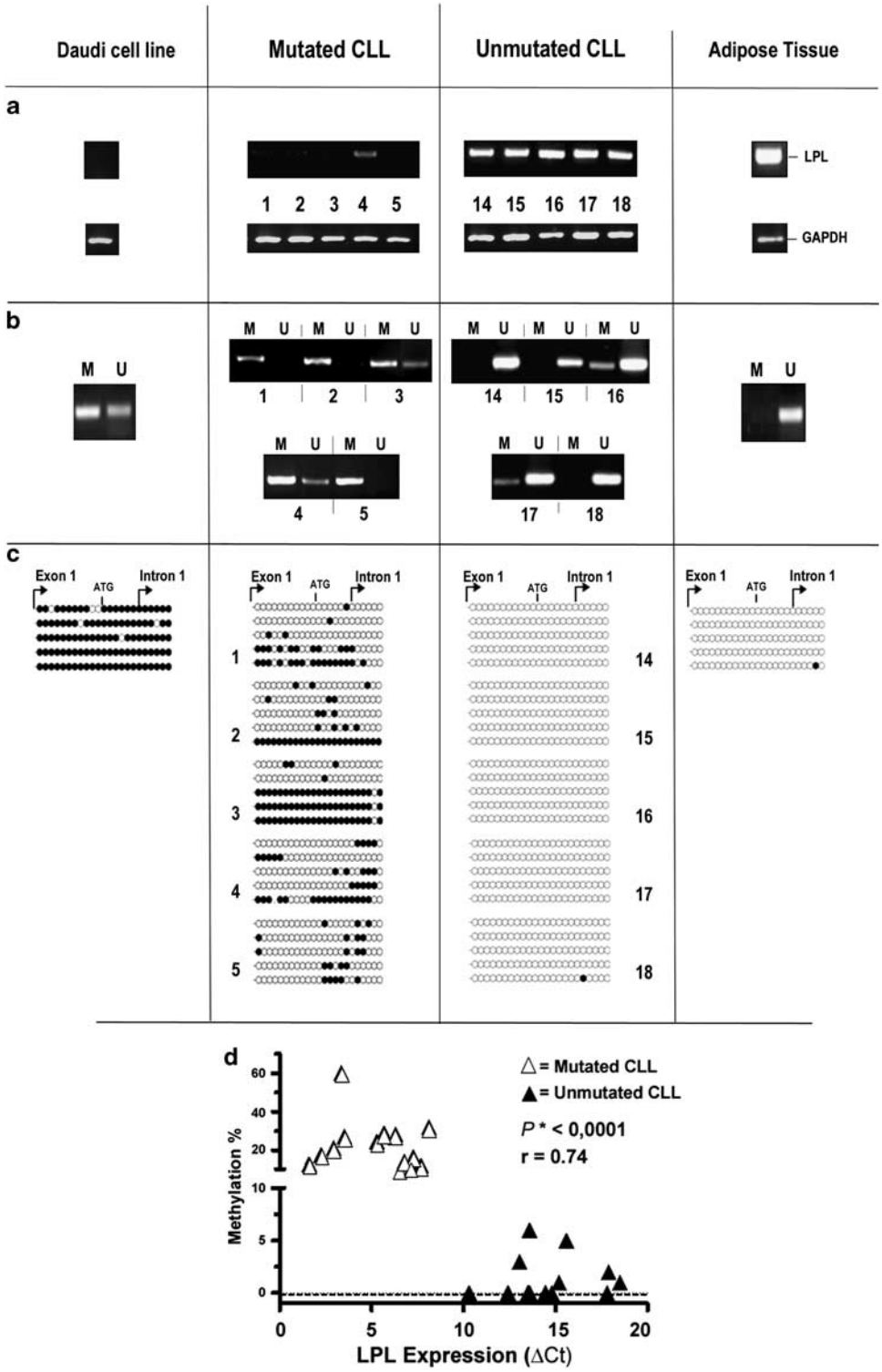

Figure 1. Differential expression of *LPL* gene and methylation status in Mut and Um CLL patients. (**a**–**c**) Results from five Mut (1–5) and five Um (14–18) representative CLL patients, Daudi cell line and adipose tissue samples as negative and positive controls, respectively, are depicted. (**a**) LPL mRNA expression evaluated by RT-PCR is shown in agarose gel stained with ethidium bromide. GAPDH was amplified in all cases as internal control. (**b**) Methylation-specific PCR analysis for R1-LPL region. U, unmethylated and M, methylated. (**c**) Results of bisulphite sequencing of R1-LPL region. Each row represents one bacterial clone in which black and white circles represent methylated and unmethylated CpG dinucleotides, respectively. (**d**) Correlation between LPL mRNA expression of 14 Mut and 12 Um CLL patients evaluated by quantitative reverse transcription PCR and methylation percentage in R1-LPL region is shown. Statistical analysis indicating a significant correlation (*) by Spearman's rank test where *P*-values are $\leqslant 0.001$ is shown. In this case for LPL, expression correlated to methylation status $P < 0.00015$; Spearman's rank coefficient $P = 0.72$.

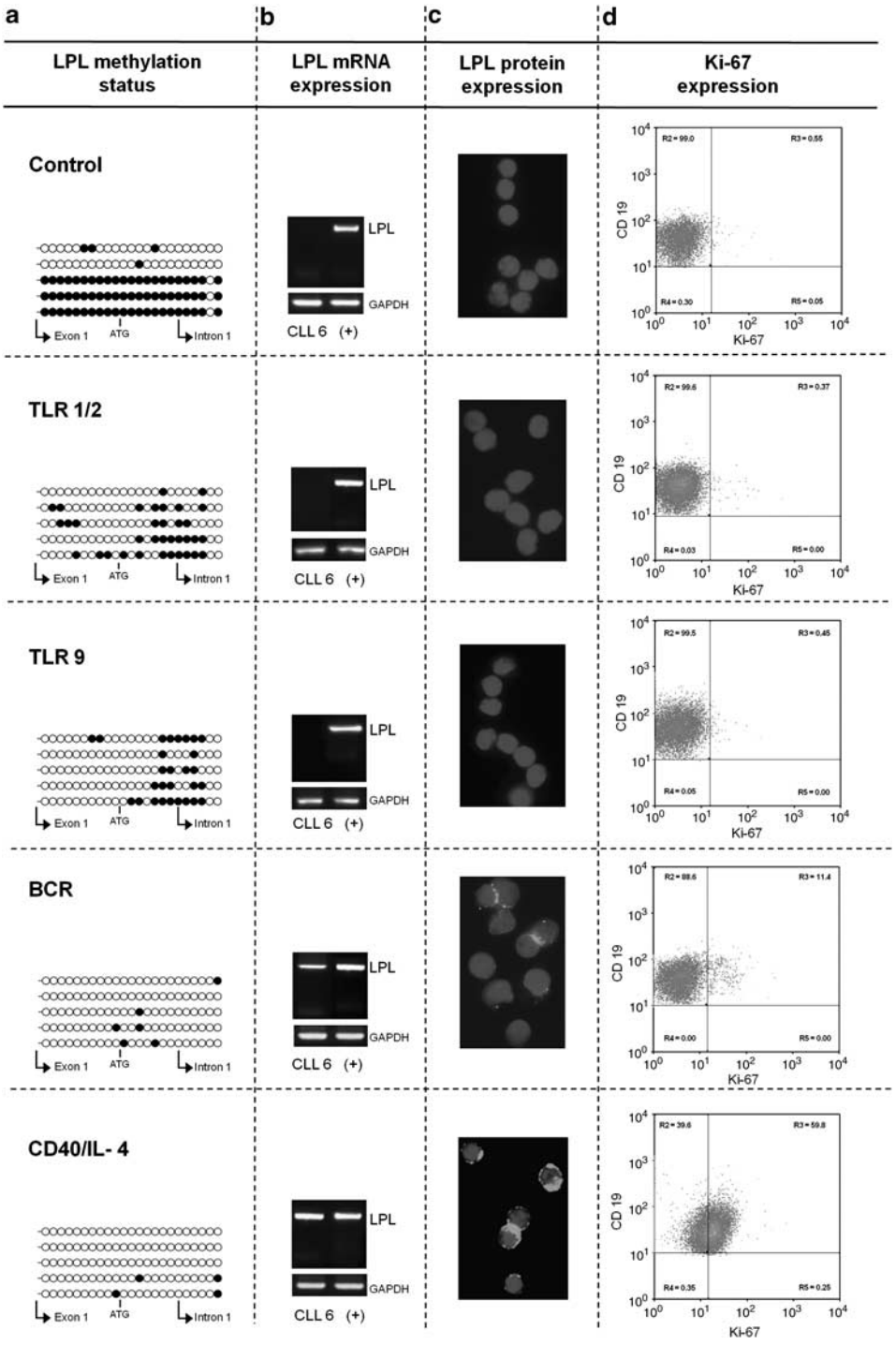

Figure 2. Representative CLL patient after stimulation with different microenvironment signals. (**a**) DNA methylation profile of R1-LPL region of CLL number 6 before and after different activation signals. Each row represents one bacterial clone in which black and white circles represent methylated and unmethylated CpG dinucleotides, respectively. (**b**) LPL mRNA expression by RT-PCR. LPL expression is depicted in agarose gel stained with ethidium bromide. Um/LPL$^{(pos)}$ CLL was used as positive control and GAPDH was used as endogenous control. (**c**) LPL protein expression in CLL patient. Protein expression was visualized by epifluorescence microscopy in Mut/LPL$^{(neg)}$ CLL B cells and in the same CLL case after different stimulations. Green: antibody anti-LPL, blue dye: DAPI. (**d**) Evaluation of Ki-67 expression. Cytometry assays displaying Ki-67 and CD19 expression in CLL patient (Mut/LPL$^{(neg)}$ number 6). Cell populations were discriminated by forward scattering and later B lymphocytes were discriminated by gating CD19 subset. The color reproduction of this figure is available at the *Leukemia* journal online.

expression in CLL B cells could be also affected by their interaction with autologous-activated T cells. To this aim, PBMC from two negative LPL CLL samples and one weak positive CLL sample were stimulated with immobilized anti-CD3 for 4 days. We found that both negative cases became positive for LPL mRNA after autologous T-cell activation, whereas the weakly positive CLL sample slightly enhanced LPL expression. Moreover, the methylation status of R1-LPL region turned into a mostly unmethylated

pattern of CpG dinucleotides (methylation % in Supplementary Figure 3D), confirming previous results obtained with recombinant CD40L and IL-4. Graphics and statistical analysis of the six stimulated CLL samples with the different signals and of the three CLL activated with autologous T cells are provided in the Supplementary Figure 3. Overall, these results link tumoral cell proliferation to a demethylation process in the CpG island of LPL DNA and suggest that expression of this gene in CLL could be related to specific proliferative microenvironment signals.

Evidences indicate that CLL evolution results from the balance between proliferating cells in specialized tissue microenvironment and circulating cells resisting apoptosis.[2] This equilibrium is finely tuned by a set of surface molecules expressed by CLL B cells and modulated in response to environment signals.[14] High expression of *LPL* gene in Um CLL B cells constitutes an unexpected observation. This specific and anomalous expression constitutes not only a suitable prognostic marker in CLL, but could also help to understand the heterogeneous behavior of this disease. LPL has a bridging function in the formation of a trimolecular complex (lipoprotein particle, LPL and heparan sulfate proteoglycans).[9] This role is a very interesting characteristic, because in addition to its catalytical function, LPL expression in Um CLL patients might be associated with the migratory capacity of a tumoral proliferative cell subset. If true, LPL might also act as a crosstalk factor facilitating specific interactions with accessory cells in the tissue microenvironments. The expression of this protein in concert with integrins, such as CD49d, antiapoptotic molecules (BCL2) as well as chemokines (CCL3, CCL4, CXCL12), implicated in the activation of CLL proliferative pool,[14,15] could be responsible for a circular activation loop in which the leukemic clone is continuously nourished.

The role that abnormal LPL expression could have in disease evolution, has been also addressed by previous work from Pallash *et al.*,[10] demonstrating that lipase-associated genes and triglyceride-specific lipase activity were increased when comparing CLL B cells to normal $CD5^+$ B cells. The same authors suggest that lipid metabolism and lipase activity may be functionally relevant in aggressive CLL.[10] Our results showing proliferation of the tumoral clone associated with demethylation and subsequent LPL expression support these results and highlight the idea that *LPL* gene could constitute a potential therapeutic target in Um CLL cases.

In conclusion, by comparing methylation changes in the LPL-CpG island between Um and Mut CLL patients, we demonstrate a clear association between LPL expression and a demethylation process in the CpG island of the *LPL* gene. This process can be induced in the leukemic clone by specific microenvironment signals, delivered by CD40L/IL-4 and anti-IgM, but not by T-independent related signals delivered through Toll-like receptors. Overall, these results suggest that an epigenetic mechanism, triggered by the microenvironment, regulates LPL expression in CLL B cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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