

## 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<sup>+</sup> 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.<sup>1</sup> This leukemic B-cell accumulation results from a complex balance between activation of cell proliferation and inhibition of apoptotic death.<sup>2</sup> 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.<sup>3</sup> 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*.<sup>4</sup> Despite the usefulness of *LPL* for CLL prognosis,<sup>5–8</sup> its functional role and the molecular mechanism regulating its expression remain elusive as yet.

**Q1**  
**Q2**  
*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.<sup>9</sup> 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.<sup>10</sup>

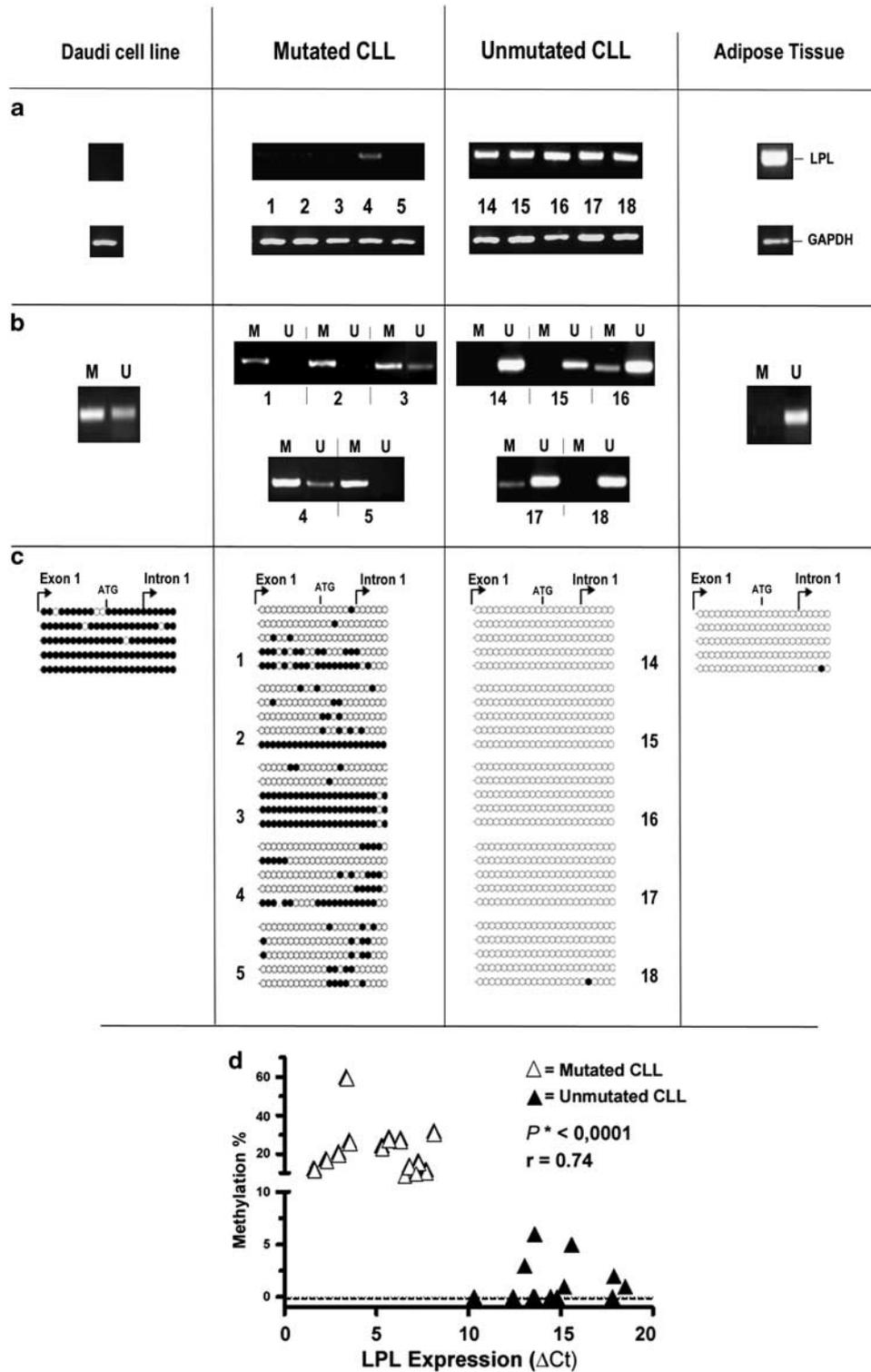
There is increasing evidence that regulation of gene expression during normal lymphocyte development is mediated through changes in chromatin structure and/or through the methylated patterns of CpG islands. Tissue-specific patterns of methylated cytosine residues can be altered by environmental factors,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

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.

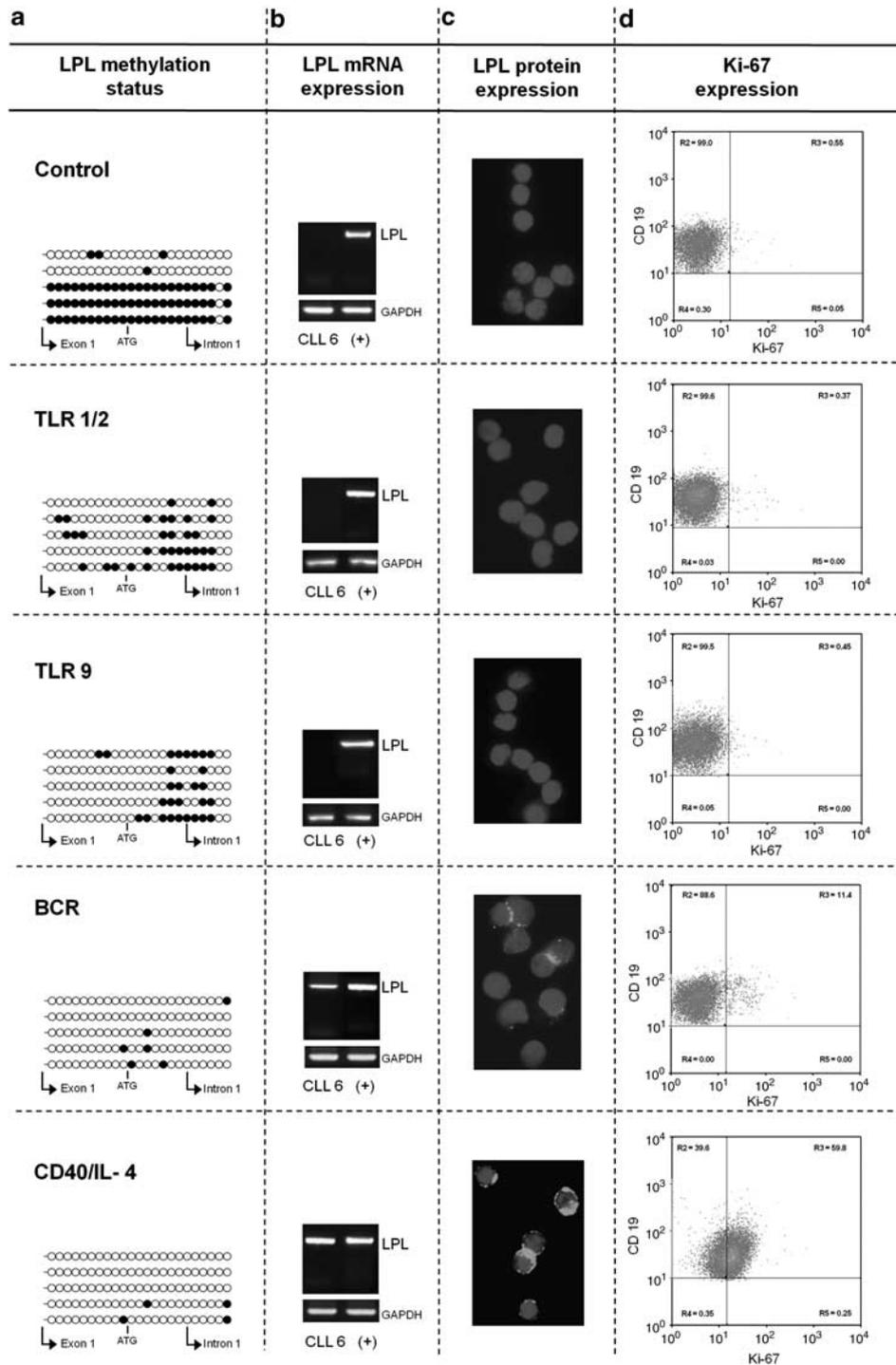
In a first step, we analyzed the CpG sites in the *LPL* gene. This analysis revealed CpG-rich sequences encompassing a CpG island of 1163 bp with 112 CpG dinucleotides. This area includes a region within the first exon and the first intron of the *LPL* gene. To better characterize this CpG island, we 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 transcriptional gene expression.<sup>11</sup> 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.<sup>13</sup> 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* expression in CLL B cells could be also affected by their interaction with autologous-activated T cells. To this aim, PBMC from two



**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  $\leq 0.001$  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<sup>(pos)</sup> 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<sup>(neg)</sup> 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<sup>(neg)</sup> 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.

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.<sup>2</sup> This equilibrium is finely tuned by a set of surface molecules expressed by CLL B cells and modulated in response to environment signals.<sup>1,4</sup> 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).<sup>3</sup> This role is a very interesting characteristic, because in addition to its catalytic 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,<sup>14,15</sup> 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.*,<sup>10</sup> demonstrating that lipase-associated genes and triglyceride-specific lipase activity were increased when comparing CLL B cells to normal CD5<sup>+</sup> B cells. The same authors suggest that lipid metabolism and lipase activity may be functionally relevant in aggressive CLL.<sup>10</sup> 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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#### REFERENCES

- Dighiero G, Hamblin TJ. Chronic lymphocytic leukaemia. *Lancet* 2008; **371**: 1017–1029.
- Caligaris-Cappio F. Role of the microenvironment in chronic lymphocytic leukaemia. *Br J Haematol* 2003; **123**: 380–388.
- Zenz T, Fulda S, Stilgenbauer S. More (on) prognostic factors in chronic lymphocytic leukemia. *Leuk Lymphoma* 2010; **51**: 5–6.
- Oppezzo P, Vasconcelos Y, Settegrana C, Jeannel D, Vuillier F, Legarff-Tavernier M *et al.* The LPL/ADAM29 expression ratio is a novel prognosis indicator in chronic lymphocytic leukemia. *Blood* 2005; **106**: 650–657.
- Heintel D, Kienle D, Shehata M, Krober A, Kroemer E, Schwarzingler I *et al.* High expression of lipoprotein lipase in poor risk B-cell chronic lymphocytic leukemia. *Leukemia* 2005; **19**: 1216–1223.
- Sevov M, Kaderi M, Kanduri M, Mansouri, Buhl A, Cahill N *et al.* A comparative study of RNA-based markers in chronic lymphocytic leukemia reveals LPL as a powerful predictor of clinical outcome. *Haematologica* 2009; **94**(Suppl 3): 1–95.
- Van Bockstaele F, Pede V, Janssens A, Callewaert F, Offner F, Verhasselt B *et al.* Lipoprotein lipase mRNA expression in whole blood is a prognostic marker in B cell chronic lymphocytic leukemia. *Clin Chem* 2007; **53**: 204–212.
- Kaderi MA, Kanduri M, Buhl AM, Sevov M, Cahill N, Gunnarsson R *et al.* LPL is the strongest prognostic factor in a comparative analysis of RNA-based markers in early chronic lymphocytic leukemia. *Haematologica* 2011; **96**: 1153–1160.
- Kolset SO, Salmivirta M. Cell surface heparan sulfate proteoglycans and lipoprotein metabolism. *Cell Mol Life Sci* 1999; **56**: 857–870.
- Pallasch CP, Schwamb J, Konigs S, Schulz A, Debey S, Kofler D *et al.* Targeting lipid metabolism by the lipoprotein lipase inhibitor orlistat results in apoptosis of B-cell chronic lymphocytic leukemia cells. *Leukemia* 2008; **22**: 585–592.
- Brenet F, Moh M, Funk P, Feierstein E, Viale AJ, Socci ND *et al.* DNA methylation of the first exon is tightly linked to transcriptional silencing. *PLoS One* 2011; **6**: e14524.
- Watanabe M, Ogawa Y, Itoh K, Koiwa T, Kadin ME, Watanabe T *et al.* Hypomethylation of CD30 CpG islands with aberrant JunB expression drives CD30 induction in Hodgkin lymphoma and anaplastic large cell lymphoma. *Lab Invest* 2008; **88**: 48–57.
- Bilban M, Heintel D, Scharl T, Woelfel T, Auer MM, Porpaczky E *et al.* Deregulated expression of fat and muscle genes in B-cell chronic lymphocytic leukemia with high lipoprotein lipase expression. *Leukemia* 2006; **20**: 1080–1088.
- Burger JA. Chemokines and chemokine receptors in chronic lymphocytic leukemia (CLL): from understanding the basics towards therapeutic targeting. *Semin Cancer Biol* 2010; **20**: 424–430.
- Palacios F, Moreno P, Morande P, Abreu C, Correa A, Porro V *et al.* High expression of AID and active class switch recombination might account for a more aggressive disease in unmutated CLL patients: link with an activated microenvironment in CLL disease. *Blood* 2010; **115**: 4488–4496.