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EFFECTS OF DNMT1, DNMT3A AND DNMT3B GENE EXPRESSION ON CHRONIC LYMPHOCYTIC LEUKEMIA

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Effects of *DNMT1, DNMT3A* and *DNMT3B* gene expression on Chronic Lymphocytic Leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) is the most prevalent adult leukemia in Western World, characterised by the accumulation of monoclonal B lymphocytes with low proliferation rate and impaired apoptosis in blood, bone marrow and secondary lymphoid organs. The disease has a notable clinical course diversity, with different prognosis and distinct treatment needs. However, the exact aetiology is still unknown.

Since cancer cells have global DNA hypomethylation and local hypermethylation, there are evidences that epigenetic modifications have an important role in CLL pathogenesis. DNA methyltransferases (DNMT), including DNMT1, DNMT3A and DNMT3B, are responsible for DNA methylation and changes in the expression of these enzymes were observed in other hematological malignancies.

This study evaluated the expression levels of *DNMT1*, *DNMT3A* and *DNMT3B* genes in CLL patients in order to identify their role in the development of this leukemia and as new potential biomarkers of diagnosis and prognosis.

A total of 67 CLL patients and 23 controls (CTL) were enrolled in the study. Informed consent was obtained from all samples in accordance with the Helsinki Declaration. *DNMT1*, *DNMT3A*, *DNMT3B* and *GUSB* (endogenous control) genes expression levels were evaluated by qPCR. Statistical analysis was performed considering a level of significance of 95%.

CLL patients had a significant lower *DNMT3B* gene expression levels [median: 0.023, interquartile range (IR): 0.040] compared with controls (median: 0.038, IR: 0.267; p=0.002). Additionally, patients with 17p deletion [del(17p)] had higher levels of *DNMT3B* gene expression levels (presence: median: 0.032, IR: 0.026; absence: median 0.014, IR: 0.031; p=0.047). Using ROC analysis, *DNMT3B* expression levels had shown to be a possible diagnosis biomarker (cut-off<0.02639, sensitivity: 62.7%, specificity: 78.3%; p=0.002).

In conclusion, this study suggests that the decrease in *DNMT3B* gene expression levels may contribute to CLL development constituting a new potential diagnostic biomarker. Furthermore,

the correlation of the increase in the expression levels of this *DNMT* with the del(17p), a cytogenetic marker of poor prognosis, suggests that *DNMT3B* gene may have a role in CLL prognosis.

Keywords

Chronic Lymphocytic Leukemia; *DNMT1*; *DNMT3A*; *DNMT3B*; Epigenetics; Diagnostic biomarker; Prognostic biomarker

Resumo

A Leucemia Linfocítica Crónica (LLC) é a leucemia mais comum no Ocidente e é caracterizada pela acumulação de linfócitos B monoclonais, por diminuição da proliferação e comprometimento da apoptose, no sangue, medula óssea e gânglios linfáticos. A doença manifesta-se de uma forma marcadamente heterogénea, com prognóstico variável e diferentes necessidades de tratamento. Deste modo, a sua etiologia permanece desconhecida.

O DNA das células B tumorais apresenta hipometilação global com regiões de hipermetilação, sugerindo que as alterações epigenéticas têm um importante papel na patogénese da LLC. As DNA metiltransferases (DNMT), nomeadamente a DNMT1, a DNMT3A e a DNMT3B, são responsáveis pela regulação da metilação do DNA, tendo sido descritas alterações da sua expressão em algumas doenças hematológicas malignas.

Deste modo, o presente estudo avaliou a expressão génica dos genes *DNMT1*, *DNMT3A* e *DNMT3B* em doentes com LLC com o objetivo de compreender o seu papel no desenvolvimento desta patologia e o seu potencial como marcadores de diagnóstico e prognóstico.

Com este propósito, foi extraído RNA de 67 doentes e 23 controlos (CTL), tendo sido solicitado o seu consentimento informado de acordo com a Declaração de Helsinki. A expressão génica dos genes *DNMT1*, *DNMT3A*, *DNMT3B* e *GUSB* (controlo endógeno) foi avaliada através de qPCR. A análise estatística foi realizada considerando um intervalo de confiança de 95%.

Assim, os nossos resultados demonstraram que os doentes apresentam uma menor expressão génica de *DNMT3B* (mediana: 0.023, amplitude interquartil: 0.040) comparativamente com os controlos (mediana: 0.038, amplitude interquartil: 0.267; p=0.002). Para além disso, doentes com deleção do braço curto do cromossoma 17 [del(17p)] possuem níveis mais elevados de expressão do gene *DNMT3B* (presença: mediana: 0.032, amplitude interquartil: 0.026; ausência: mediana: 0.014, amplitude interquartil: 0.031; p=0.047). A partir da análise da curva ROC, a expressão génica de *DNMT3B* surgiu como um potencial marcador de diagnóstico da LLC (cut-off<0.02639, sensibilidade: 62.7%, especificidade: 78.3%; p=0.002).

Concluindo, este estudo sugere que a expressão do gene *DNMT3B* poderá contribuir para a patogénese da LLC e constituir um potencial marcador de diagnóstico da doença. Por conseguinte, o aumento dos níveis de expressão o gene *DNMT3B* com a presença da del(17p), um marcador de mau prognóstico da doença, sugere que este gene poderá também ter impacto no prognóstico da LLC.

Palavras-chave

Leucemia Linfocítica Crónica; *DNMT1*; *DNMT3A*; *DNMT3B*; Epigenética; Marcador de diagnóstico; Marcador de prognóstico

Abbreviations

CD, Cluster of differentiation

cDNA, Complementary deoxyribonucleic acid

CLL, Chronic Lymphocytic Leukemia

CpG, Cytosine Guanine dinucleotide

CTL, Controls

Del, Deletion

DNA, Deoxyribonucleic acid

DNMT, DNA Methyltransferase

EDTA, Ethylenediaminetetraacetic acid

FISH, Fluorescence in situ hybridization

GUSB, Glucuronidase Beta

Hb, Hemoglobin

IGHV, Immunoglobulin Heavy Chain Variable

IPI, International Prognostic Index

IQ, Interquartile range

MBL, Monoclonal B-cell lymphocytosis

NPV, Negative Predictive Value

OD, Optical density

OS, Overall survival

PPV, Positive Predictive Value

qPCR, Real Time Polymerase Chain Reaction

RNA, Ribonucleic acid

ROC, Receiver operating characteristic

TP53, Tumor protein p53

TET, Ten-eleven translocation

TDG, Thymine DNA glycosylase

Tri, Trisomy

ZAP, Zeta chain associated protein kinase 70

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western World, affecting mainly the elderly individuals with a median age at diagnosis of 71 years old and a male to female ratio of 2:1.[1-3] The disease is characterised by the accumulation of monoclonal B lymphocytes with low proliferation rate and impaired apoptosis in bone marrow, blood and secondary lymphoid.[4,5] CLL cells have a mature cell morphology appearance but are immunologically immature. CLL can be preceded by a premalignant precursor state denominated monoclonal B-cell lymphocytosis (MBL).[2]

The presence of lymphocytosis (≥5000 monoclonal B lymphocytes/μl) is required to establish the diagnosis and the clonality needs to be confirmed by flow cytometry.[2,3] They express CD5, CD19, CD20 and CD23 surface antigens, however levels of surface immunoglobulins, CD20 and CD79b are lower than in normal B cells.[2,3] Furthermore, peripheral blood smear should show cells typically small, mature-appearing lymphocytes with a dense nucleus lacking discernible nucleoli and a narrow border of cytoplasm and chromatin partially aggregated.[2,3]

The exact aetiology of CLL is still unknown[4] and patients have a notable clinical course diversity.[6-9] Although there are patients that can live more than 10 years without any therapeutic intervention, there are also those with a median overall survival lower than 3 years, even using effective chemotherapy.[9] Consequently, CLL patients have different prognosis and distinct treatment needs[5]. The main staging systems used to predict evolution and survival are Binet and Rai (Table 1), and more recently the CLL-International Prognostic Index (CLL-IPI).[2,3,10] However they are manifestly insufficient to predict the management and prognosis in a biologically and clinically heterogeneous disease.

Cytogenetic analysis is a helpful tool to study somatic alterations that have prognostic impact and therapeutic consequences, such as the deletion of short arm of chromosome 17 [del(17p)], the deletion of long arm of chromosomes 11 [del(11q)] and 13 [del(13q)], and trisomy 12 [tri(12)], between other events.[1-4,6,11] These genetic anomalies are found in more than 80% of CLL patients.[3,4,9] Other molecular analysis should be performed including *TP53* mutations and the immunoglobulin heavy chain variable region (IGHV) mutation status.[2,4] Patients who have CLL

cells with del(17p) and/or *TP53* mutation and del(11q) are associated with poor outcomes.[3] More recently, new molecular alterations present in CLL were described as prognostic biomarkers, such as *NOTCH1*, *SF3B1*, *BIRC3* and *ATM* mutations.[10] In this way, FISH and conventional karyotyping can help to define CLL prognosis,[2,3] although the impact of these cytogenetic abnormalities and others molecular alterations in pathogenesis still needs further research.[2]

Table 1. Staging systems and prognosis for chronic lymphocytic leukemia.

Risk Group	Staging systems				Prognosis (in years)
	Binet		Rai		
Low-risk	**A**	Hb ≥ 10.0 g/dL Thrombocytes ≥ 100×10^9/L < 3 Lymphadenopathy	**0**	Lymphocytes >15×10^9/L	>10
Intermediate-risk	**B**	Hb ≥ 10.0 g/dL Thrombocytes ≥ 100×10^9/L ≥ 3 Lymphadenopathy	**I**	Lymphocytes >15×10^9/L Lymphadenopathy	>8
			II	Lymphocytes >15×10^9/L Hepatomegaly/Splenomegaly	
High-risk	**C**	Hb < 10.0 g/dL Thrombocytes < 100×10^9/L	**III**	Lymphocytes >15×10^9/L Hb < 11.0 g/dL	6,5
			IV	Lymphocytosis Thrombocytes < 100×10^9/L	

Hb, hemoglobin. Adapted from Eichhorst *et al* (2015).[2]

The role of epigenetic in cancer has been investigated, including in CLL.[4,7,8,12] These mechanisms are responsible for the regulation of gene expression without changing DNA sequence[1,13] and allow a correct embryonic development, cellular differentiation and genomic stability.[14,15] DNA methylation, histone modification and microRNA expression are the main mechanisms capable of changing DNA accessibility.[13,16] Methylation consists in the addition of a methyl group, from the S-adenylmethionine donnor, to the C5 position of cytosines, characteristically in CpG dinucleotides, creating a 5-methylcytosine.[1,4,7,13,17-20] DNA methylation is controlled by three main DNA methyltransferases.[1,5,12,13,17,18] DNMT1 is a maintenance enzyme, capable of recognizing the hemimethylated DNA and restoring the symmetry.[12,13,17-19] DNMT3A and DNMT3B are *de novo* methyltransferases, responsible to methylate previously unmethylated DNA.[12,13,17,18] Methylation pattern in body cells is bimodal, with more than 80% of

CpG sites methylated and less than 10% unmethylated.[16] Demethylation can result from a passive mechanism which consist in a lack of functional DNA methylation maintenance machinery or from the TET-TDG pathway, an active process.[20] In this way, the dynamic balance between methylation and demethylation is very important to manage gene expression and protects chromosomes from instability, genetic translocation and gene disruption.[5]

Studies observed that cancer cells, comparatively with healthy cells, have a global DNA hypomethylation, activating oncogenes and transposons, and a regional hypermethylation, frequently associated with tumour suppressor genes.[6-8,11] This phenomenon is also present in CLL cells but how this affects disease pathogenesis and progression is not completely understood.[1,4,6,12,19-22]

In this context, changes in *DNMT* expression were verified in many cancer types, including hematologic malignancies, such as acute and chronic myelogenous leukemia and diffuse large B-cell lymphomas.[15,16,23,24] Therefore, this study analysed the expression levels of *DNMT1*, *DNMT3A* and *DNMT3B* genes in order to identify their role in CLL development and determinate their potential as biomarkers of diagnosis and prognosis.

Materials and Methods

Ethical Statement

Informed consent was obtained from all samples in accordance with the Helsinki Declaration. The protocol was approved by the Ethics Committee of the Faculty of Medicine of University of Coimbra.

Study Population

A total of 67 CLL patients and 23 controls (CTL) were enrolled in the study. Individuals were followed in the Hematology Service of Centro Hospitalar e Universitário de Coimbra, EPE (CHUC, EPE) and Hospital Distrital da Figueira da Foz, EPE (HDFF, EPE), since 2010 to 2017. Controls were selected from a group of healthy volunteers without evidence of neoplasm. Demographic features for patients and controls (gender and age) and patient's clinical description (B symptoms, Binet and Rai classification, cytogenic abnormalities and survival) were collected.

RNA isolation

Peripheral blood samples were collected from CLL patients and controls with EDTA tubes and instantly storage at 4ºC. After erythrocyte lysis, leukocytes were isolated and RNA was extracted using tripleXtractor reagent (Grisp, Portugal), according to manufacturer instructions. Thereafter, total RNA concentration and purity (OD260/OD280) was quantified using *Nanodrop 1000* (*NanoDrop Technologies, Wilmington, DE*). Finally, it was stored at -80ºC until cDNA synthesis.

cDNA Synthesis

To synthesize cDNA from RNA previously extrated, Xpert cDNA Synthesis Mastermix (Grisp, Portugal) was used according manufacturer instructions, with oligo(dT) and random *hexamers* as primers. Lastly, cDNA was stored at -20ºC until qPCR analysis.

Real Time PCR

The genes expression levels of *DNMT1*, *DNMT3A* and *DNMT3B* were analysed by real time PCR (qPCR) using Xpert Fast SYBR (Grisp, Portugal), according manufacturer instructions, and normalised to *GUSB* expression (endogenous control).

The following primers were used: housekeeping *GUSB* (forward: 5'-CACCAGGGACCATCCAATACC-3'; reverse: 5'- GCAGTCCAGCGTAGTTGAAAAA-3'), *DNMT1* (forward: 5'- ACCATCAGGCATTCTACCA-3'; reverse: 5'-TCTCCTTGTCTTCTCTGTCAT-3'), *DNMT3A* (forward: 5'-CGCTAATAACCACGACCAG-3'; reverse: 5'-CGATTCCATCAAAGAGAGACA-3') and *DNMT3B* (forward: 5'-ACTTGGTGATTGGCGGAAG-3'; reverse: 5'-GTGAGTAATTCAGCAGGTGGTAA-3').

The qPCR was performed using QuantStudio3 Thermo Fisher Scientific, in 96-well plates. All samples were in duplicate. The thermocycling parameters were one cycle of 20 seconds at 90ºC and 40 cycles of 3 seconds at 95ºC and 30 seconds at 60ºC. The relative expression was calculated with $2^{-\Delta Ct}$.

Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics® version 25. Normality was assessed by Kolmogorov-Smirnov and Shapiro-Wilk analysis. For non-normally distributed variables, Mann-Whitney and Kruskal-Wallis tests were performed to evaluate clinical significant differences between two groups (patients and controls; each clinical characteristic and *DNMT*s expression) and more than two groups (*DNMT*s expression and Rai stage classification and risk groups), respectively. The receiver operating characteristics (ROC) curve analysis was performed to evaluate *DNMT*'s accuracy as diagnostic biomarker and predictor of death and del(17p) presence. Overall survival (OS) was defined as the time interval from diagnosis to the last follow-up or to death and it was analysed using Kaplain-Meier method. A p value ≤0.05 was considered statistically significant.

Results

Characterization of Study Population

The present study included a group of chronic lymphocytic leukemia patients (*n*=67) and a control group (*n*=23), their characteristics are described in Table 2. CLL group was composed by 35 men (53.8%) and 30 women (46.2%), between 48 and 101 years old, with a median age of 73 years old. Control group was composed by 8 men (34.8%) and 15 women (65.2%), between 58 and 88 years old, with a median age of 72 years old. To confirm adequate matching between these two groups we assessed differences in demographic characteristics and there was no statistical differences in terms of age (*p*=0.148) and gender (*p*=0.596).

According Rai classification, CLL patients group included 44 individuals with stage 0 (73.3%), 5 with stage I (8.3%), 7 with stage II (11.7%), 3 with stage III (5.0%) and 1 with stage IV (1.7%). In this way, the study was composed by 44 patients with low risk (73.3%), 12 with intermediate risk (20.0%) and 4 with high risk (6.7%). B symptoms were found in 7 patients (11.9%). The distribution of cytogenetic abnormalities at diagnosis shows the presence 22 patients with del(13q) (40.7%), 12 with del(17p) (22.2%), 7 with del(11q) (13.0%) and 6 with tri(12) (11.1.0%). Most CLL patients were still alive (*n*=32; 53.3%) and 28 died during follow-up period (46.7%), considering a median survival of 78.72 months (range: 6.70-290.30).

Table 2. Demographic and clinical characteristics of chronic lymphocytic leukemia patients and controls.

		CLL patients (*n*=67)		**Controls** (*n*=23)	
		n	%	*n*	%
Gender		*n*=65		23	
	Male	35	53.8	8	34.8
	Female	30	46.2	15	65.2
Age (years)					
	Median age	73.00		72.00	
	Range	48-101		58-88	
B Symptoms		*n*=59			
	Presence	7	11.9		
	Absence	52	88.1		
Rai		*n*=60			
	0	44	73.3		
	I	5	8.3		
	II	7	11.7		
	III	3	5.0		
	IV	1	1.7		
Risk Group		*n*=60			
	Low	44	73.3		
	Intermediate	12	20.0		
	High	4	6.7		
Cytogenic Abnormalities		*n*=54			
Del(13q)	Presence	22	40.7		
	Absence	32	59.3		
Del(17p)	Presence	12	22.2		
	Absence	42	77.8		
Del(11q)	Presence	7	13.0		
	Absence	47	87.0		
Tri(12)	Presence	6	11.1		
	Absence	48	88.9		
Survival Rates		*n*=60			
	Death	28	46.7		
	Alive	32	53.3		

CLL, Chronic Lymphocytic Leukemia. Del, deletion. *n*, number of cases. Tri, trisomy. %, percentage.

Evaluation of *DNMT1*, *DNMT3A* and *DNMT3B* expression

The expression level of *DNMT1*, *DNMT3A* and *DNMT3B* genes were analysed by qPCR in CLL patients and controls. In this study, we observed that CLL patients had a slight increase in the expression of *DNMT1* gene [median: 0.691, interquartile range (IR): 0.990, Figure 1] and similar expression of *DNMT3A* (median: 0.283, IR: 0.268, Figure 2) in comparison to controls (*DNMT1*: median: 0.603, IR: 0.995; *DNMT3A*: median: 0.281, IR: 0.284). However, as observed in Figure 3, a significant lower expression of *DNMT3B* gene was found in CLL patients (median: 0.023, IR: 0.040) when compared to controls (median: 0.038, IR: 0.267, p=0.002). Additionally, in 12 CLL patients (18%) no expression of *DNMT3B* was detected.

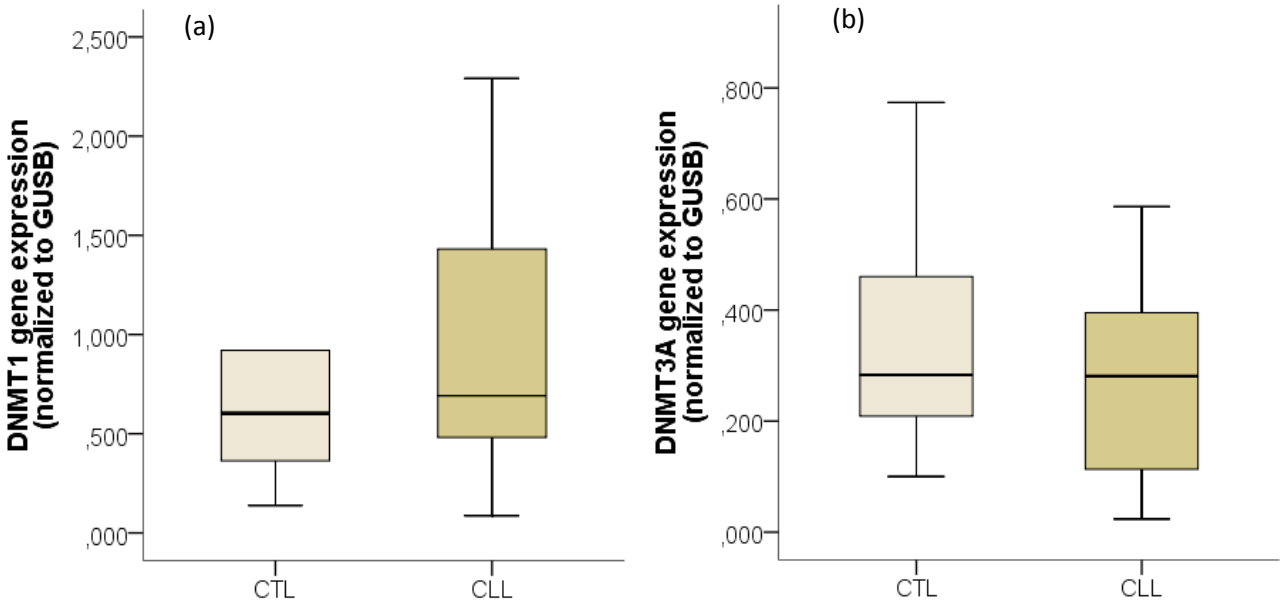

Figure 1. Analysis of *DNMT1* (a) and *DNMT3A* (b) genes expression levels in controls and chronic lymphocytic leukemia patients by real time PCR. CLL, Chronic Lymphocytic Leukemia. CTL, Controls. p>0.05.

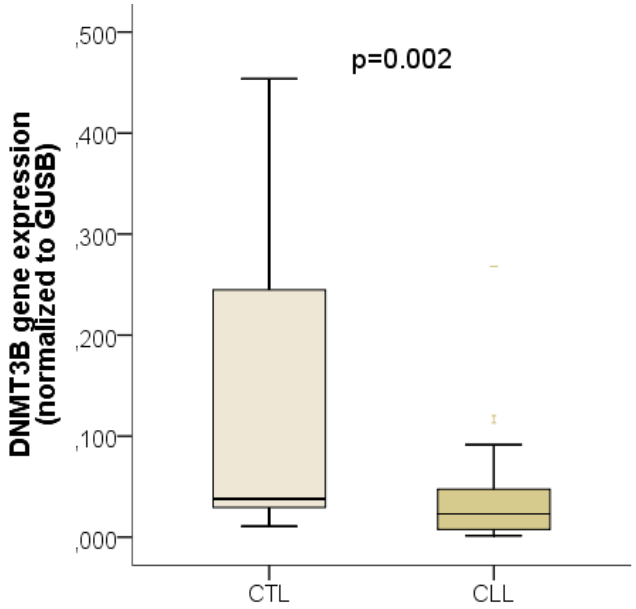

Figure 2. Analysis of *DNMT3B gene* expression levels in controls and chronic lymphocytic leukemia patients by real time PCR. Gene expression was absent in 12 samples (18%). CLL, Chronic Lymphocytic Leukemia. CTL, Controls.

Correlation between *DNMT*s expression levels and clinical characteristics of CLL patients

Next, we correlated the *DNMT*s expression levels with CLL patient's clinical parameters. First, we did not find any significant association between the *DNMTs genes* expression levels and the presence of B symptoms. Similarly, no significant association between Rai stages or risk groups and *DNMT*s expression levels was found (p>0.05). Furthermore, this study did not find any significant correlation between tri(12), del(11q) and del(13q) and each *DNMT* gene expression levels (p>0.05). However, patients with del(17p) showed significant high expression levels of *DNMT3B* (median: 0.032, IR: 0.026) than patients without this cytogenetic abnormality (median 0.014, IR: 0.031, p=0.047, Figure 4). We analysed ROC curves to determinate if DNMT3B gene expression could discriminate the presence or absence of del(17p) and no statistical significance was found. The expression of *DNMT1* and *DNMT3A* genes did not have significant correlation with the presence or absence of del(17p).

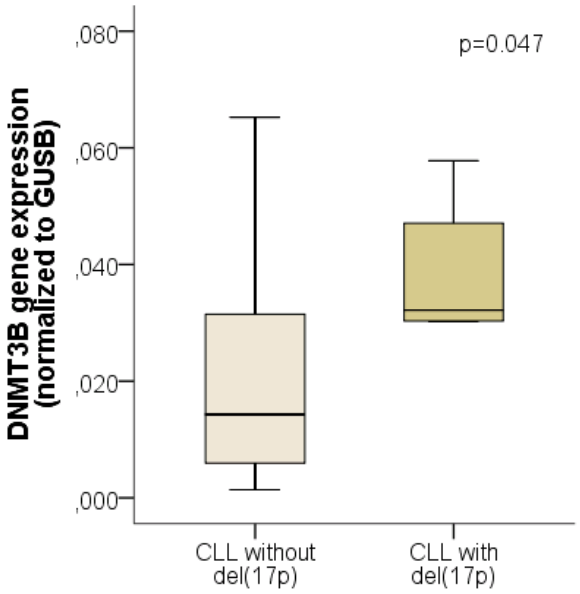

Figure 3. Analysis of *DNMT3B* expression levels according to cytogenetic status of chromosome 17. Del, deletion.

Correlation between *DNMT*s expression levels and CLL diagnosis and prognosis

In order to evaluate if *DNMT*s could be potential biomarkers of CLL diagnostic, we analysed ROC curves, to determinate a gene expression cut-off for each *DNMT* gene capable of discriminate controls from patients. As observed in Figure 5, we found that *DNMT3B* expression could be considered a good diagnostic biomarker, with an area under the curve value of 0.716 (95% confidence interval: 0.592-0.841; *p*=0.002). *DNMT3B* levels lower than 0.02639 was able to discriminate CLL patients from controls with a sensitivity of 62.7%, a specificity of 78.3%, a Positive Predictive Value (PPV) of 89.4% and a Negative Predictive Value (NPV) of 41.9%.

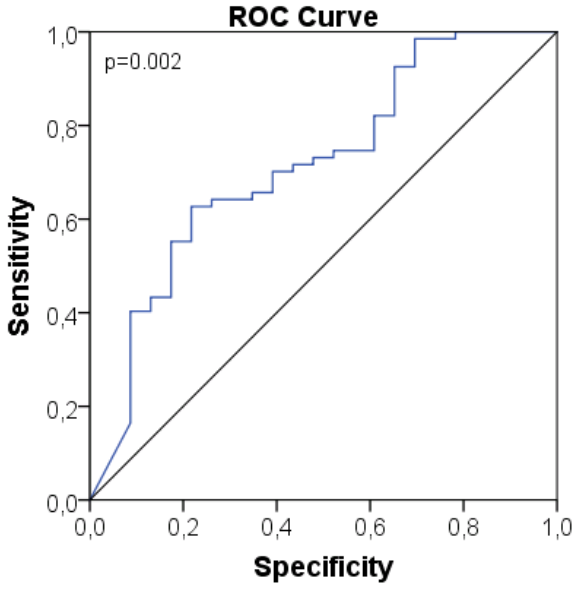

Figure 4. Receiver operating characteristic curve for prediction of chronic lymphocytic leukemia based on *DNMT3B* expression levels. ROC, receiver operating characteristic. DNMT, DNA Methyltransferase.

To study the influence of *DNMT*3B expression levels in survival of CLL patients, we stratified patients according to the cut-off previously obtained by ROC curve. Despite the initial propensity for higher levels of *DNMT3B* expression to reveal worse survival outcomes, no statistical significance was found.

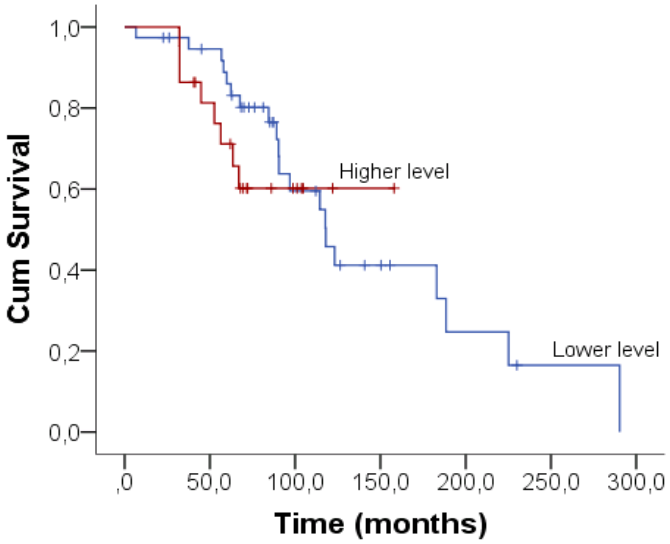

Figure 5. Survival of chronic lymphocytic leukemia patients, according *DNMT3B* expression levels by Kaplan Meier analysis. Higher level: >0.02639. Lower level: <0.02639. ROC, receiver operating characteristic. *p*>0.05.

Discussion

The DNA methylation status in CLL has been established by several studies.[1,4,6,7,12,21] In general, the DNA from CLL patients is globally hypomethylated, activating regulatory sequences, such as Alu, SAT-α and LINE-1, which results in chromatin instability and overexpression of oncogenes.[1,6] Paradoxically, regional hypermethylation leads to the silencing of tumor suppressors, transcription factors, microRNA genes and genes involved in survival and proliferation, such as *DLEU7*, *hTERT*, *HOX4A* and *DAPK1*.[1,12] DNA methylation is a complex mechanism regulated mainly by DNMT1, DNMT3A and DNMT3B.[1,12-18] Previous studies correlated *DNMTs* expression levels with the development of solid tumours, namely breast,[15,23] colon,[15,23] esophageal,[15,23,25] gastric,[23] kidney,[15,23] liver,[23] pancreatic[23] and prostate,[15,23,26] considering it a helpful target to improve patient management and treatment effectiveness.

In the field of haematological malignancies, Mizuno *et al.* (2001) showed that blood cells from patients with acute myeloid leukemia presented *DNMT1* overexpression and patients with chronic myeloid leukemia in acute phase had an overexpression of *DNMT1* and *DNMT3A* genes.[16] Additionally, Amara *et al* (2010) observed that all *DNMT*s genes were overexpressed in patients with diffuse large B-cell lymphomas.[24] In these studies, the increased *DNMT*s expression was associated with hypermethylation of tumour suppressors, inhibition of apoptosis and worse prognosis.

The present study investigated the expression levels of *DNMT*s genes in CLL patients. According to our study, DNMT1 seems to be overexpressed in CLL patients, even though this result was not statistically significant. Kn *et al* (2004), observed that *DNMT1* expression may be near normal in CLL[27] and Bagacean *et al* (2017) showed, in contrast, downregulation.[28] Although we could not find correlation between the expression of *DNMT3A* in CLL patients and controls, this is not supported by Kn *et al* (2004) and Bagacean *et al* (2017) results that observed reduction and upregulation of *DNMT3A* in CLL patients, respectively.[27,28] These differences may be correlated with the use of a low number of samples and with its collection timing.

Although we did not detect any *DNMT3B* gene expression in 12 CLL patients, our results showed that patients had lower expression of this gene when comparing with controls. This finding is not supported by studies in other neoplasms as mentioned before. However, it is in

agreement with other authors that observed a *DNMT3B* downregulation in CLL.[27,28] Some reports analysed the impact of the loss of DNMT3B function in mice models of MYC-induced lymphomagenesis and concluded that knockout of *DNMT3B* in B and T cells accelerated cellular proliferation.[29,30] In this way, Hlady *et al* (2012) observed that the *Gm128* (*C1ORF56* in humans) gene, a proto-oncogene methylated and silenced in normal thymocytes, is demethylated and overexpressed in these *DNMT3B*$^{-/-}$ malignancies.[30] Other gene promoters methylation status appears to depend on DNMT3B function, which suggested that DNMT3B plays a role in the maintenance of promoter methylation and its loss can result in hypomethylation of the genome with subsequent genomic instability and deregulation of proto-oncogenes.[29,30] Therefore, *DNMT3B* seems to have tumor suppressor functions.[29,30] Moreover, *DNMT3B* is expressed in over thirty different isoforms which can be catalytically inactive or active, resulting from alternative splicing and promoter usage,[31,32] such as *DNMT3B7,* which is one of the most commonly expressed catalytically inactive variant.[33] Alkebsi *et al* (2013) correlated DNMT3B7 overexpression with demethylation and overexpression of *Gm128* in diffuse large B cell and mantle cell lymphomas,[33] in contrast to Hlady *et al* (2012) findings.[30] In this way, *DNMT3B* underexpression and hypomethylation may have an important role in CLL pathogenesis. However, since controversial results in different malignancies are found and the mechanisms are not completely known, more studies are needed to clarify how *DNMT3B* and its isoforms expression influences the DNA methylation pattern and how it is correlated with enzyme translation, functionality and degradation.

In order to confirm the potential useful of *DNMT3B* as a biomarker for cancer diagnosis, we need to evaluate two fundamental criteria: the sensitivity and specificity. We demonstrated that *DNMT3B* gene expression levels could be used as diagnostic biomarker, using a threshold of 0.02639. Therefore, expression levels equal or under this cut-off could diagnose CLL with 62.7% of sensitivity and 78.3% of specificity.

Several cytogenetic alterations have been described as having a prognostic impact in CLL. Among them, deletion of short arm of chromosome 17 is found in 2-4% of CLL cases at diagnosis but it can be also acquired during disease development.[34] This cytogenetic anomaly is associated with rapid disease evolution, poor response or refractoriness to treatment and shortest overall survival and thereby unfavourable prognosis.[34-36] Some studies correlate the negative impact of del(17p) in CLL evolution with the loss of one allele of *TP53* gene, which has an important role in DNA repair, cell cycle arrest and cell apoptosis.[35,36] More than 80% of CLL

patients who carry del(17p) in one allele have *TP53* mutation on the other allele, which results in a complete failure of protein activity.[35] Our study showed that del(17p) may be related with *DNMT3B* since CLL patients with this abnormality have higher levels of gene expression when compared with patients without the same anomaly. Other studies demonstrated the suppressed effect of *TP53* in *DNMT* transcription in naïve embryonic stem cells and lung cancer cells.[37,38] According to this, when *TP53* gene is lower expressed, due to his deletion or mutation, *DNMT*s are overexpressed, suggesting that p53 may restrict *DNMT*s expression,[37,38] which might explain our results. Although not statistically significant, our results showed lower survival rates in patients with higher *DNMT3B* expression levels which were also correlated with a poor prognosis deletion. This suggests that this gene expression may have a role in CLL prognosis however more studies are need to confirm this finding. Additionally, we evaluated 12 patients with del(17p), which represent 22.2% of CLL patients, a much higher percentage than the prevalence described. This may be influenced by methodological differences when accessing this cytogenetic abnormality. Consequently, more studies are required to enhance this association, to clarify the mechanisms and understand the impact in the methylation pattern.

Conclusion

Concluding, our study demonstrated that *DNMT3B* gene expression levels may have an important role in CLL pathogenesis and therefore might present a potential diagnostic biomarker. Additionally, our results show that del(17p) may be related with *DNMT3B* expression and this association may contribute to disease development and prognosis.

Relevant limitations and future approaches

Nevertheless, the evaluation of a small control group and the limited access to clinical information of CLL patients might have compromised the relevance of our results. In the light of this, further studies with higher number of samples and more complete clinical data of CLL patients may present stronger results.

Additionally, this study did not evaluate DNA methylation pattern, which is necessary to understand the impact of *DNMT* expression levels on methylation status. Moreover, our results

only included general *DNMT*s transcriptional analysis and we did not evaluate isoforms or enzyme activity, highlighting the need for further investigation in order to clarify the role of DNA mechanisms and particularly the impact of DNMTs in CLL pathogenesis and progression.

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Conflict of interest

Authors do not have any conflict of interest to declare.

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