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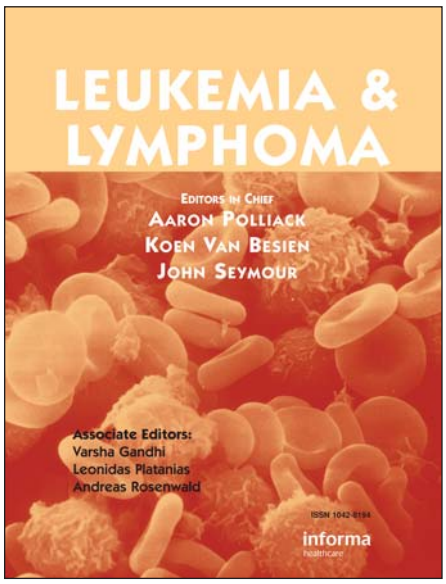

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Methylation status regulates LPL expression in Chronic Lymphocytic Leukemia

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doi: 10.3109/10428194.2013.796057

Abstract

Among different prognostic factors in Chronic Lymphocytic Leukemia (CLL), we previously demonstrated that lipoprotein lipase (LPL) is associated with an unmutated immunoglobulin profile and clinical poor outcome. Despite the usefulness of LPL for CLL prognosis, its functional role and the molecular mechanism regulating its expression are still open questions. Interaction of CLL B-cells with the tissue microenvironment favors disease progression by promoting malignant B-cell growth. Since tissue methylation can be altered by environmental factors, we investigated the methylation status of LPL gene and the possibility that overexpression could be associated with microenvironment signals. Our results show that a demethylated state of the LPL gene is responsible for its anomalous expression in unmutated CLL cases and that this expression is dependent on microenvironment signals. Overall, this work proposes that an epigenetic mechanism, triggered by the microenvironment, regulates LPL expression in CLL disease.

Methylation status regulates LPL expression in Chronic Lymphocytic Leukemia

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Abstract

Among different prognostic factors in Chronic Lymphocytic Leukemia (CLL), we previously demonstrated that lipoprotein lipase (LPL) is associated with an unmutated immunoglobulin profile and clinical poor outcome. Despite the usefulness of LPL for CLL prognosis, its functional role and the molecular mechanism regulating its expression are still open questions. Interaction of CLL B-cells with the tissue microenvironment favors disease progression by promoting malignant B-cell growth. Since tissue methylation can be altered by environmental factors, we investigated the methylation status of LPL gene and the possibility that overexpression could be associated with microenvironment signals. Our results show that a demethylated state of the LPL gene is responsible for its anomalous expression in unmutated CLL cases and that this expression is dependent on microenvironment signals. Overall, this work proposes that an epigenetic mechanism, triggered by the microenvironment, regulates LPL expression in CLL disease.

Keywords: CLL, Methylation, prognostic factors

Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease, with survival ranging from months to decades. The development of the Rai and Binet staging systems has allowed the division of patients with CLL into three prognostic groups: good, intermediate and poor prognosis. The two staging systems have improved the identification of patients who need immediate treatment. However, neither the Rai nor the Binet staging system can predict which patients among the good prognosis group will have progressive disease [1]. Major progress has been made to identify molecular and cellular markers that predict disease progression in CLL patients. Among these markers, cytogenetic abnormalities including 11q or 17p deletions in the leukemic B cells, or the presence of an unmutated (Um) status of the immunoglobulin heavy chain genes (*IGHV*) are the better predictors of rapid progression and shorter survival [1].

Gene-expression profiling (GEP) has introduced a new dimension into our understanding of CLL biology and clinical behavior. Results from GEP in CLL led us to propose that aberrant over-expression of the lipoprotein lipase (LPL) gene is a surrogate marker of the mutational status of *IGHV* [2]. This observation was extensively confirmed by other groups [3-7]. Thus, LPL mRNA over-expression is currently demonstrated to be associated with Um status and clinical poor outcome and also it appears to be the most powerful prognostic tool among RNA-based markers in CLL[8].

Previous work suggests a role for LPL expression in CLL disease, not only in gene expression changes but also in functional pathways related to fatty acid degradation and signaling, which may influence CLL cell behavior [9]. Despite these studies and though the prognostic value of LPL gene is well established, the functional consequences of LPL over-expression in CLL pathogenesis as well as the molecular mechanism regulating its expression remain elusive.

Aberrant DNA methylation has been shown to play a strong role in tumorigenesis, where genome-wide hypomethylation and regional hypermethylation of tumor suppressor gene promoters are characteristic hallmarks of many cancers. Tissue specific patterns of methylated cytosine residues can be altered by environmental factors, and are often abnormal in tumor disorders [10].

The main goal of this investigation was to obtain deep insight into the molecular mechanisms responsible for the high expression of LPL in Um CLL B-cells. In this line, we investigated the methylation status of the LPL gene promoter region, as well as the possibility that its expression could be related to specific signals delivered by the microenvironment.

Our results suggest that demethylated status of LPL gene is responsible for the anomalous expression of this prognostic marker in Um CLL and that this epigenetic mechanism, can be induced in the leukemic clone by microenvironment signals.

Materials and Methods

Patient samples and clinical data.

Peripheral blood mononuclear cells (PBMC) were obtained from 26 patients with CLL. Samples were characterized at a molecular level through CD38, LPL expression, and *IGHV* status as well as fluorescence in-situ hybridization (FISH) analysis. Supplementary Table 1. All patients were followed at the Hospital Maciel, Montevideo and provided an informed consent in accordance with the ethical regulations from Uruguay and the Helsinki Declaration.

RNA Extraction and analysis of LPL transcripts by PCR.

Genomic DNA, RNA extraction, cDNA synthesis, reverse transcription–polymerase chain reaction (RT-PCR) and quantitative PCR (Q-PCR) for LPL amplification were performed as described [2].

Methylation Analysis and 5-Aza-2´-deoxycytidine treatment.

CpG island in the 5'-region of LPL gene was identified by EMBOSS/CpGPlot/CpGReport/Isochore software. Five hundred ng of genomic DNA was treated with sodium bisulphite and amplified using Bisulphite-sequencing primers. Five clones of each CLL patients were cloned and sequenced to evaluate the methylation status of CpG island. In order to confirm the specificity of methylation changes in the LPL methylation status Daudi cell line was cultured over 3 and 5 days in supplemented RPMI media containing 10μM 5-Aza-2´-deoxycytidine (5-Aza-dC) (Sigma Aldrich, USA). Medium was changed every 24 hours and bisulphite analysis was carried out as describe above.

CLL B-cells stimulation with different microenvironment signals.

Stimulation of PBMCs on negative LPL expression patients with autologous T-cells or with recombinant CD40L and IL-4 were performed as described in [11] and [12], respectively. In turn, B-Cell receptor (BCR) stimulation was achieved by anti-IgM (Jackson ImmunoResearch, USA) immobilized to culture plates at 15μg/ml and the cells collected after 4 days.

Flow cytometry analysis.

$1x10^6$ PBMCs were incubated for 45 minutes at 4°C with anti-CD19-PerCP, anti-CD25-PE, anti-CD80-PE, and anti-CD86-FITC, (BD Biosciences, CA, USA). The cells were then washed and analyzed. Negative isotype controls were performed with irrelevant antibody in the same conditions. Results were analyzed by Summit v4.3 software (Dako Inc., CA, USA).

Epifluorescence microscopy.

For intracellular detection of LPL, indirect immunofluorescence technique was performed using mAb anti-LPL, 5D2 antibody (kindly provided by Dr. J. Brunzell, University of Washington). PBMC from CLL patients were isolated by B-Cell Isolation Kit for B-CLL (http://www.miltenyibiotec.com), washed with PBS and 5U/ml heparin (Sigma Aldrich, USA), fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Nonspecific binding was blocked with 5% FBS in PBS, incubated with anti-LPL antibody in overnight at 4ºC, and finally with Alexa® Fluors 488-labeled goat anti-mouse antibody (Invitrogen,

Carlsbad, CA, USA) for 1hr. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). After washes, preparations were visualized (100X) and photographed in Olympus inverted microscope CKX31.

Statistical analyses.

Expression of LPL mRNA, CD25, CD80 and CD86 as activation control proteins and methylation percentage of R1-LPL region were compared between control samples and different activations subsets using either paired Wilcoxon Signed Rank Test or two tailed unpaired Student's *t*-test. Variables with *P* values of less than 0.05 were considered to be significant. The Spearman rank correlation coefficient was calculated to determine the strength of association between LPL expression and methylation percentage. *P* values ≤ 0.01 are considered significant. All analyses were done using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA).

Results

The first exon/intron within the core promoter of LPL gene is differentially methylated in Mutated and Unmutated CLL patients.

In order to address whether LPL gene can be regulated epigenetically through CpG methylation, we analyzed the CpG sites in the LPL gene. This analysis revealed CpG-rich sequences encompassing a classical CpG-island of 1163 bp with 112 CpG dinucleotides. This region is located 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** = 248bp, from +87 bp to +335bp and **R2** = 261bp, from + 446bp to +707bp) (Figure 1A-C). Methylation of the first exon is critical for transcriptional silencing [10]. Accordingly with this, our initial results comparing methylation changes between these two regions in 6 CLL samples showed that main differences appeared to be restricted to R1 (exon 1, CpG dinucleotides number 1-18) and to R2 (first region of intron 1, CpG dinucleotides number 19-23) (Figure 1C). Following this, 26 CLL patients (14 Um expressing LPL and 12 Mut negative for LPL mRNA, Supplementary table 1) were analyzed by bisulphite DNA conversion and sequencing methodology focusing on the R1 of LPL-CpG island (Figure 1C). We found that Mut CLL cases which did not express, or expressed minimal levels of LPL mRNA had a methylated R1 profile. In contrast, Um CLL cases expressing higher levels of LPL mRNA, displayed a demethylated profile of this region (Figure 1D). Overall, these results suggest that differential methylation status is responsible for LPL gene expression in Mut and Um CLL patients.

LPL expression correlates with the methylated status of CpG island and depends on microenvironment signals.

To confirm previous results linking LPL expression with a demethylation status, we studied the LPL mRNA expression of these 26 CLL patients by RQ-PCR. A significant correlation ($p < 0.0001$) between LPL expression and a demethylated status in Um CLL patients and absence of LPL expression and a methylated status in Mut CLL patients (Figure 2A) was found. To further analyze

the epigenetic mechanism of LPL expression we exposed the LPL-negative Daudi cells to 5-Aza-dC, a DNA methyltransferase inhibitor. Result showed that exposure to this drug triggered mRNA LPL expression at significant levels compared with untreated cells (3 and 5 days, $p < 0.01$), and was also able to induce a demethylation of R1-LPL region, Figure 2B. These data confirm that demethylation in Exon 1/Intron 1 of LPL gene is correlated with the expression of this prognostic marker in leukemic CLL B-cells. Additionally, we asked whether LPL anomalous expression in leukemic B-cells could be related to the microenvironment signals. To obtain deep insight in this hypothesis, PBMC from three negative LPL samples were incubated with or without autologous T-cells, activated through TCR cross-linking. After 4 days, CD19/CD5 positive cells were purified and mRNA LPL expression and the methylation status of R1-LPL region were evaluated. As depicted in figure 2C, we found that following autologous T cell activation the leukemic clone expressed LPL and altered the methylation status of R1 LPL-region into a mostly unmethylated pattern. To confirm this result we also stimulated another 6 LPL negative patients through CD40 and IL-4 receptors or through the B cell receptor (BCR). Both activation protocols were able to induce LPL mRNA and protein expression and it was associated with DNA demethylation of R1-LPL region. One representative LPL negative CLL patient with or without stimulations is shown (Figure 2D). Overall, these results suggest that LPL expression in CLL is related with proliferative microenvironment signals that appear to induce a demethylation process in the leukemic clone.

DISCUSSION

Although it has traditionally been assumed that CLL is the consequence of long lived tumor cell accumulation, evidence indicates that disease evolution results from the balance between proliferating cells in specialized tissue microenvironment and circulating cells resisting apoptosis [13]. This equilibrium is finely tuned by a set of surface molecules expressed by CLL B cells and modulated in response to environment signals [14].

Since there is no expression of LPL in normal B cells, the presence of high levels of LPL in Um CLL B-cells is a very interesting issue. This anomalous expression constitutes not only a suitable prognostic marker for the disease but could also be helpful to understand the heterogeneous proliferative behavior of CLL-B cells.

At physiological level, the largest expression of LPL gene has been reported in adipose tissue, skeletal muscle, heart tissue, dendritic cells and CD33 myeloid cells [9]. LPL is a protein located on the luminal side of the wall vessels, where it is anchored to heparan sulfate proteoglycans and contains binding sites for heparan sulfate chains and apoproteins [15]. Furthermore, LPL has a bridging function in the formation of a trimolecular complex including a lipoprotein particle, LPL and heparan sulfate proteoglycans from different cells [15]. This is an interesting characteristic of LPL because, independently of its catalytic function, LPL expression in Um CLL patients may be associated with cell spreading and migratory capacity of the proliferative cell subset [5,15]. If it is

the case, LPL might also act as a crosstalk factor facilitating specific interactions with accessory cells in the tissue microenvironment.

The results present here connect tumor cell proliferation with epigenetic changes on the CpG island of LPL DNA suggesting that LPL expression in CLL is related with microenvironment signals that appear to induce a demethylation process in the leukemic clone. This data is supported by the fact that anomalous LPL expression is a hallmark of Um CLL patients.

In conclusion, this work shows that an epigenetic mechanism, triggered by the microenvironment, is responsible for the anomalous LPL expression in Um CLL patients.

Acknowledgements: This work was supported by grants from CHLCC (Comisión Honoraria de Lucha contra el Cáncer). We wish to thank to Dr. Miquel Llobera by critical comments, to Dr. J. Brunzell by anti-LPL antibody and Mrs. Ivana Faccini for helpful secretarial assistance.

Conflict of Interest Disclosures: The authors declare no competing financial interest

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Figure Legends

Figure 1

CpG island analysis of LPL gene in CLL patients. (A) Localization of the CpG island of LPL gene. CpG island was defined as the region containing over 50% of CpG dinucleotides by EMBOSS/CpGPlot/CpGReport/Isochore software. **(B)** Schematic representation of exon 1 and intron 1 within CpG island of LPL gene. The position of exon1, intron1, the transcription start site (TSS) and the PCR primers for R1 and R2 are depicted as a, b, c and d. **(C)** R1 and R2 profile methylation differences. Methylation analysis of R1 and R2-LPL region was performed in 6 CLL cases (3 Mut and 3 Um) in order to visualize the main changes in the methylation status of both subsets. Shadow squares highlight the fact that the major differences concerning the methylation profile are placed in R1 and not in R2. Each row represents one bacterial clone in which black and white circles represent methylated and unmethylated CpG dinucleotides, respectively**. (D)** Differential expression of LPL gene and methylation status in Mut and Um CLL patients. 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. 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. Methylation status evaluated by bisulphate sequencing of R1-LPL region is shown as lollipop diagrams. Each row represents one bacterial clone in which black and white circles represent methylated and unmethylated CpG dinucleotides, respectively.

Figure 1

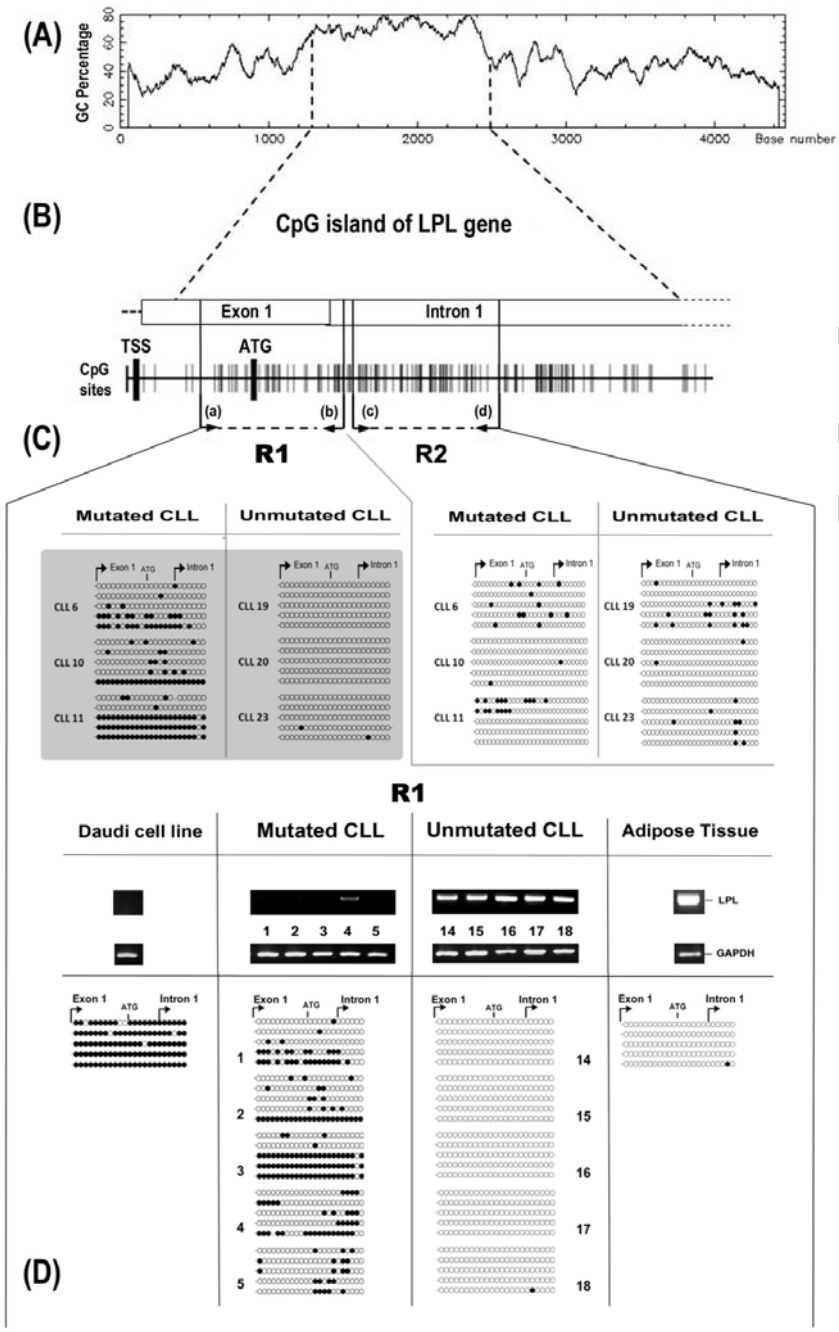

Figure 2

LPL expression and methylation changes analysis after incubation with demethylated agents or microenvironment signals. (A) 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. Statistical analysis indicating a significant correlation by Spearman's rank (*P*-values ≤ 0.001) is shown. In this case LPL expression was correlated to methylation status ($P < 0.00015$; Spearman's rank coefficient $P = 0.72$) in 26 CLL samples. **(B)** Activation of LPL expression and DNA demethylation after 5-Aza-dC treatment. LPL expression levels ($2^{-\Box\Box Ct}$) by QRT-PCR after 5-Aza-dC treatment on Daudi cell line is depicted (error bars indicate range factor difference). The histogram represents mean of three replicates. (** = $P < 0.01$ by unpaired two-tailed Student's t-test). DNA methylation status of R1-LPL region in untreated and treated cells by 5 days is shown. Black and white circles represent methylated and unmethylated cytosine respectively and each row represents one bacterial clone. **(C)** Autologous T-cell activation on CLL B-cells. Black bars depict LPL expression at mRNA levels in control cultures (Ctrol) and in stimulated T-cell cultures (Act). Quantification was performed by QRT-PCR ($2^{-\Box\Box Ct}$). Expression levels of control samples were normalized to 1 as expression relative unit. White bars represent the percentage of methylated CpG dinucleotides in control and activated T-cell cultures. **(D)** Representative CLL sample after stimulation with different microenvironment signals. *LPL Methylation status*: DNA methylation profile of R1-LPL region 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. *LPL mRNA expression*: LPL expression by RT-PCR is depicted in agarose gel stained with ethidium bromide. Um/LPL(pos) CLL sample was used as positive control and GAPDH was used as endogenous control. *LPL protein expression*: Protein expression was visualized by epifluorescence microscopy, Green: antibody anti-LPL, Blue dye: DAPI. Successful activations with autologous T-cells, CD40L/IL-4 and anti-BCR were corroborated by cytometry flow analysis with anti-CD19-PerCP, anti-CD25-PE, anti-CD80-PE and anti-CD86-FITC.

Figure 2