Evaluation of miR-15a, miR-16-1, ZAP-70, Ang-2, and Bcl-2 as potential prognostic biomarkers in chronic lymphocytic leukemia

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Chronic lymphocytic leukemia (CLL) is a blood cancer characterized by the accumulation of clonal B-lymphocytes. This study evaluated the mRNA gene expression of miR-15a, miR-16-1, ZAP-70, and Ang-2 by qPCR, as well as the plasma levels of Bcl-2 by Elisa immunoassay, in CLL patients and healthy controls. Significant differences were observed when comparing patients and controls regarding miR-15a ($p < 0.001$), miR-16-1 ($p < 0.001$) mRNA, Ang-2 gene expression, and Bcl-2 plasma levels ($p < 0.001$). When stratified by risk, differences were maintained with a significantly reduced expression in high-risk patients. A positive correlation was observed between miR-15a and platelets ($R^2 = 0.340$; $p = 0.009$) as well as between Bcl-2 and leukocytes ($R^2 = 0.310$; $p = 0.019$). Conversely, negative correlations were observed between ZAP-70 and platelets ($R^2 = -0.334$; $p = 0.011$), between miR-15a and lymphocytes ($R^2 = -0.376$; $p = 0.004$), as well as between miR-16-and lymphocytes ($R^2 = -0.515$; $p = 0.00004$). The data suggest that a reduction in miR-15a and miR-16-1 expressions, in addition to an overexpression of Bcl-2, are associated with the reduction in apoptosis and, consequently, to a longer survival of lymphocytes, thus contributing to lymphocyte accumulation and aggravation of the disease. By contrast, Ang-2 expression was significantly higher in A than in B + C Binet groups. This context leads to the speculation that this biomarker should be investigated in more robust studies within populations with a still relevantly indolent form of the disease in an attempt to identify those patients with a greater potential for an aggravation of the disease.

Keywords: CLL. miR-15a. miR-16-1. Biomarkers.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a type of cancer characterized by B-lymphocyte accumulation in the blood, bone marrow, and lymphoid organs. These lymphocytes tend to have great longevity, which, in most cases, is determined by the inhibition of apoptosis (Dighiero, 2005).

The leukemia staging systems of Binet and Rai, at the time of diagnosis, do not enable any identification of patients who will present a rapid progression of the disease. Among patients in the early stages of the disease, nearly 50% will evolve aggressively with a rapid progression and premature death caused by B-cell CLL (B-CLL). Thus, in addition to the clinical stage, other prognostic factors are important to identify patients at a higher risk of rapid disease progression even if they do not present a clinically advanced stage of the disease (Rodrigues *et al.*, 2016; Gaidano, Foà, Dalla-Favera, 2012).

The mutational status of the gene that encodes the expression of the variable region of the immunoglobulin

Tatiane V. Braga, Fernanda C. G. Evangelista, Marie G. Santiago, Aline L. M. Ferrão, Tamara D. Almeida, Bárbara L. F. Barbosa, Sergio S. S. Araujo, Glaciano N. Ribeiro, Maria G. Carvalho, Adriano P. Sabino

heavy chain (IgVH) is an important prognostic marker. Patients with a favorable prognosis present mutations in the IgVH gene, while those patients without mutations present an unfavorable evolution of their disease (Gonçalves *et al.*, 2009; William, 2002).

One gene located on the short arm of chromosome 2 encodes a tyrosine kinase protein of 70 kDa (ZAP-70), which is essential in the signal transduction of T-lymphocytes. This intracellular protein is associated with the zeta chain of the T-cell receptor complex (TCR), from which the ZAP name comes (zeta-associated protein). The ZAP-70 protein, though not found in normal B-lymphocytes, is expressed in CLL cells with no mutated IgVH genes. CLL lymphocytes with mutations in IgVH genes rarely maintain the expression of this protein. Studies suggest that, in prognostic evaluations, ZAP-70 could be used as a substitute for the unmutated IgVH status, which correlates with the more aggressive form of the disease (Gomes *et al.*, 2017).

As reviewed by Mirzaei *et al.* (2018), miRNAs have appeared as new diagnostic and therapeutic biomarkers in CLL patients and their deregulation may well be associated with pathological events present in this disease. Recently, Ahmadvand *et al.* (2019), reported that miR-125a downregulation is correlated with P53, NRG1 (neuregulin-1), and ERBB2 (also known as HER2, *neu*, and NGL) expressions, genes known to be involved in cancer development. These authors suggested that the miR-125a expression level could be a novel potential biomarker for CLL prognosis.

The miRNAs exert post-transcriptional regulation according to the degree of complementarity with the target mRNA. Evaluation of the differential miRNA expression in normal and malignant B-cells may suggest its potential in hematopoiesis and leukogenesis. The miR-15a and miR-16-1 genes are located on chromosome 13q14, a region deleted in more than half of the B-CLL cases (Braga *et al.*, 2017; Mirzaei *et al.*, 2017; Calin *et al.*, 2008). Furthermore, reports have shown that a germline mutation in the gene locus of miR-15a/miR-16-1 decreases the expression of these mature miRNAs in CLL cells. These findings suggest that miR-15a and miR-16-1 act as tumor suppressor genes in humans (Cimmino *et al.*, 2005). Indeed, in normal lymphocytes, miR-15a and miR-

16-1 are expressed at high levels, whereas in patients with CLL, survival of B-lymphocytes is increased as a result of the inappropriate expression of Bcl-2 family proteins (Roberts *et al.*, 2016; Zanette *et al.*, 2007; Packham, Stevenson, 2005).

CLL alterations in the apoptosis process can also lead to changes in the mechanism of angiogenesis. *In vitro* CLL cells are able to increase the secretion of angiopoietin-2 (Ang-2) when subjected to hypoxic conditions. The prolonged reduction of oxygen tension in normal tissue triggers a cascade of compensatory responses, resulting in the formation of new vessels, thereby improving tissue oxygenation. In normal individuals, angiogenesis participates in the growth and development of the organism. However, in malignant diseases, angiogenesis is essential for the expansion of solid tumors and hematological malignancies, including CLL. Ang-2 is a 75 kDa glycoprotein, and its expression can be induced by such factors as the vascular endothelium growth factor (VEGF) and hypoxia (Aguirre *et al.*, 2016; Kopparapu *et al.*, 2015; Xia, Lu, Li, 2012; Maffei *et al.*, 2010).

The evaluation of potential biomarkers applied to the prognosis in CLL becomes extremely important for the early identification of individuals who will most likely suffer a rapid disease progression and may benefit from more aggressive, individualized treatment protocols. To investigate the potential role of miR-15a, miR-16-1, ZAP-70, and Ang-2 gene expressions, in addition to Bcl-2 plasma levels, such as prognostic markers in CLL, peripheral blood samples from CLL patients were analyzed and compared to a control group. Variations in biomarker expressions may indicate differences between normal cells and malignant B-cells, since changes in expression often play an important role in the genesis and evolution of lymphoid malignancies.

MATERIAL AND METHODS

Patients

Patients were selected by hematologists from the Hematology Unit of the Clinical Hospital, Federal University of Minas Gerais (UFMG), Brazil, from August 2014 until February 2015. Patients' clinical data

were obtained from the medical records. Forty-three patients with confirmed CLL, rated by Binet criteria, were included in the study: 27 low-risk patients, and 16 moderate and high-risk patients. The control group consisted of 15 subjects that were clinically and laboratorily healthy, with no hematological dyscrasia. The 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 (World Medical Association, 2013).

Blood Sample

Whole blood samples were obtained by venipuncture using EDTA vacuum system tubes (Becton Dickson, New Jersey, USA). Blood tests were performed by an automated system (T890 Beckman Coulter Inc, USA). Samples were processed immediately after collection and stored at −80 ºC for further analysis. Information on patient therapeutics was obtained from patients' medical records.

RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis were performed using Trizol LS and the High-Capacity cDNA Reverse Transcription Kit, respectively (ThermoFisher Scientific®, Carlsbad, California, USA), following manufacturer's instructions.

mRNA and miRNA expression analysis – Comparative real-time PCR

Real-time qPCR assays for miR-15a and miR-16-1 were carried out in duplicate, in a 10µL volume, using TaqMan® microRNA assays (ThermoFisher Scientific®, Carlsbad, California, USA). Relative miR-15a and miR-16-1 mRNA expression was calculated from normalized ΔCT (cycle threshold) related to the housekeeping gene (RNU48). Real-time PCR assays, ZAP-70, were carried out in duplicate in a 10µL volume using SYBR Green (ThermoFisher Scientific®, Carlsbad, California, USA) and the following primers: protein tyrosine kinase 70kDa (ZAP-70) forward 5′-CGCTGCACAAGTTCCTGGT-

3′ and reverse 5′-GACACCTGGTGCAGCAGCT- 3′ (Wiestner *et al.*, 2002), along with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-GGTCGGAGTCAACGGATTTG-3' and reverse 5'ATGAGCCCCAGCCTTCTCCAT-3' (Zeng *et al.*, 2016). Relative ZAP-70 mRNA expression was calculated from normalized ΔCT (cycle threshold) related to the housekeeping gene (GAPDH). Real-time PCR assays for Ang-2 were carried out in duplicate in a 10µL volume using TaqMan® mRNA assays (ThermoFisher Scientific®, Carlsbad, California, USA). Relative Ang-2 mRNA expression was calculated from normalized ΔCT (cycle threshold) related to the housekeeping gene (GAPDH.). To detect changes in gene expression in CLL patients, the normalized ΔCT values for each sample were compared with the mean ΔCT level of the control group, and changes in the miR-15a, miR-16-1, ZAP-70, and Ang-2 gene (ΔΔCT) expressions were calculated.

The obtained values were converted to a linear scale ($2^{-\Delta\Delta CT}$) and reported as the fold-change in expression (arbitrary units).

In vitro evaluation of miR15a and miR-16-1 gene expression after treatment with fludarabine

After peripheral blood collection from untreated patients (low-risk patients), blood mononuclear cells (PBMCs) were extracted using Ficoll (Sigma Diagnostics, Inc., Missouri, USA). To evaluate the cytotoxic activity of fludarabine, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. Fludarabine was incubated together with 1×10^7 cells/well at a previously determined IC50 concentration (23.25 µM) for 48 hours. RNA cells were then extracted using Trizol LS. In addition, cDNAs were made and real-time PCR reactions were performed to analyze miR-15a and miR-16-1 gene expressions, as previously described.

Enzyme-linked Immunosorbent Assay (ELISA)

Bcl-2 plasma levels were determined by a commercial ELISA kit (Human Bcl-2 Platinum ELISA–eBioscience © 2017 Thermo Fisher Scientific Inc.) according to manufacturers' instructions.

Tatiane V. Braga, Fernanda C. G. Evangelista, Marie G. Santiago, Aline L. M. Ferrão, Tamara D. Almeida,
Bárbara L. F. Barbosa, Sergio S. S. Araujo, Glaciano N. Ribeiro, Maria G. Carvalho, Adriano P. Sabino

Statistical analysis

Statistical analysis was performed using variance analysis (ANOVA), followed by multiple comparison tests using Sigma Stat Software (SPSS) for Windows, version 2.03. Spearman's test was used to investigate the possible correlation between variables. GraphPad ABI Prism software, version 3.0, was used to plot graphs. Differences were considered significant when $p < 0.05$. Outliers were calculated using the GraphPad QuickCalcs Web site: http://www.graphpad.com/quickcalcs/Grubbs1.cfm

RESULTS

Age and number of individuals by gender were not significantly different among the analyzed groups. Significant differences were observed for blood analysis when comparing patients classified according Binet staging versus the control group (Table I).

Table I - Clinical and Laboratory (blood analysis) of patients with CLL and control

Age (years)*	Binet A	Binet B+C	Control	p
	67.95 ± 13.28	68.49 ± 13.29	67.71 ± 15.75	0.987
M/F	16/11	13/3	9/6	0.913
Erythorcytes**(10^{12}/L)	4.47 (3.99 – 4.99)	3.63 (3.21 – 4.21)	4.70 (4.50 – 5.13)	< 0.050 [a,c]
Hemoglobin*(g/L)	130.43 ± 2.08	100.94 ± 2.14	130.43 ± 1.57	< 0.050 [a,c]
Platelet** (10^9/L)	157.00 (132.00 – 197.00)	106.00 (72.50 – 139.25)	230.00 (185.00 – 251.00)	< 0.050 [a,b,c]
Leukocytes**(10^9/L)	20.65 (8.55 – 41.92)	17.90 (6.97 - 48,52)	5.8 (0.3 – 6.6)	< 0.050 [a,c]
Lymphocyte**(10^9/L)	16.19 (4.76 –35.67)	16.63 (4.87 – 39.76)	2.51 (2.17 – 2.65)	< 0.050 [b,c]
LDT	2 (7.41%)	3 (18.75%)	na	0.600

a: A vs B+C; b: A vs Control; c: B+C vs Control; M: male; F: female; *: mean ± SD; **: median and interquartile range; LDT: lymphocyte doubling time; na: not applicable.

Erythrocyte counts and hemoglobin levels were significantly reduced in the Binet B + C group, when compared to the A and control groups ($p < 0.050$), while the leukocyte count was significantly elevated in the B + C group, when compared to the control group ($p < 0.050$). Concerning the number of platelets, a significant reduction was observed in the B + C group, when compared to both the A and the control groups ($p < 0.050$). Finally, the lymphocyte count was higher in both the A and the B + C groups, when compared to the control group ($p < 0.050$). By contrast, when the A and B + C groups were compared as regards the lymphocyte doubling time over 6 months, no difference was observed.

At the time of collection, 18.5% of Binet A and 25% of Binet B + C patients were on chlorambucil or fludarabine treatment, while 7.41% of the Binet A patients and 18.75% of the Binet B + C patients presented lymphocyte doubling over a 12-month period.

Data of miR-15a, miR-16-1, ZAP-70, and Ang-2 mRNA gene expressions are presented in Figures 1, 2, 3, and 4, respectively. A similar profile can be observed for miR-15a and miR-16-1. Significantly lower expressions were observed when comparing patients and controls for miR-15a ($p < 0.001$) and miR-16-1 ($p < 0.001$) gene expressions. By contrast, no significant difference was observed for ZAP-70 mRNA gene expression. However, a descending expression of Ang-2 was observed from the controls to CLL Binet A and B+C groups. Significant differences were observed in the Ang-2 expression of CLL Binet A when compared to CLL Binet B + C,

and CLL Binet B + C when compared to the controls ($p < 0.050$).

A positive correlation between the results of miR-15a and platelets count ($R^2 = 0.340$; $p = 0.009$) was observed. By contrast, negative correlations were observed between the results of ZAP-70 and platelets counts ($R^2 = -0.334$; $p = 0.011$), between miR-15a and lymphocytes ($R^2 = -0.376$; $p = 0.004$), as well as between miR-16-1 and lymphocytes ($R^2 = -0.515$; $p = 0.00004$). When comparing the Binet A group and the Binet B + C group, Bcl-2 levels (Figure 5) proved to be significantly higher in the latter group ($p < 0.001$), as would be expected given the greater severity of the disease. A positive correlation was observed between the results of Bcl-2 and the leukocyte count ($R^2 = 0.310$; $p = 0.019$). No significant differences were observed for miR-15a and miR-16-1 gene expression after treatment with fludarabine.
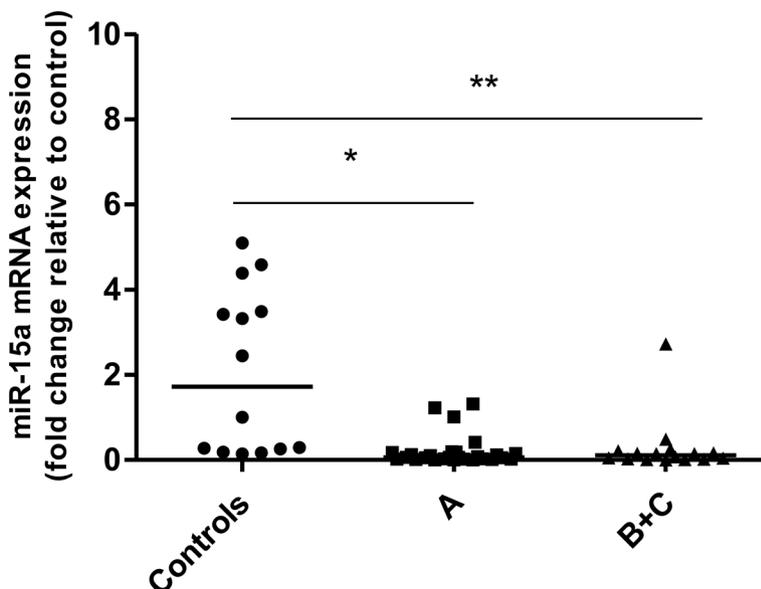

FIGURE 1 - miR-15a mRNA gene expression in the Controls, CLL Binet A (A) and Binet B+C (B+C). *p<0.001: A versus controls. **p<0.001: B+C versus controls. No significant difference was found when comparing Binet A and Binet B+C. Data was presented as median values generated by ANOVA, followed by the Kruskal-Wallis test.
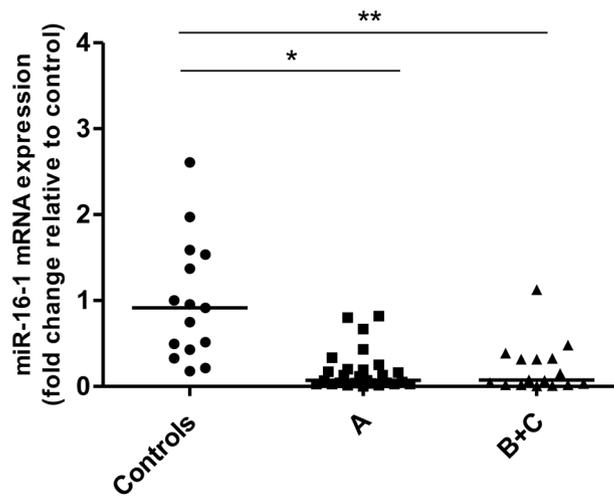
Tatiane V. Braga, Fernanda C. G. Evangelista, Marie G. Santiago, Aline L. M. Ferrão, Tamara D. Almeida, Bárbara L. F. Barbosa, Sergio S. S. Araujo, Glaciano N. Ribeiro, Maria G. Carvalho, Adriano P. Sabino

FIGURE 2 - miR-16-1 mRNA gene expression in the Controls, CLL Binet A (A) and Binet B+C (B+C). *p<0.001: A versus controls. **p<0.001: B+C versus controls. No significant difference was found when comparing Binet A and Binet B+C. Data was presented as median values generated by ANOVA, followed by the Kruskal-Wallis test.
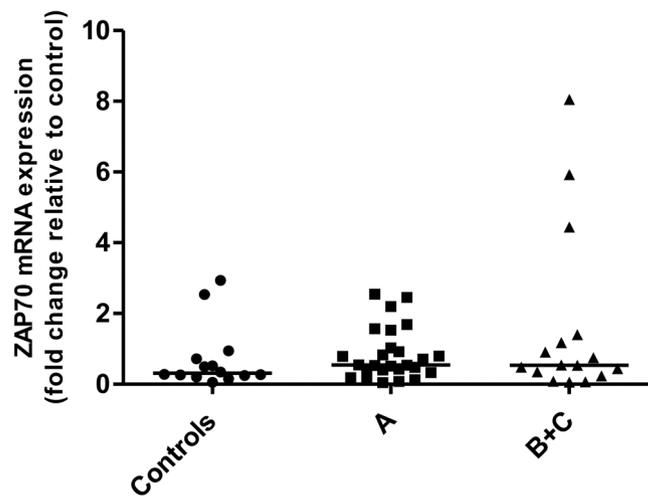
FIGURE 3 - ZAP-70 mRNA gene expression in the Controls, CLL Binet A (A) and Binet B+C (B+C). The transcript levels of ZAP-70 were similar among the studied groups. Data was presented as median values generated by ANOVA, followed by the Kruskal-Wallis test.
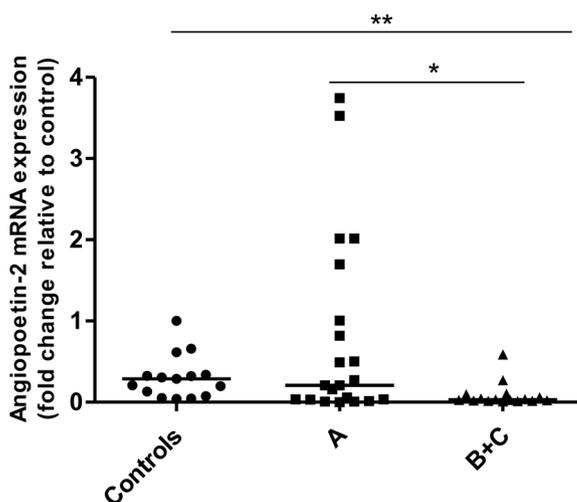
FIGURE 4 - Ang-2 mRNA gene expression in the Controls, CLL Binet A (A) and Binet B+C (B+C). *p=0.002: A versus B+C. **p=0.002: Controls versus Binet B+C. No significant difference was found when comparing Controls and Binet A. Data was presented as median values generated by ANOVA, followed by the Kruskal-Wallis test.
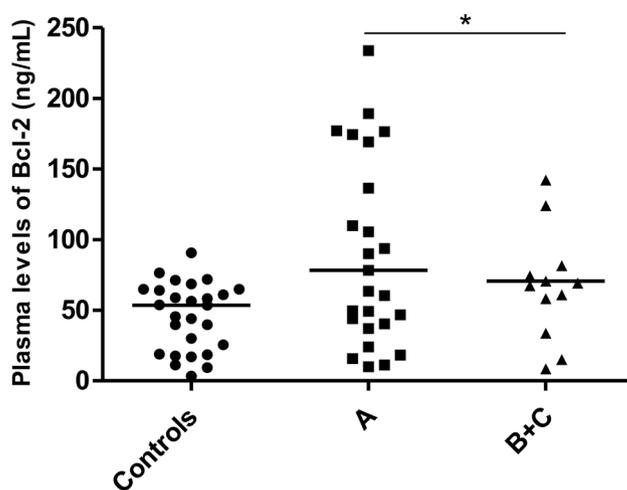

FIGURE 5 - Plasma levels of Bcl-2 in the Controls, Binet A (A) and Binet B+C (B+C) groups. *p < 0.001: A versus B+C. No significant difference was found when comparing Control and Binet A and Binet B+C. Data was presented as median values generated by ANOVA, followed by the Kruskal-Wallis test.

DISCUSSION

The occurrence of CLL before the age of 30 is very rare and more commonly affects males, as observed in the present study. CLL in older patients tends to be less favorable because of their comorbidities. When patients are evaluated according to sex, male patients usually present a less favorable prognosis when compared to female patients (Vasconcelos, 2005).

Confirming previous data and clinical characteristics of the disease, the present study observed different degrees of anemia in patients according to Binet staging. It is known that one of the changes related to the onset of anemia in these patients is extensive bone

marrow involvement that leads to progressive anemia in patients with CLL. In 10–15% of the patients, anemia is of the autoimmune hemolytica type. B-lymphocytes in CLL often produce autoantibodies (e.g., polyreactive antibodies), which are frequently observed in normal lymphocytes in the early stages of maturation. These autoantibodies are part of the characterization of the autoimmune phenomena that is often observed in these patients, causing anemia and autoimmune thrombocytopenia. Approximately 2% of the patients have immune thrombocytopenic purpura, which responds positively to treatment with corticosteroids (Bordin, 2005). However, these types of events were not observed in the present study. By contrast, a marked leukocytosis, due to a very high number of lymphocytes, occurred in these patients. In addition, it was observed that moderate to high-risk patients showed a tendency toward increased lymphocyte doubling rates, when compared with low-risk patients. Lymphocyte doubling time is referred to as the duplication of the absolute number of lymphocytes in the blood count within a period of less than or equal to 12 months. The presence of lymphocyte duplication is associated with disease progression. Such subclones can develop genetic abnormalities and can present higher associations with cell proliferation and death rates (Defoiche *et al.*, 2008; Hallek, 2008). In the present study, the Binet B + C group had a higher percentage of patients with lymphocyte duplication over a 12-month period, which is consistent with more aggressive characteristics of the disease in this group. However, no statistically significant difference was observed, perhaps due to the small number of analyzed samples.

A reduced expression of miR-15a and miR-16-1 was observed in the patients from the present study. The miR-15a and miR-16-1 genes act as tumor suppressors and are expressed at high levels in normal lymphocytes, which is not observed in CLL, since they present a low expression profile (Bartels, Tsongalis, 2009; Hanlon, Rudin, Harries, 2009; Cimmino *et al.*, 2005). However, when comparing the groups of patients with CLL, the Binet A group presented a lower miR-15a expression than did the Binet B + C group. This finding is in agreement with previous studies, suggesting that the deletion of

miR-15a and miR-16-1 is indeed the most frequent genetic alteration in indolent CLL, which indicates patients of a lower risk group (Binet A). In patients with deletions of the long arm of chromosome 13 (13q14), the loss of miR-15a /16-1 expression not only shifts the balance towards increased levels of Bcl-2, an anti-apoptotic protein, but also towards higher levels of p53 (with pro-apoptotic action). Consequently, the number of apoptotic cells decreases due to the high levels of Bcl-2, while the p53 mechanism maintains relatively low tumor cell growth (Dighiero, 2005). To the best of our knowledge, this explanation is consistent with the observed inverse correlations between of miR-15a/16-1 expressions and lymphocyte counts in these patients.

Chemotherapy for CLL is recommended, primarily in patients with an aggressive form of the disease. Patients with clinical conditions and laboratory findings indicative of low risk are generally not treated or receive a less aggressive therapy. However, patients with moderate to severe CLL should be managed with the main available therapeutic regimens: chemotherapy (Fludarabine®, Ciclofosfamide®), immunotherapy (Rituximab® and Alentuzumab®), radiotherapy, and "target therapy" (Ibrutinib®) (Scarfò, Ferreri, Ghia, 2016).

In many cases, fludarabine is used to treat CLL patients. The present study assessed whether or not this drug could be interfering in miR-15a and miR-16-1 gene expressions. No statistically significant difference was found in the expression of miR-15a and miR-16-1 after *in vitro* treatment with fludarabine, suggesting that this treatment does not interfere at the molecular level, in this case, the evaluated microRNAs.

ZAP-70 is an intracellular protein that is associated with the zeta chain of the T- cell receptor complex (TCR). The protein ZAP-70, though not found in normal B-lymphocytes, is expressed in CLL cells with no mutated IgVH genes. CLL lymphocytes with mutations in IgVH genes rarely maintain this protein's expression. Some studies have suggested that ZAP-70 is a good biomarker in CLL (Chakupurakal *et al.*, 2012; Huang *et al.*, 2012; Richardson *et al.*, 2006; Chen *et al.*, 2002). Previous data from our research group has found no difference in the expression of ZAP-70 that points to it as a biomarker in CLL (unpublished data), and similar

results were observed in this study. It is interesting to note that the association between these potential biomarkers and CLL has been widely discussed by different research groups (Wang *et al.*, 2012). Thus, it has been shown that the mutational state of IgVH genes and chromosomal abnormalities seem to be correlated, despite being different parameters of prognostic relevance. In relation to progression free survival (PFS), our study shows that, among the untreated patients, those with unmutated IgVH exhibit a more rapid disease progression. According to previous reports, in patients with unmutated IgVH and high expression of ZAP-70 (poor prognosis), both miR-15a and miR-16-1 expression levels are higher than in patients with better prognoses (Wang *et al.*, 2012; Calin *et al.*, 2008; Del Principe *et al.*, 2006; Chauffaille, 2005). This fact may well explain how the 13q deletion is associated with indolent or less aggressive CLL, thereby justifying the lower levels of miR-15a expression in the Binet A patients.

Ang-2 is produced by leukemic cells that induce angiogenesis. Ang-2 activates endothelial cells that produce VEGF, which increases the proliferation and migration of endothelial cells and stimulates the emergence of new blood vessels. Thus, it is related to solid tumors and their angiogenesis, in addition to being involved in poor prognoses in patients with CLL (in addition to the significant association between their expression and the unmutated IgVH status) (Sagatys, Zhang, 2012; Vrbacky *et al.*, 2010).

According to the literature, patients classified as Binet B or C generally present a higher Ang-2 expression than patients classified as Binet A. However, no association was made between levels of Ang-2 expression and disease severity in this study, since the group of low-risk patients (Binet A) presented a higher median of gene expression than did those patients with a moderate and high risk (Binet B + C).

Among the studied patients, Binet A and Binet B + C groups presented lower miR-15a and miR-16-1 expressions and higher plasma concentrations of Bcl-2, when compared to the control group. Positive correlation was also found between Bcl-2 and leukocytes, indicating an inhibition of the apoptosis process. The downregulation of miR-15a and miR-16-1 as part of

13q14 deletion contributes to an increase in Bcl-2 expression. Bcl-2 protein levels are controlled by miR-15a and miR-16-1; therefore, a lower expression of these miRNAs generates an increase in Bcl-2 expression. In patients with CLL, an increase in Bcl-2 levels leads to a reduction in apoptosis and consequently to a longer lymphocyte survival. By contrast, the reduction of miR-15a and miR-16-1 also reduces tumor growth via p53. This may explain how the 13q deletion region, where miR-15a and miR-16-1 are located, is associated with indolent CLL (Garg *et al.*, 2012; Shahjahani *et al.*, 2015; Degheidy *et al.*, 2013).

In summary, the data analyzed together allow us to conclude that the miR-15a and miR-16-1, in addition to Bcl-2, are associated with a reduction in the apoptosis process, which contributes to lymphocyte accumulation and aggravation of the disease. As Ang-2 expression was significantly higher in A than in B + C Binet groups, this biomarker should be investigated in more robust studies within populations with a still relevantly indolent form of the disease in an attempt to identify those patients with a greater potential for an aggravation of the disease.

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