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Article

Chronic Lymphocytic Leukemia (CLL): evaluation of AKT protein kinase and microRNA gene expression related to disease pathogenesis

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The present study evaluated 56 patients diagnosed with Chronic Lymphocytic Leukemia (CLL) and a control group of 44 clinically healthy subjects with no previous history of leukemia. Genetic expressions of AKT and microRNAs were evaluated by quantitative PCR (qPCR). A significant increase in AKT gene expression in patients when compared to controls was observed (p = 0.017). When the patients were stratified according to Binet subgroups, a significant difference was observed between the subgroups, with this protein kinase appearing more expressed in the B+C subgroup (p = 0.013). Regarding miRNA expression, miR-let-7b and miR-26a were reduced in CLL patients, when compared to controls. However, no significant differences were observed in these microRNA expressions between the Binet subgroups (A versus B+C). By contrast, miR-21 to miR-27a oncogenes showed no expression difference between CLL patients and controls. AKT protein kinase is involved in the signaling cascade that occurs with BCR receptor activation, leading to increased lymphocyte survival and protection against the induction of cell death in CLL. Thus, increased AKT protein kinase expression and the reduction of miR-let-7b and miR-26a, both tumor suppressors, may explain increased lymphocyte survival in CLL patients and may be promising markers for the prognostic evaluation of this disease.

Keywords: Chronic Lymphocytic Leukemia. AKT protein kinase. microRNAs. Apoptosis.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) can be recognized by a heterogeneous clinical course and cannot be accurately predicted by clinical staging systems. This finding led to the investigation of prognostic markers that can add predictive value to staging systems. Among the clinical and biological markers suggested in the literature, chromosomal alterations, ZAP-70 tyrosine kinase protein, the presence of CD38, and the mutational state of immunoglobulin genes have proven to be promising in prognostic evaluations in CLL (Gonçalves *et al*., 2009). However, the techniques employed for the detection of these biomarkers are laborious and inaccessible to most oncohematology services, which have motivated the search for other substitute markers.

High levels of AKT protein kinase expression in CLL B lymphocytes are associated with unfavorable prognosis and appears to be related to a higher cell activation state (Singh *et al*., 2017). Alterations in cell signaling pathways contribute not only to tumor development, but also to resistance to conventional treatment. Thus, it is important to understand the AKT role in CLL pathogenesis and progression in order to devise strategies to exploit it therapeutically.

Alterations in microRNAs (miRNAs) have been studied in many tumors. The ability of miRNAs to

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modulate gene expression is essential in order to control various cell processes; therefore, any imbalance of these can lead to the development and progression of various diseases, such as CLL (Balatti *et al*., 2015). Deregulation of miRNAs can affect cell cycles, proliferation, and especially apoptosis pathways of leukemic B lymphocytes. Many miRNAs have not yet been related to CLL, and several of them still have an unknown mechanism of action. Thus, additional studies involving other miRNAs with a potential involvement in this disease are desirable.

A comprehensive literature review revealed that some miRNAs, such as miR-let-7b, miR-26a, and miR-27a have not yet been explored in hematological diseases including CLL. Within this context, the hypothesis of the present study admits that alterations in the expression of the protein kinase AKT and miRNAs may be related to the greater aggressiveness and progression of CLL.

PATIENTS AND METHOD

Patients and controls

In this study, a total of 100 individuals, 56 patients with CLL and 44 healthy subjects (controls) were enrolled after strictly observing the inclusion and exclusion criteria at the time of blood collection. Participants were distributed into two groups as follows. Group 1: 56 individuals with a diagnosis of CLL, selected by hematologists in the Hematology Unit of the Clinical Hospital of the Federal University of Minas Gerais (UFMG), Brazil. Patients with a confirmed diagnosis of CLL were included according to criteria established by the World Health Organization (WHO), Matutes Scoring System, and International Working Group Classification (Binet System); and Group 2: 44 apparently healthy individuals were selected from a local community with no diagnosis and history of hematological malignancies, along with age and sex paired with the CLL patients. The exclusion of diseases was conducted by self-report, in addition to the filling-out of clinical records and laboratory tests for the screening of major diseases. For a greater reliability, hemograms were taken of these individuals, whose parameters proved to be within the reference intervals (control group).

The institutional UFMG Ethics Committee approved this study, and informed consent was obtained from all participants. This study was carried out in accordance with the Declaration of Helsinki (WMA, 2013).

Methods

Blood count

EDTA blood samples were analyzed in the Coulter T-890 hematology analyzer to obtain red blood cell, platelet, leukocyte, and absolute and percent lymphocyte counts, as well as hemoglobin levels at most 90 minutes after venipuncture.

RNA extraction

RNA extraction from whole blood was performed using the TRIzol® kit (Invitrogen™, Carlsbad, California, USA), following manufacturer's instructions strictly.

RNA quantification

A 1μL aliquot of each total RNA sample was quantified using the NanoVue Plus spectrophotometer (GE Healthcare Life Sciences, Little Chalfont, UK), at wavelengths of 260nm and 280nm.

The purity of the samples was verified from the 260/280nm ratio, considering the ratio (260/280) of optical densities (OD) equal to or greater than 1.8 as a good quality reference for use.

cDNA synthesis

The cDNA was obtained using the High-Capacity cDNA Reverse Transcription kit (Life Technologies®, Carlsbad, California, USA) for protein kinase AKT mRNA expression. For miRNAs, the miRNA 1st-Strand cDNA Synthesis Kit (Agilent Technologies, Santa Clara, California, USA) was used to make cDNA, following all of the appropriate steps: polyadenylation, RNA purification, and synthesis, according to the manufacturer's instructions.

Real time qPCR

Once synthesized, cDNA was amplified by real-time polymerase chain reaction (qPCR), AKT protein kinase mRNA gene expression (FW: GGTGATCCTGGTGAAGGAGA; RV: CTTACTGTGCCCGTCCTTGT; Chen et. al., 2014), and the following microRNAS provided by IDT (Integrated DNA Technologies - Coralville, Iowa - USA): hsa-let-7b-5p miRNAs (TGAGGTAGTAGGTTGTGTGGTT), has-miR-26a-5p (TTCAAGTAATCCAGGATAGGCT), has-miR-21-5p: (TAGCTTATCAGACTGATGTTGA), hsa-miR-27a-3p (TTCACAGTGGCTAAGTTCCGC), and U6 (CGCTTCGGCAGCACATATAC - endogenous control) for relative quantification, using the Sybr Green system (Agilent Technologies - Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix – fast mode), according to manufacturer's protocol. PCR was performed on the Applied Biosystems StepOne - Real Time PCR System (Thermo Fisher™, Waltham, USA) for the AKT analyses and the QuantStudio 3 - Real Time PCR System (Thermo Fisher™, Waltham), for miRNA analysis. GAPDH (FW: GAAGGTGAAGGTCGGAGTC; RV: GAAGATGGTGATGGGATTTC; Di Nicolantonio *et al*., 2005) was used as an endogenous reaction control for AKT and U6 for miRNAs, in addition to a reference sample chosen from the control group as a parameter of normality and used for all analyses.

Statistical analysis

The results were analyzed using the software "Sigma Stat" version 2.03 and presented as mean and standard deviation for normal distribution, and as median and interquartile range for non-normal distribution. For variables with normal distribution, analysis of variance (ANOVA), followed by Tukey's multiple comparisons test, was used for comparison of three groups, and Student's T test for both groups. For non-normal variables, the Mann Whitney test was used to compare the two groups, while the Kruskal-Wallis test, followed by the Dunn test, was used to compare more than two groups. Correlation between the parameters was investigated by the Pearson and Spearman correlations for quantitative and qualitative variables, respectively. Differences were considered significant when $p < 0.05$.

RESULTS

Table I shows demographic data of the entire study population. No significant differences were observed in the groups concerning men, women, and age among patients with CLL and the control group.

TABLE I - Characterization of patients with CLL and control subjects in relation to gender and mean age for the groups

Groups	*n*	Men	Women	Age* (years)
CLL	56	31	25	67 (56-77)
CT	44	17	27	71 (66-76)
Total	100	48	52	-
***p*-value**	-	0.099	0.099	0.122

CLL: Chronic Lymphocytic Leukemia; CT: Controls. *Median. Statistical test: Normality, Shapiro-Wilk; Comparison of two groups, Mann-Whitney.

As shown in Table II, the number of platelets and red blood cells, in addition to hemoglobin levels, were significantly reduced in patients with CLL. However, as expected, the overall number of leukocytes and lymphocytes was significantly higher in patients with CLL, when compared to the control group.

TABLE II - Blood cell analysis from patients and control subjects

Groups*	Platelets (10^3/µL)	Lymphocytes (10^3/µL)	Leukocytes global (10^3/µL)	Hemoglobin (g/dL)	Red blood cells (10^3/µL)
CLL (*n* = 56)	144.00 (110.00-187.00)	13.78 (4.65-37.39)	19.40 (7.40-48.30)	12.30 (10.60-13.70)	4.06 (3.42-4.77)
CT (*n* = 44)	198.50 (167.00-232.50)	2.53 (1.20-3.12)	5.55 (4.10-6.30)	14.70 (13.30-16.40)	5.195 (4.57-5.55)
***p*-value**	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

CLL: chronic lymphocytic leukemia; CT: Controls. * Results in median and interquartile range. Statistics test: Normality, Shapiro-Wilk; Comparison of two groups, Mann-Whitney.

Patients with CLL were stratified and analyzed according to the Binet classification. Of all the patients studied, 36 were classified as low risk (Binet A) and 20 as moderate/severe risk (Binet B+C).

When the patients were analyzed according to disease stage, significant differences were observed in the number of platelets and red blood cells between subgroups A and B+C. Differences were also found when they were compared to controls, which were reduced in patients with CLL ($p < 0.05$). A difference was also identified in the number of leukocytes and lymphocytes, higher in both subgroups A and B+C, when compared to the controls ($p < 0.05$). Regarding hemoglobin, levels were significantly reduced in patients regardless of the Binet subgroup when compared to the controls ($p < 0.05$) (Table III).

TABLE III - Characterization of patients according to Binet staging

Groups*	Platelets (10^3/µL)	Lymphocytes (10^3/µL)	Global Leucocytes (10^3/µL)	Hemoglobin (g/dL)	Red blood cells10^3/µL)**
A (*n* = 36)	170.00 (126.00-200.00)	15.49 (5.78-36.22)	20.00 (8.78-44.95)	12.75 (11.45-14.43)	4.23 (±1.06)
B+C (*n* = 20)	106.00 (63.00-140.00)	11.66 (4.26-44.79)	17.20 (5.20-50.90)	10.90 (9.80-13.00)	3.63 (±0.92)
CT (*n* = 44)	198.50 (167.00-232.50)	2.53 (2.00-3.12)	5.55 (4.10-6.30)	14.70 (13.30-16.40)	5.16 (±0.72)
***p*-value**	$< 0.05^{a,b,f}$	$< 0.05^{c,d}$	$< 0.05^{c,d}$	$< 0.05^{a,b}$	$< 0.05^{a,b,f}$

[a]CT x B+C; [b]CT x A; [c]A x CT; [d]B+C x CT; [e]B+C x A; [f]A x B+C; * Results in median and interquartile range; ** Results in mean and standard deviation. CT: Controls; Statistics test: Normality, Shapiro-Wilk; comparison of three groups, ANOVA: Kruskal-Wallis followed by multiple comparison, Dunn's and Holm-Sidak.

Despite the difference in hemoglobin reference values between men and women, it was observed that when its values were analyzed separately, the difference in hemoglobin levels remained significant among men [$p < 0.001$; CLL: median = 12.35g/dL (10.13-14.15); CT: median = 14.40g/dL (13.25-15.75)] and women [$p < 0.001$; CLL: median = 12.20g/dL (11.00-13.60); CT: median = 14.35g/dL (13.30-16.08)], which proved to be higher in the controls when compared to the CLL patients.

AKT protein kinase mRNA expression

Significant differences for AKT mRNA gene expression were observed when CLL patients were compared to the controls ($p = 0.015$) and among all three subgroups, with higher values for Binet B+C. When stratified according to disease staging, significantly higher values were found in subgroup B+C, followed by subgroup A and controls (Figure 1).

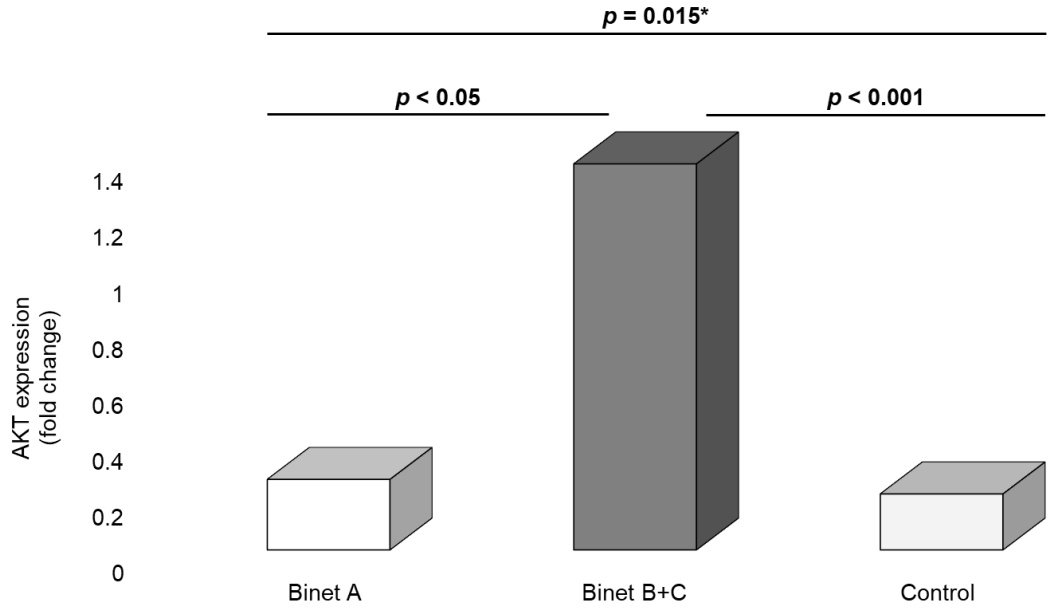

FIGURE 1 - AKT gene expression in Binet A, Binet B+C, and control groups; *p-value among CLL patients and controls. CLL: Chronic Lymphocytic Leukemia; CT: Controls.

miRNA gene expression

Table IV shows the gene expression values for each miRNA. Significant differences were observed in the gene expression of miRNA-let-7b ($p < 0.001$) and miRNA-26a ($p < 0.001$), both tumor suppressors, which was significantly reduced in CLL patients when compared to the controls. Considering miRNA-let-7b gene expression, patients presented a twenty-fold lower expression than did the controls. Some Binet B+C patients had zero or very close to zero expression. Regarding miRNA-26a, patients presented a seven-fold lower expression than did the controls.

TABLE IV - miRNA gene expression in patients with CLL and controls

Groups*	miR-let-7b	miR-26a	miR-21	miR-27a
CLL (*n* = 56)	0.07 (0.03-0.27)	0.21 (0.05-0.44)	0.27 (0.08-3.47)	0.25 (0.08-1.09)
CT (*n* = 44)	1.36 (0.38-2.43)	1.370 (0.500-3.320)	0.36 (0.12-1.80)	0.64 (0.16-2.00)
***p*-value**	< 0.001	< 0.001	0.614	0.125

Results in median and interquartile range; CLL: Chronic Lymphocytic Leukemia; CT: Controls. Statistics test: Normality, Shapiro-Wilk; comparison of two groups: Mann Whitney.No significant differences were found when comparing patients and controls for miRNAs-21 and miRNAs-27a, both oncogenes. However, the expression of such oncogenes was considerably different in some patients as compared to others. Considering miRNA-21 gene expression, the majority of patients presented a value close to 8, while two patients of Binet A reached the values of 144.76 and 243.17 and one Binet B+C patient reached the value of 145.48.

Regarding miRNA-27a, the majority of Binet A patients presented low expression, except for some patients with expression values of 15.66, 27.68, and 38.01, while two patients of the Binet B+C subgroup presented higher expressions of 102.61 and 687.90. No significant difference was observed when comparing the patients among the Binet subgroups.

Figures 2 and 3 illustrate the median of gene expression values of each miRNA, according to CLL patients and controls.

To identify patients with a higher potential for worse outcomes, correlations were investigated between the molecular parameters AKT and miRNAs (some of which were significantly altered) and between molecular parameters and laboratory tests of blood indicative of poor prognosis (hemoglobin, leukocyte count, lymphocyte count, among other parameters). The following correlations were significant (Spearman, $p < 0.05$): AKT versus Binet staging, $r = 0.584$ and $p = 0.0258$, miR-27a versus miR-21, $r = 0.400$ and $p = 0.004$; miR-27a versus miR-let-7-b, $r = -0.331$ and $p = 0.01$; and miR-let-7b versus miR-26a, $r = 0.327$ and $p = 0.010$.

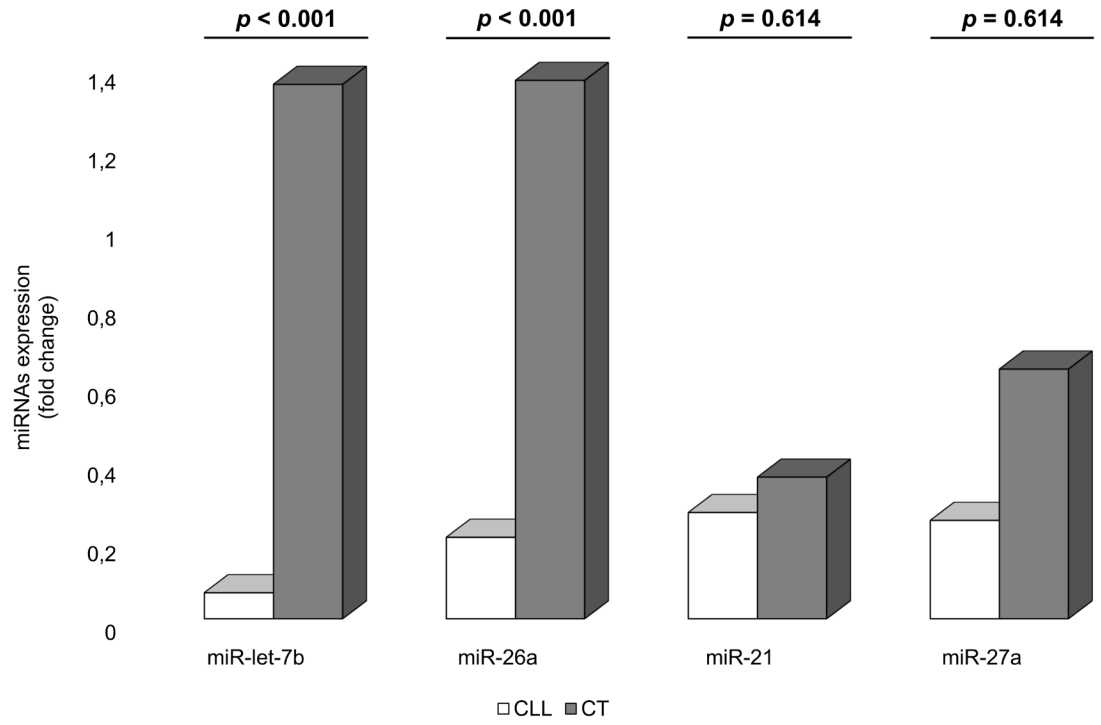

FIGURE 2 - Median values of gene expression of each miRNA according to CLL patients as a whole and controls; CLL: Chronic Lymphocytic Leukemia; CT: Controls.

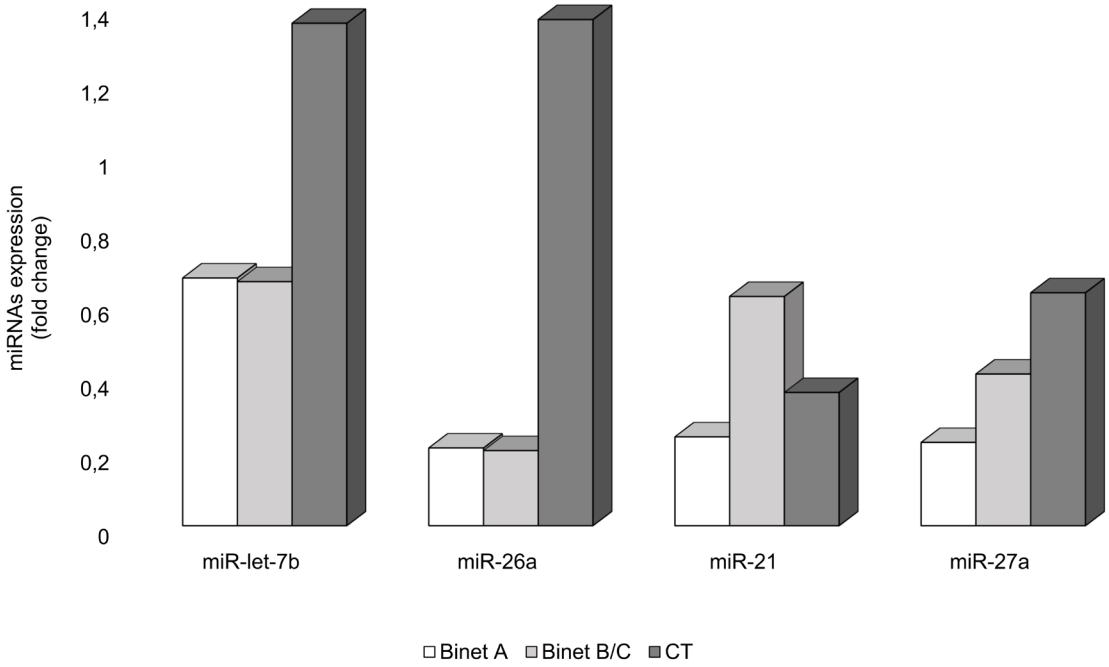

FIGURE 3 - Median values of gene expression of each miRNA according to Binet A or B+C subgroup and controls; **p*-value between Binet subgroups; CT: Controls.

DISCUSSION

In the present study, CLL patients stratified in different stages of Binet were evaluated for the expression of the protein kinase AKT and mRNAs related to disease pathogenesis. As expected, Binet's advanced stage (B+C) presented indications of anemia and trombocytopenia, in addition to a significant increase in the global leucocyte count due to relative and absolute lymphocytosis. Most patients involved in the study were classified as Binet A.

Increased Protein kinase AKT gene expression in patients with CLL

Analysis of AKT data revealed increased mRNA expression in patients with CLL as a whole. When AKT data were stratified according to Binet staging, the subgroup B+C showed significantly higher values in relation to controls and A subgroup. Thus, it can be assumed that the increase in AKT expression was consistent with the most severe forms of CLL, signaling to an unfavorable prognostic value of this biomarker. However, it should be noted that some patients in the A subgroup also had a higher AKT expression (Q3 = 1.655), suggesting that such patients should be followed with greater frequency and clinical accuracy in order to identify those whose disease progresses unfavorably and early.

Higher values of AKT mRNA expression in the B+C subgroup coexisted with lower hemoglobin and platelet values, as well as higher overall counts of leukocytes and lymphocytes. By contrast, in the A subgroup, AKT values were not as high, coexisting with less pronounced changes in hemoglobin, platelet, total leukocyte, and lymphocyte values. In view of these findings, a potential prognostic value can be inferred from the evaluation of AKT mRNA expression, since, at first, this new biomarker appears to be closely related to the hemoglobin rates and the number of leukocytes, lymphocytes, and platelets of the patients.

Our data related to AKT mRNA expression are corroborated by the findings of Singh *et al.* (2017), who demonstrated an inhibition of PI3k and AKT signaling in cell lines of CLL mice, leading to a significant reduction in cell survival. This same study and others (Cheng *et al.*, 2014; Palacios *et al.*, 2015) reported that the treatment of these cell lines with novel therapeutic targets, such as small molecules specific for Btk (Ibrutinib) or PI3k (Idelalisib), which are related to AKT pathway inhibition, lead to reduced cell viability, fibronectin dependent adhesion, and proliferation.

Still related to our findings, Chapman *et al.* (2017) reported the role and importance of AKT in the survival, growth, and proliferation of CLL B lymphocytes by stimulation of CD40. This study also confirmed that cell activation is accompanied by a reduction in PTEN (Phosphatase and tensin homolog), an important cell suppressor, through miRNA-22 activation, which also promotes AKT activation (Palacios *et al.*, 2015; Chapman *et al.*, 2017; Liu *et al.*, 2015).

Choudhary *et al.* (2015) reported the importance of combining chemotherapeutic agents with AKT signaling pathway inhibitors. These authors proved that AKT activation led to increased expression of Mcl-1 and bcl-xL (antiapoptotic proteins), with BIM sequestration (pro-apoptotic protein) and prevention of ABT199 cell inhibition (bcl-2 inhibitor). In addition, Larsen *et al.* (2017) tested another AKT inhibitor, MK-2206, in combination with Bendamustine and Rituximab in a phase I/II study in patients with refractory CLL. In this study, MK-2206 was administered in 30 patients one week before the other drugs, showing an overall response rate of 92% and a disease-free and treatment-free survival of 16 and 24 months, respectively. Thus, the authors concluded that treatment with the AKT inhibitor, combined with chemoimmunotherapy, is promising and merits further study.

As previously mentioned, an expressive and significant increase in expression was observed among the Binet subgroups, which was higher in the B+C subgroup, strongly suggesting that the expression of AKT should increase with the disease worsening. In addition, there was a moderate and significant correlation between AKT expression and Binet staging ($r = 0.584$ and $p = 0.0258$). Such findings highlight its role in cell proliferation and resistance to apoptosis, which is in agreement with studies found in the literature. As already known, CLL cells exhibit aberrant BCR receptor signaling, promoting tyrosine kinase Bruton kinase (Btk) activation, which

is essential for the activation of various cell survival pathways, such as AKT, ERK, and NF-êB. With the activation of these pathways, there is an increase in cell proliferation and survival, a mechanism characteristic of CLL pathogenesis (Cheng *et al.*, 2014).

Considering the findings, associating the performance of AKT with a critical role in cell survival, AKT has become an important object of study of mechanisms and potential therapeutic target.

MiRNAs: reduced miR-let-7b and miR-26a gene expression in CLL patients

An analysis of Figures 2 and 3 shows a large heterogeneity in the miRNA results, and miR-let-7b and miR-26a were less expressed in the patient group than in the controls, while miR-21 showed a tendency to increase in patients, especially in the B+C subgroup. However, individual patient characteristics should be evaluated to explain such discrepant findings, as should the search for association with other prognostic factors. Thus, it was hypothesized that miRNAs investigated in this study, miR-let-7b and miR-26a, may suggest an unfavorable prognosis.

The expression of miR-let-7 in most human cancers, including hematological cell cancer, is significantly reduced. The main function of this miRNA is to suppress tumors (Esquela-Kerscher, Slack, 2006). MiR-let-7 has proven to be a direct regulator of K-ras expression in human cells. One study (Nair, Maeda, Ioanidis, 2012) reported that let-7 expression was decreased in lung cancer, whereas K-ras was increased in cancer cells and vice versa in normal cells. Thus, in many cancer types, the decrease in miR-let-7 is associated with a poorer prognosis for patients (Nair, Maeda, Ioanidis, 2012).

In the present study, a significant difference was observed between CLL patients and controls, with the lowest expression in CLL patients for miR-let-7b. This fact suggests an association between the reduction of miR-let-7b and the development of malignant disease, as found in other studies with cancer cells.

One study (Admoni-Elisha *et al.*, 2016) investigating new CLL-related proteins reported for the first time an increased expression of mitochondrial antiviral signaling protein (MAVS), a mitochondrial protein that modulates the activity of anti-apoptotic proteins leading to resistance to apoptosis. After searching the miR Base and Star Base databases (mirbase.org, starbase.sysu.edu.cn), as well as databases of miRNAs and their targets, it was observed that miR-let-7b is correlated with MAVS, suggesting that this miRNA may play an important role in the modulation of this protein.

In summary, the results of the present study referring to miR-let-7b may be the signaling to another biomarker with potential unfavorable prognostic value in CLL, since this miRNA expression correlated inversely with miR-27a, an oncogene, and directly with the miR-26a, also a tumor suppressor. Since miR-let-7b has a tumor suppressor function, it can be hypothesized that a low expression of this tumor inhibitor may favor cell proliferation, particularly of lymphocytes. In light of the knowledge that miR-let-7b works as a tumor suppressor, that is, rarely expressed in patients with several cancer types, a considerable reduction of its expression in CLL would be expected.

MiR-26 plays the role of a tumor suppressor and is important in the regulation of carcinogenesis and tumor progression, which are reduced in many cancer types (Gao, Liu, 2011). It appears that this miRNA acts by inhibiting proliferation and inducing cell apoptosis. An inverse relationship was observed between miR-26 and IL-6, and may be explained by the deregulation of this interleukin (Yang *et al.*, 2013; Zhang *et al.*, 2013). Another study (Chen *et al.*, 2016) reported that miR-26 decreased IL-6 production and TNF-α/NF-êB signaling, and it was concluded that the reduction of miR-26 implies a worse prognosis in patients with pulmonary adenocarcinoma.

Qiu *et al.* (2017) showed that miR-26 was decreased in gastric cancer and that this reduction was significantly associated with poor patient survival. Instead, miR-26 high expression led to the suppression of proliferation, migration, invasion, and formation of cell colonies, and induced cell apoptosis when compared to the controls. Thus, this miRNA expression has proven to be a potential independent prognostic biomarker for predicting survival in patients with gastric cancer. In agreement with the previous study, Jayaraman *et al.* (2017) also found a reduction of miR-26 expression in endometrial cancer.

C-Myc deregulation is a common alteration in several types of cancers, including CLL. One study (Li *et al.*, 2017), conducted with squamous cell carcinoma of the esophagus, one of the most lethal cancers in the world, observed a relationship between miR-26 and c-Myc alterations. In patients with this type of tumor, the authors observed a reduction of more than 50% in the miR-26 expression, even in early disease stages.

In the present study, the expression of miR-26a was significantly lower in patients with CLL than in controls. A direct correlation between the expression of miR-26a and miR-let-7b, another tumor suppressor, was observed. It can be noted that miR-26a expression was lower than other miRNAs included in this study, and even lower in advanced-stage (Binet B+C) patients. Although no report on miR-26 has been found in the field of onco-hematological diseases, more robust studies on this miRNA would be desirable because of its involvement with the Notch, c-Myc, and NF-êB pathways, pathways known to be involved with CLL.

Admoni-Elisha *et al.* (2016), in their study with new potential biomarkers in CLL, reported some new proteins that would not yet have been correlated with CLL, including VDAC1. VDAC1 is a protein related to the metabolism of mitochondria and interacts with apoptosis and caspase proteins. According to this study, VDAC1 levels are highly correlated with the number of leukemic cells in CLL. By means of careful search in miR Base and Star Base (mirbase.org, starbase.sysu.edu.cn), as well as in databases of miRNAs and their targets, it was observed that miR-26a is associated with VDAC1. Considering that this protein may be deregulated in CLL, the modulation of this miRNA in patients may be altered, thus contributing to the pathogenesis of this disease.

Extrapolating the above for our study, it seems that the levels of miR-26a would already be very low at the beginning of CLL, when still in its indolent form (Binet A subgroup), which could contribute to the development of the disease, since this miRNA presents a tumor suppressor function. Its low expression would then favor cell proliferation and the inhibition of apoptosis, contributing to the disease's characteristics as relative and absolute lymphocytosis. Thus, miR-26a, as a therapeutic strategy, could theoretically bring benefits to the newly diagnosed patient, who still presents the indolent from of the disease.

MiRNAs: Unchanged miR-21 and miR-27a expression in patients with CLL

In most tumors, miR-21 presents increased expression, participates in different oncogenic processes, and is associated with a worse prognosis. These references increase in breast, lung, colon, stomach and pancreas cancers, among others (Fu *et al.*, 2011). The miR-21 is more commonly expressed in activated B cells, especially in the germinal center and memory B cells. Increased expression of this miRNA can be induced by IL-4 and CD40L or even by BCR stimulation, which allows B lymphocytes to become hyperactive and prone to malignant transformations. In CLL, miR-21 is increased in most cases and is related to fludarabine resistance, low survival, and an increased risk of disease progression (Ferracin *et al.*, 2010; Rossi *et al.*, 2010; Saleh *et al.*, 2017). In this context, Carabia *et al.* (2017) reported that stimulation by IL-4 of the CLL cell microenvironment led to an increased miR-21 expression and mRNA reduction of their target genes, tumor suppressors PTEN, PDCD4, and PIAS3, via ZAP-70 signaling pathway, MAPK, and STAT3, resulting in increased cell survival. Which is consistent with a previous study in which Saleh *et al.* (2017) observed that miR-21 was increased 10-fold in CLL cells when compared to normal B cells.

B cells play an essential role in immune response control, the production of antibodies, the presentation of antigens, as well as the production and stimulation of the cytokine pathway. Thus, regulatory B lymphocytes produce IL-10 and inhibit autoimmunity. Thses can also lead to transplant rejection and promote tumor growth (Mauri, Menon, 2015). Wang *et al.* (2017) first showed that IL-10 is the target of miR-21 and that this miRNA is able to down-regulate regulatory B cells. IL-10 is associated with autoimmune disease development.

Although there were no significant differences in the expression of miR-21 between the subgroups in our study, a discrete increase was observed, especially in Binet B + C patients. It is noteworthy that a Binet B + C patient had an expression of miR-21 145.48 fold,

when compared to the control group, in addition to an increase in miR-27a, another oncogene, and a reduction in the tumor suppressor miR-26a. Another patient in the Binet A subgroup presented a miR-21 expression of 144.76 fold, when compared to the control group, as well as an increase in miR27-a, indicating that the patient is most likely prone to a more rapid evolution of disease. Due to its oncogenic role, this miRNA is believed to be involved in the worsening of the disease, also supported by its relationship with AKT, which increased in CLL patients in the present study, corroborating resistance to apoptosis. Perhaps the low number of samples tested in our study and a larger number of patients belonging to the Binet A subgroup can justify the absence of significant differences between groups for this miRNA.

MiR-27 is often altered in several types of cancer, such as colon and breast cancers, osteosarcoma, and gastric adenocarcinoma, and can act as an oncogene or tumor suppressor (Mertens-Talcott *et al.*, 2007). Li *et al.* (2016) evaluated the function of miR-27 in renal cell carcinoma and showed that miRNA is related to the suppression of cell proliferation and the induction of cell apoptosis, suggesting that miR-27 has a tumor suppressor action in this cancer type. The data from this study showed that the tumor suppressor action of this miRNA is due to the endothelial growth factor receptor (EGFR), which constitutes a new target of miR-27. Therefore, a dysregulation of the cell cycle, caused by EGFR inhibition, was observed. However, another study (Liu *et al.*, 2009) involving miR-27 in gastric adenocarcinoma reported that this miRNA presented an oncogenic profile inducing cell growth. Thus, these authors have suggested that miR-27 may function as an oncogene. Another study by Salah *et al.* (2015) reported that miR-27 was increased in osteosarcoma and promoted the formation of lung metastases, suggesting that miRNA plays a role in developing metastasis.

In the present study, no significant differences were found in miR-27a levels when comparing patients and controls, or Binet subgroups, most likely due to the limited number of samples tested. Thus, our data do not allow us, at first, to infer whether miR-27a functions as a tumor suppressor or an oncogene. However, it is possible to observe a tendency to increase the expression of this miRNA in Binet B+C patients when compared to the Binet A patients. In our study, an inverse correlation was found between the expression of miR-27a and miR-let-7b. It is also important to note that a patient with a high expression of miR-27a (687.9) also showed a significant increase in AKT and a reduction in miR-let-7b. Such findings call attention to the relationship between such biomarkers as well as to a need for the closer monitoring of this patient, who may evolve more rapidly. In other words, a high expression of miR-27a could signal the worsening of the disease. Although no study has been found on oncohematological diseases, it is suggested that miR-27a also plays an oncogenic role in CLL.

A miRNA may take on an oncogenic or tumor suppressive role, depending on the type of cell involved, and this may explain the difference in miR-27a expression in different tumor types, cell types, and targets. The data obtained in the present study, that is, a slight increase in miR-27a in controls and with a tendency to increase in the subgroup B+C, suggest that miR-27a would be implicated in oncogenesis, favoring cell proliferation and the antiapoptotic mechanism.

According to miRBase and StarBase (mirbase.org, starbase.sysu.edu.cn), miRNAs databases and their targets, it was observed that miR-27a, as well as miR-let-7b, are also associated with the MAVS protein. This protein was studied by Admoni-Elisha *et al.* (2016), who reported, for the first time, its increase in CLL and its relation to resistance to cell apoptosis. Extrapolating to our study, the presumed and inverse correlation between miRNAs, miR-27a, and miR-let-7b with the MAVS protein would signal a resistance to cell apoptosis. Considering that some patients stood out with the increase of miR-27a, the idea is reinforced that this miRNA can influence the progression of the disease. Thus, the follow up of such patients that could evolve more quickly, especially those classified with Binet A, and it would be advisable for measures to contain the disease to be adopted.

Finally, it is noteworthy that among patients in the Binet B+C stage, one patient presented high AKT expression (around 74.25), a significant increase in miR-27a and miR-21, both oncogenes, in addition to a significant reduction in miR -let-7b, a tumor suppressor.

A current assessment of various laboratory parameters revealed that the same patient had a significant increase in urea and creatinine levels, a significant increase in LDH, total direct and indirect bilirubin, a considerable reduction in the number of platelets, as well as global leukocytes and lymphocytes, and maintained the number of red blood cells and hemoglobin levels, probably reflecting the recent use of chemotherapy. Another patient with AKT expression of 200.53 also presented a high expression of miR-21. Thus, according to full analysis of our data, it can be concluded that the expression of the prognostic markers evaluated in the present study may add value to the available prognostic tools to discriminate those at highest risk of disease progression.

Although our results *a priori* are promising to differentiate those patients with unfavorable prognosis, it should be noted that the majority of circulating lymphocytes are T-type cells in the control subjects analyzed by us, whereas in CCL patients showed B- type cells, which may be a limitation of our study.

CONCLUSION

In short, it can be assumed that the increased expression of the AKT and reduced miRNA expression, such as miR-let-7b and miR-26a, were consistent with the most severe forms of CLL, thus signaling to an unfavorable prognostic value of this biomarker. However, it should be noted that some A subgroup patients also presented a higher expression of AKT and a reduced mRNA expression, suggesting that such patients should be monitored with greater frequency and clinical rigor in order to identify those whose disease will progress unfavorably early.

In light of the knowledge of the profiles related to AKT protein kinase and mRNA gene expression related to CLL pathogenesis, it would be desirable to follow patients with greater disease severity (Binet B+C) in order to determine the survival rate of those with unfavorable profiles. Particularly, it would be very important to follow patients in the Binet A subgroup, focusing up on those who, although still indolent, already present unfavorable biomarkers, such as increased expression of AKT, miR-27a and miR-21, and a low expression of miR- let-7b and miR-26a in an attempt to identify those at a higher risk of disease progression, who should be monitored longitudinally. If an unfavorable profile for such biomarkers is predictive of the worsening of CLL, such analyses could be added to biomarkers with proven prognostic importance, such as certain chromosomal alterations, ZAP-70, CD38, mutational status of IgVH, among others, besides the conventional parameters used in the Binet staging classification, such as hemoglobin and global counts of leukocytes, lymphocytes, and platelets.

An analysis of the data obtained from CLL patients suggests that increased AKT mRNA expression, as well as a significant reduction of miR-let-7b and miR-26a, both tumor suppressors, corroborates increased cell proliferation and survival, which is of great importance in the disease pathogenesis.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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REFERENCES

Admoni-Elisha L, Nakdimon I, Shteinfer A, Prezma T, Arif T, Arbel N, et al. Novel Biomarker Proteins in Chronic Lymphocytic Leukemia: Impact on Diagnosis, Prognosis and Treatment. PLoS One. 2016;11(4):e0148500.

Balatti V, Pekarky Y, Croce CM. Role of microRNA in chronic lymphocytic leukemia onset and progression. J Hematol Oncol. 2015;8(12).

Carabia J, Carpio C, Abrisqueta P, Jimenez I, Purroy N, Calpe E, et al. Microenvironment regulates the expression of miR-21 and tumor suppressor genes PTEN, PIAS3 and PDCD4 through ZAP-70 in chronic lymphocytic leukemia. Sci Rep. 2017;7(1):12262.

Chapman EA, Oates M, Mohammad IS, Davies BR, Stockman PK, Zhuang J, et al. Delineating the distinct role of AKT in mediating cell survival and proliferation induced by CD154 and IL-4/IL-21 in chronic lymphocytic leukemia. Oncotarget. 2017;8(61):102948-64.

Chen CY, Chang JT, Ho YF, Shyu AB. MiR-26 down-regulates TNF-alpha/NF-kappaB signalling and IL-6 expression by silencing HMGA1 and MALT1. Nucleic Acids Res. 2016;44(8):3772-87.

Chen X, Wang K, Xu Z, Li S, Liu Q, Fu D, et al. MiR-133b regulates bladder cancer cell proliferation and apoptosis by targeting Bcl-w and Akt1. Cancer Cell Int. 2014;14(70).

Cheng S, Ma J, Guo A, Lu P, Leonard JP, Coleman M, et al. BTK inhibition targets in vivo CLL proliferation through its effects on B-cell receptor signaling activity. Leukemia. 2014;28(3):649-57.

Choudhary GS, Al-Harbi S, Mazumder S, Hill BT, Smith MR, Bodo J, et al. MCL-1 and BCL-xL-dependent resistance to the BCL-2 inhibitor ABT-199 can be overcome by preventing PI3K/AKT/mTOR activation in lymphoid malignancies. Cell Death Dis. 2015;6:e1593.

Di Nicolantonio F, Mercer SJ, Knight LA, Gabriel FG, Whitehouse PA, Sharma S, et al. Cancer cell adaptation to chemotherapy. BMC Cancer. 2005;5:78.

Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. 2006;6(4):259-69.

Ferracin M, Zagatti B, Rizzotto L, Cavazzini F, Veronese A, Ciccone M, et al. MicroRNAs involvement in fludarabine refractory chronic lymphocytic leukemia. Mol Cancer. 2010;9:123.

Fu X, Han Y, Wu Y, Zhu X, Lu X, Mao F, et al. Prognostic role of microRNA-21 in various carcinomas: a systematic review and meta-analysis. Eur J Clin Invest. 2011;41(11):1245-53.

Gao J, Liu QG. The role of miR-26 in tumors and normal tissues (Review). Oncol Lett. 2011;2(6):1019-23.

Gonçalves RP, Maia DP, Custódio RKA, Machado RPG, Duarte FB, Silva LB. Evaluation of the hematologic profile of patients with chronic lymphocytic leukemia (CLL-B) at a state hemocenter. Rev Bras Hematol Hemoter. 2009;31(4):228-34.

Jayaraman M, Radhakrishnan R, Mathews CA, Yan M, Husain S, Moxley KM, et al. Identification of novel diagnostic and prognostic miRNA signatures in endometrial cancer. Genes Cancer. 2017;8(5-6):566-76.

Larsen JT, Shanafelt TD, Leis JF, LaPlant B, Call T, Pettinger A, et al. Akt inhibitor MK-2206 in combination with bendamustine and rituximab in relapsed or refractory chronic lymphocytic leukemia: Results from the N1087 alliance study. Am J Hematol. 2017;92(8):759-63.

Li J, Liang Y, Lv H, Meng H, Xiong G, Guan X, et al. miR-26a and miR-26b inhibit esophageal squamous cancer cell proliferation through suppression of c-MYC pathway. Gene. 2017;625:1-9.

Li Y, Li J, Sun X, Chen J, Zheng J, Chen R. MicroRNA-27a functions as a tumor suppressor in renal cell carcinoma by targeting epidermal growth factor receptor. Oncol Lett. 2016;11(6):4217-23.

Liu T, Tang H, Lang Y, Liu M, Li X. MicroRNA-27a functions as an oncogene in gastric adenocarcinoma by targeting prohibitin. Cancer Lett. 2009;273(2):233-42.

Liu TM, Ling Y, Woyach JA, Beckwith K, Yeh YY, Hertlein E, et al. OSU-T315: a novel targeted therapeutic that antagonizes AKT membrane localization and activation of chronic lymphocytic leukemia cells. Blood. 2015;125(2):284-95.

Mauri C, Menon M. The expanding family of regulatory B cells. Int Immunol. 2015;27(10):479-86.

Mertens-Talcott SU, Chintharlapalli S, Li X, Safe S. The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. Cancer Res. 2007;67(22):11001-11.

Nair VS, Maeda LS, Ioannidis JP. Clinical outcome prediction by microRNAs in human cancer: a systematic review. J Natl Cancer Inst. 2012;104(7):528-40.

Palacios F, Abreu C, Prieto D, Morande P, Ruiz S, Fernandez-Calero T, et al. Activation of the PI3K/AKT pathway by microRNA-22 results in CLL B-cell proliferation. Leukemia. 2015;29(1):115-25.

Qiu X, Zhu H, Liu S, Tao G, Jin J, Chu H, et al. Expression and prognostic value of microRNA-26a and microRNA-148a in gastric cancer. J Gastroenterol Hepatol. 2017;32(4):819-27.

Rossi S, Shimizu M, Barbarotto E, Nicoloso MS, Dimitri F, Sampath D, et al. microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. Blood. 2010;116(6):945-52.

Salah Z, Arafeh R, Maximov V, Galasso M, Khawaled S, Abou-Sharieha S, et al. miR-27a and miR-27a* contribute to metastatic properties of osteosarcoma cells. Oncotarget. 2015;6(7):4920-35.

Saleh LM, Wang W, Herman SE, Saba NS, Anastas V, Barber E, et al. Ibrutinib downregulates a subset of miRNA leading to upregulation of tumor suppressors and inhibition of cell proliferation in chronic lymphocytic leukemia. Leukemia. 2017;31(2):340-9.

Singh SP, Pillai SY, de Bruijn MJW, Stadhouders R, Corneth OBJ, van den Ham HJ, et al. Cell lines generated from a chronic

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lymphocytic leukemia mouse model exhibit constitutive Btk and Akt signaling. Oncotarget. 2017;8(42):71981-95.

Wang H, Xu W, Shao Q, Ding Q. miR-21 silencing ameliorates experimental autoimmune encephalomyelitis by promoting the differentiation of IL-10-producing B cells. Oncotarget. 2017;8(55):94069-79.

World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013;310(20):2191-4.

Yang X, Liang L, Zhang XF, Jia HL, Qin Y, Zhu XC, et al. MicroRNA-26a suppresses tumor growth and metastasis of human hepatocellular carcinoma by targeting interleukin-6-**S**tat3 pathway. Hepatology. 2013;58(1):158-70.

Zhang Y, Zhang B, Zhang A, Li X, Liu J, Zhao J, et al. IL-6 upregulation contributes to the reduction of miR-26a expression in hepatocellular carcinoma cells. Braz J Med Biol Res. 2013;46(1):32-8.

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