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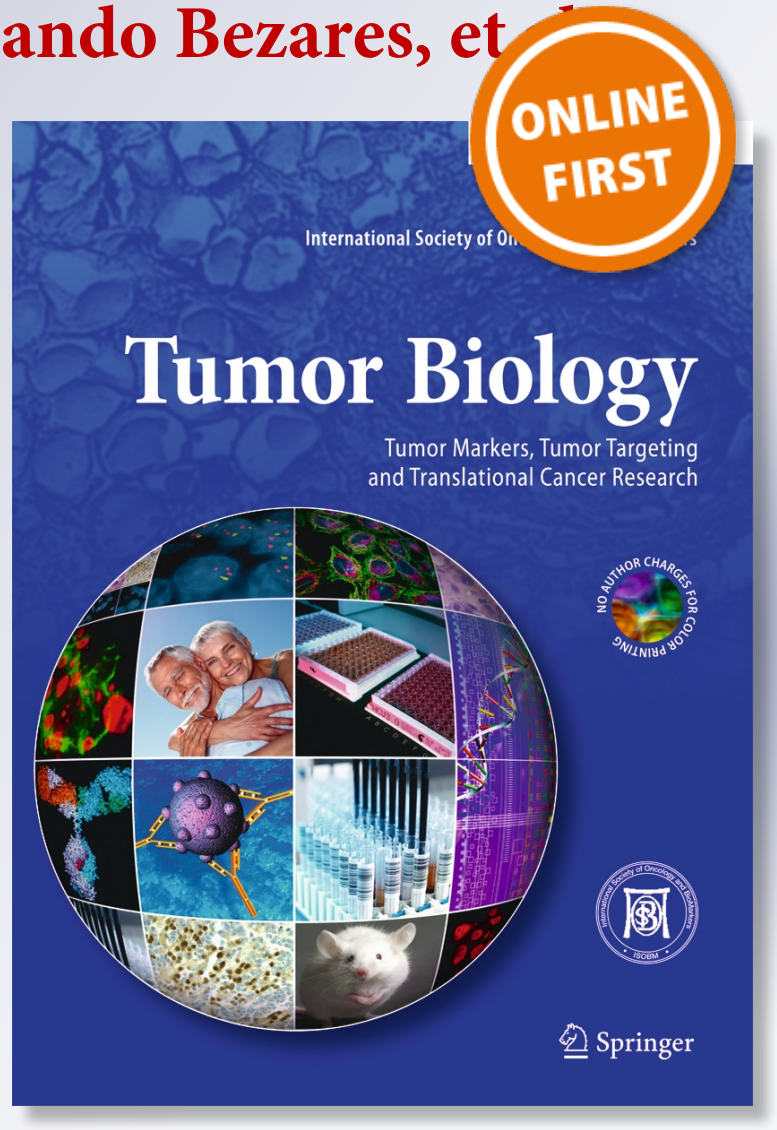

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RESEARCH ARTICLE

SOX11 expression in chronic lymphocytic leukemia correlates with adverse prognostic markers

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Abstract The transcription factor *SOX11* plays an important role in embryonic neurogenesis and tissue remodeling. Recent studies have shown aberrant expression of *SOX11* in various types of aggressive B cell neoplasms. In this study, we have analyzed *SOX11* transcription levels in 86 patients with diagnosis of chronic lymphocytic leukemia (CLL). Results were correlated with well-known prognostic factors such as immunoglobulin heavy chain variable (*IGHV*) gene mutational status, cytogenetics risk groups and clinicopathological characteristics of the disease. Overall, 35 % of cases showed *SOX11* expression; meanwhile, the remaining 65 % lacked gene expression. The analysis taking into account the *IGHV* mutational status showed significant differences in *SOX11* transcripts levels between mutated (0.004±0.0001) and unmutated CLL patients (0.405±0.011) ($p<0.0001$), as well as a positive correlation between *SOX11* mRNA expression and the percentage of *IGHV* homology ($p=0.0001$). Furthermore, significantly lower *SOX11* mRNA expression was detected in patients with deletion 13q14 as a single alteration (0.016±0.008) than those observed in cases with deletions 11q/17p (0.35±0.017) ($p=0.02$). The correlation of gene expression with clinical evolution showed shorter treatment free survival ($p=0.043$) and overall survival ($p=0.047$) in *SOX11* positive patients compared to *SOX11* negative cases. Our findings show for the first time an association between *SOX11* expression and some CLL poor prognostic factors. These results suggest *SOX11* as a possible biomarker that adds new biological information that could contribute to a better understanding of this pathology.

Keywords Chronic lymphocytic leukemia · *SOX11* · Gene expression profile · *IGHV* mutational status · Molecular cytogenetics

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in the Western world, accounting for nearly 30 % of all leukemias. The clinical course of the disease is extremely heterogeneous, with patients that require therapy immediately after diagnosis while others living for decades without ever requiring treatment [1]. In the last decades, besides clinical parameters, a number of biological features have been shown to correlate with prognosis [2–6]. Among them, the mutational status of the immunoglobulin heavy chain

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variable (*IGHV*) genes and genomic abnormalities have demonstrated to have independent prognostic value, being a reflection of the biological characteristics of the disease. Fluorescence in situ hybridization (FISH) analysis allowed the identification of distinct cytogenetic risk groups, in which patients with deletion 13q14 as a single alteration have a better outcome, while cases with deletions 11q22 or 17p13 show the shortest median survival, and patients with trisomy 12 have an intermediate prognosis [6]. The mutational status of *IGHV* genes [2, 3] defines two disease subgroups, one expressing mutated (M) *IGHV* segments with a more favorable clinical course and the other expressing unmutated (UM) *IGHV* segments, associated to a poor outcome. However, these known prognostic factors do not entirely explain the marked clinical heterogeneity of the disease or the development of severe complications.

Members of the SOX (SRY box-containing) gene family encode a group of transcription factors that accomplish important tasks during determination of cell fate and differentiation [7]. To date, more than 20 SOX gene family members have been identified, and SOX genes are divided into eight subgroups (A–H) according to the degree of homology within and outside the DNA-binding high-mobility group (HMG) domain. Among them, the *SOX11* [SRY (sex-determining region Y)-box 11] gene, mapped on chromosome 2p25.3, belongs to the subgroup C of SOX gene family and is commonly associated with embryonic neurogenesis and tissue remodeling and is not expressed in any adult normal tissue [8–10]. Human SOX11 is homologous to SOX4 with 55 % amino acid identity within the C-terminal transactivation domain and 86 % identity for the HMG domain [11]. SOX4 is a prominent transcription factor in lymphocytes of both B and T cell lineage and is crucial for mice B lymphopoiesis [12]. In contrast, SOX11 has no known lymphopoietic function and is not usually expressed in B cells. However, it has been shown to be expressed in different solid tumors [13–15] as well as in specific subtypes of B cell lymphoid malignancies. Thus, several studies have shown aberrant SOX11 nuclear protein expression and mRNA levels in mantle cell lymphoma (MCL) patients, establishing that this gene could be a reliable biomarker in this pathology, beyond the cyclin D1 status, that distinguish MCL from other lymphoid neoplasms [16–18]. In addition, different authors have shown *SOX11* expression in patients with lymphoblastic lymphoma, Burkitt's lymphomas and T cell prolymphocytic leukemia [17, 18], indicating that the deregulation of this gene is not completely restricted to MCL. Moreover, a recent study [19] found CLL/PL (chronic lymphocytic leukemia/prolymphocytic leukemia) positive cases in their cohort, indicating a wider role for *SOX11* in lymphomagenesis than previously reported. In addition, Cao et al. [20] showed that ZAP70-positive CLL patients had higher *SOX11* mRNA levels than ZAP70-negative CLL cases, suggesting that *SOX11* is expressed in CLL patients with poor prognosis. In this report, we have analyzed the *SOX11* expression profile in M-CLL and UM-CLL patients, and the results were correlated with cytogenetic risk groups and clinicopathological characteristics of the disease.

Methods

Patients

Our cohort included 86 unselected CLL patients (54 males; median age, 62.5 years; range, 36–86 years), consecutively referred to our institution. Patients were diagnosed according to the World Health Organization criteria [21]. From the total cohort, 51 cases (59 %) had newly diagnosed CLL; the remaining patients were diagnosed more than 1 year before the start of our study (range 12–144 months). All cases were previously untreated. Stage was assessed according to Rai classification [22]. Clinical stages were available in 82 patients, with the following distribution: 0—20 cases (24.4 %), I—24 (29.3 %), II—22 (26.8 %), III—9 (11 %), and IV—7 (8.5 %). FISH analysis was performed in 62 cases. The study was approved by the local Ethics Committee. All individuals provided their informed written consent.

RNA isolation

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) from mononuclear cells isolated on a Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden) density gradient from peripheral blood samples of patients. RT-PCR was performed using 1X RT Buffer (Promega, Madison, WI, USA), 200 U/μL of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI, USA), 250 ng/μL of random primer (Promega, Madison, WI, USA), and 10 mM each dNTP (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was carried out from 1 μg of total RNA previously treated with DNAse (Ambion, Carlsbad, CA, USA) for 10 min at 95 °C, 60 min at 37 °C, and 10 min at 95 °C to inactivate the enzyme in a 20 μL final volume. cDNA obtained was stored at −20 °C until use.

SOX11 mRNA expression

The analysis of *SOX11* mRNA expression was performed using quantitative real time PCR (qRT-PCR) in a LightCycler system (Roche Diagnostics, Mannheim, Germany), based on TaqMan methodology. The housekeeping gene *GUSB* (β-glucoronidase) (Hs00939627_m1, Applied Biosystems, Foster City, CA, USA) was used to normalize sample-to-sample differences in cDNA input, RNA quality, and RT efficiency. Briefly, the PCR reaction was performed by using 4 μL of each RT reaction, 5X TaqMan Master Mix (Roche

Diagnostics, Mannheim, Germany), 1X TaqMan Gene Expression Assays for SOX11 (Hs00846583_s1, Applied Biosystems, Foster City, CA, USA) in a 20 μL final volume reaction. The cycle conditions were: 2 min at 50 °C, 10 min at 95 °C to activate the enzyme, followed by 45 cycles at 95 °C for 15 s, 60 °C for 1 min. All measurements were performed in triplicate and included a determination of a negative control, in which cDNA was substituted by water. Standard curves were constructed with fivefold serial dilutions of the cDNA from a positive control sample for *SOX11* (5 to 0.15 μg total RNA). As negative controls, mononuclear cells from healthy normal individuals and K-562 cell line were used. As positive control, we used a human embryonic stem cell-derived from neuronal progenitors (WA09), obtained from WiCell Research Institute (Madison, USA). Additionally, 20 mantle cell lymphoma samples were also evaluated.

IGHV mutational status

The *IGHV* gene sequences were determined from RNA samples of CLL patients as previously described [23]. Briefly, amplification of IGHV regions by PCR was performed on cDNA using VH framework region 1 consensus family specific primers (VH1-VH6) and JH primers [24]. When amplifications of these primers were unsuccessful, an alternative set of primers that anneal to sequences in the leader region (LH1-LH6) and one antisense Cμ-primer [25] were used. Thermal cycling conditions were 3 min at 93 °C, followed by 33 cycles at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30s, elongation at 72 °C for 7 min, and a final step at 4 °C for 10 min. PCR products were purified in 1 % agarose gels, sequenced bidirectionally, and analyzed on an automated DNA sequence analyzer (377 ABI Prism, PE Biosystem, Foster City, CA, USA). Sequence data were analyzed using IgBLAST (http://www.ncbi.nlm.nih.gov/igblast) and the ImMunoGeneTics database (IMGT; http://imgt.cines.fr) [26]. IGHV sequences with less than 98 % homology with respect to the germline counterpart were considered as mutated while those with homology of 98 % or higher were classified as unmutated.

FISH study

FISH analysis was performed on interphase nuclei of cultured peripheral blood lymphocytes. Slides were hybridized with a panel of DNA probes to detect prognostic aberrations in CLL: trisomy 12 and deletion 13q14, LSI D13S319/LSI13q34/CEP12 (Vysis-Abbott, Downers Grove, IL, USA) or EN12 and OLE 13q14.3 D13S319/13q34 (Live-Lexel, Buenos Aires, Argentina), deletion 11q and 17p, LSI p53/LSI ATM (Vysis-Abbott, Downers Grove, IL, USA) or OLE 11q22.3 ATM/11q25 and OLE17p13 (P53)/EN17 (Live-Lexel, Buenos Aires, Argentina), according to the manufacturer's protocols. For each probe, at least 200 interphase nuclei were analyzed. The cutoff for positive values (mean of normal control+3 standard deviations), determined from samples of ten cytogenetically normal donors, were 3.02, 10.2, 7.7, and 5.1 % for trisomy 12, monosomies of D13S319, ATM, and TP53, respectively.

Statistical analysis

All statistical evaluations were performed using GraphPad Prism version 5.0 (2008). The analysis of mRNA expression data was done using the Mann–Whitney test. Spearman correlation was used to analyze *SOX11* mRNA expression and the percentage of *IGHV* homology. Groupwise comparison of the distribution of clinical and laboratory variables was performed with the Student *t* test (for quantitative variables) and the χ^2 or Fisher's exact test (for categorical variables). Receiver operating characteristic (ROC) curves analysis was applied to calculate the expression cutoff value for *SOX11* gene, with the highest sensitivity and specificity. Overall survival (OS) defined from the date of diagnosis to the death of the patient and treatment free survival (TFS) calculated from the date of diagnosis to the first CLL-specific treatment were estimated by the Kaplan–Meier method and compared with the log-rank test. For all tests, $p<0.05$ was regarded as statistically significant.

Results

Age, sex, clinical characteristics, and mutation status of 86 CLL patients are summarized in Table 1. Fifty-one (59.3 %)

Table 1 Clinical characteristics of CLL patients

	Total
No. of patients (*n*)	86
Sex (M/F)	54/30
M IGHV CLL	51
UM IGHV CLL	35
Mean age (years) (range)	62.5 (36–86)
Clinical stages (%)	
Rai 0	24.4
Rai I–II	56.1
Rai III–IV	19.5
Mean WBC count ($\times 10^9$/L) (range)	49.12 (8.9–500)
Mean lymphocyte (%) (range)	75 (54–98)
Mean Plt count ($\times 10^9$/L) (range)	183 (16.9–378)
Mean Hb (g/dL) (range)	13 (6–15.75)
Mean LDH (UI/L) (range)	383.2 (128–680)
Mean B_2M (μg/mL) (range)	2.23 (1.1–3.7)

M/F nale/female, *M* mutated, *UM* unmutated, *IGHV* immunoglobulin heavy chain variable region, *WBC* white blood cells, *Plt* platelets, *Hb* hemoglobin, *LDH* lactate dehydrogenase, *B_2M* beta 2 microglobulin

cases were M-CLL, whereas the remaining 35 (40.7 %) were classified as UM-CLL. Two patients showed double in-frame rearrangements. The distribution by *IGHV* family and gene usage is shown in Fig. 1a, b. The most commonly expressed family was IGHV3 (45.5 %), followed by IGHV1 (23.9 %) and IGHV4 (21.6 %). Among genes, *IGHV1-69* (9 cases; 10.2 %), related to UM status, was the most frequently observed, followed by *IGHV3-23* (8; 9 %) and *IGHV2-05* (6; 6.8 %), associated to mutated status. Three cases (3.4 %) showed *IGHV3-21*, all of them UM-CLL. Molecular cytogenetic studies were performed in 62 cases; 71 % (44/62) of them showed FISH alterations. The most frequent anomaly was deletion 13q14 observed in 48 % (30/62) of patients, 73 % (22/30) of them as single alteration. Trisomy 12 was detected in 19 % (12/62), deletion 11q in 13 % (8/62), and deletion 17p in 4.8 % (3/62) of patients. Nine cases (14 %) showed more than one FISH alteration.

SOX11 mRNA expression levels in CLL

SOX11 mRNA expression was evaluated in 86 cases with CLL. Analysis of data showed a wide heterogeneity among patients. For a better analysis, patients were divided into two groups: with and without expression, according to the cutoff value obtained using ROC curves analysis (0.017). Overall, 30 cases (35 %) showed *SOX11* mRNA expression (0.46± 0.07); meanwhile, the remaining 56 patients (65 %) were below the cutoff value (0.0026±0.0008). Additionally, *SOX11* transcription levels were evaluated in 12 healthy donors (0.001±0.0001) and in 20 MCL samples: 15 with classical MCL (cMCL) (4.04±1.2) and 5 with indolent MCL (iMCL) (0.005±0.001). *SOX11* negative expression CLL patients showed a similar distribution of mRNA values than healthy donors and iMCL patients (Fig. 2).

The analysis of data taking into account the mutational status showed that almost all patients from UM-CLL group (29/35; 83 %) had *SOX11* mRNA expression over our cutoff; meanwhile, all except one case from the M-CLL group (50/51; 98 %) were below the cutoff value ($p<0.0001$). In addition, significant differences in *SOX11* mRNA levels between both M (0.004±0.0001) and UM-CLL (0.405±0.011) were observed ($p<0.0001$). Figure 2 shows the distribution of patients in both groups. Besides, a positive correlation between

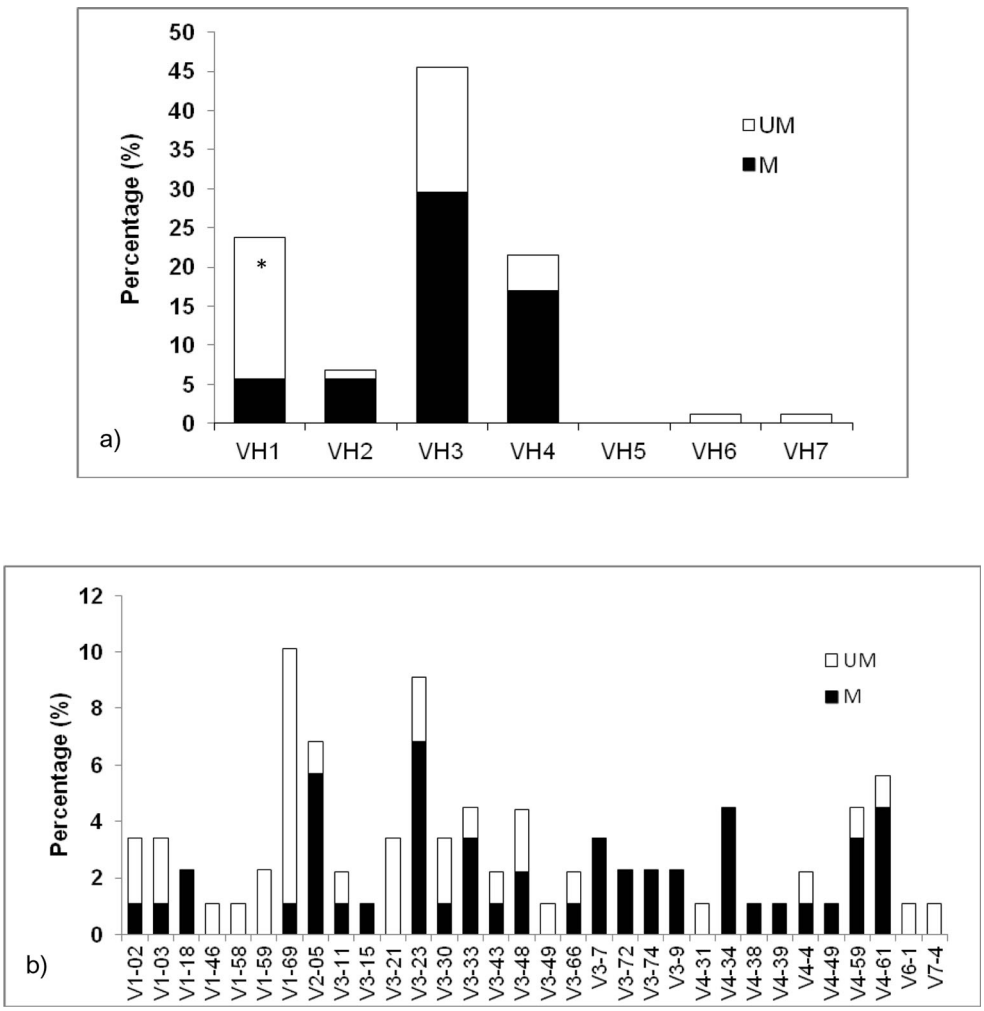

Fig. 1 Distribution of immunoglobulin heavy chain variable (*IGHV*) family and gene usage. **a** *IGHV* family usage profile of patients with mutated (*M*) and unmutated (*UM*) chronic lymphocytic leukemia. *Significant differences in the mutational status between IGHV1 and IGHV3 ($p=0.0039$) and IGHV4 families ($p=0.0012$), and IGHV1 with respect to the entire series ($p=0.0004$); **b** *IGHV* gene segment usage profile in patients with M-CLL and UM-CLL. *IGHV1-69* was preferentially associated to UM status (88.8 % of cases), all of them with 100 % sequence homology, whereas *IGHV3-7*, *IGHV3-72*, *IGHV3-74*, *IGHV3-9*, and *IGHV4-34* were always related to *M* status

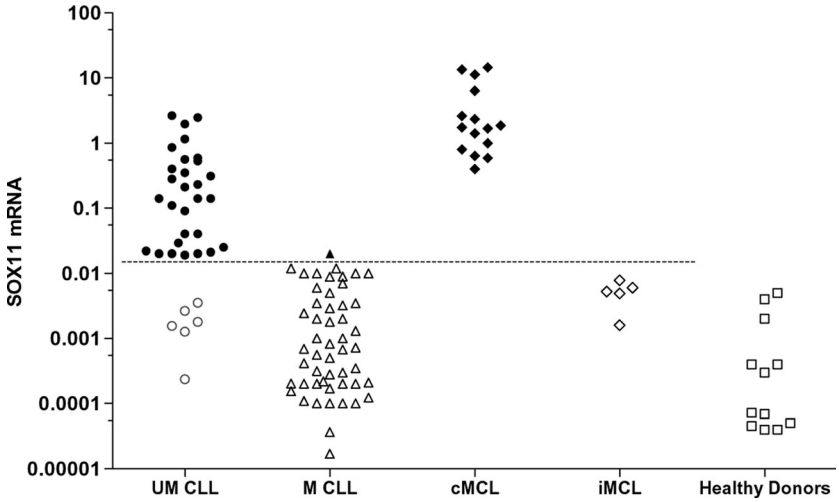

Fig. 2 Graphic representation of *SOX11* mRNA expression in mutated (*M*) and unmutated (*UM*) chronic lymphocytic leukemia (*CLL*) patients, classical (*cMCL*), and indolent mantle cell lymphoma (*iMCL*) patients and healthy donors

SOX11 mRNA expression and the percentage of *IGHV* homology was observed (r=0.638; p=0.0001). We have also analyzed *SOX11* mRNA levels in different *IGHV* genes. Thus, higher mRNA expression was observed in *IGHV1-69* (9 cases) (mean 0.4971) associated to UM-CLL; meanwhile, *IGHV3-23* (8 cases), related to M-CLL, showed the lowest values (mean 0.0122). Moreover, a high mean of *SOX11* mRNA expression (0.1245) was also observed in our patients with *IGHV3-21* gene usage, rearrangement associated to poor outcome [27].

Our data were also evaluated according to FISH risk groups. In this regard, significantly lower *SOX11* mRNA expression in patients with 13q14 deletion as a single alteration (0.016±0.008) than those observed in cases with deletions 11q/17p (0.35±0.017) (p=0.02), and also compared to patients without cytogenetic and FISH alterations (0.18±0.09) (p=0.043), was observed (Fig. 3). Patients with trisomy 12 (0.35±0.028) also showed increased levels of *SOX11* mRNA transcripts compared to cases with deletion 13q14 but without reaching significant differences. Most of cases (88 %) with deletion 13q14 as a single alteration had mutated status meanwhile 73 % of patients with deletions 11q/17p were UM-CLL (p=0.018).

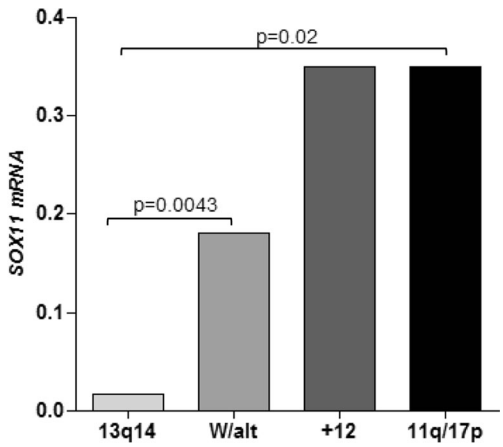

Fig. 3 Distribution of *SOX11* mRNA expression according to FISH risk groups. W/alt: without alterations

Correlation between *SOX11* expression and CLL prognostic factors

We explore for possible relationships between *SOX11* transcriptional levels and CLL prognostic factors (Table 2; Supplementary material Online Resource 1 Fig. S1). Analysis of data showed significantly higher β2 microglobulin levels (p=0.027) and a tendency to increased mean of white blood cells count (p=0.05) in patients that had *SOX11* expression compared to those without it. In addition, although no significant differences were found, increased lactate dehydrogenase serum levels and percentage of lymphocytes, as well as a decrease in platelet count and hemoglobin levels in *SOX11* positive patients compared to negative ones, were observed. The TFS was shorter in patients with *SOX11* expression (49 months) than in *SOX11* negative cases (168 months) (p=0.043). Besides, OS was significantly shorter in the first group compared to the latter (p=0.047) (Fig. 4a, b).

Discussion

Recent studies have shown that *SOX11* is an active and central regulator of cellular growth in hematopoietic malignancies [28]. In this report, we have explored *SOX11* expression levels in patients with CLL and their correlation with biological and clinical characteristics of the disease. Interestingly, we found that *SOX11* transcriptional levels were strongly associated to the mutational status of *IGHV* genes, with positive mRNA levels in UM-CLL patients and lack of *SOX11* expression in

Table 2 Clinical characteristics in *SOX11* positive and negative CLL patients

	SOX11 positive	*SOX11* negative	*p*
No. of patients (*n*)	30	56	
Sex (M/F)	16/14	38/18	0.242
M IGHV CLL	1	50	<0.0001
UM IGHV CLL	29	6	
Mean age (years) (range)	63.9 (36–88)	62.1 (36–88)	0.118
Clinical stages (%)			
Rai 0	7.3	17.1	0.54
Rai I–II	22.0	34.1	
Rai III–IV	4.8	14.6	
Mean WBC count ($\times 10^9$/L) (range)	44 (8.9–118)	29.3 (9.5–86.7)	0.05
Mean lymphocyte (%) (range)	76.2 (59–95)	73.6 (54–98)	0.395
Mean Plt count ($\times 10^9$/L) (range)	174 (24–300)	187 (16.9–378)	0.511
Mean Hb (g/dL) (range)	12.9 (6–14.7)	13.04 (7.6–15.7)	0.818
Mean LDH (UI/L) (range)	430.7 (159–642)	367.4 (198–680)	0.126
Mean B_2M (μg/mL) (range)	2.6 (1.6–3.7)	2.1 (1.1–3.3)	0.027

M/F male/female, *M* mutated, *UM* unmutated, *IGHV* immunoglobulin heavy chain variable region, *WBC* white blood cells, *Plt* platelets, *Hb* hemoglobin, *LDH* lactate dehydrogenase, B_2M beta 2 microglobulin

cases with mutated status, indicating an association between *SOX11* expression and adverse prognosis. As known, mutational status of *IGHV* genes is an important prognostic factor in CLL that defines two disease subgroups, been UM status related to poor outcome [29, 30]. In concordance with our results, Cao et al. [20] found increased *SOX11* expression in UM-CLL patients compared to M-CLL cases but without reaching significant differences, suggesting that patients expressing *SOX11* mRNA may have a poor prognosis. Moreover, studies in MCL patients have also found high *SOX11* expression in tumors with no or low *IGHV* mutations, with clinical significance [31]. In addition, when we analyzed *SOX11* taking into account FISH risk groups, we found no *SOX11* expression in cases with 13q14 deletion as single alteration, according to the more favorable prognosis for this anomaly; meanwhile, positive *SOX11* mRNA values were observed in patients who harbor deletion 11q/17p, related to poor outcome. The association between *SOX11* expression and FISH alterations would reflect a possible biological connection between these molecular markers, suggesting this transcription factor as a possible biomarker of unstable and/or progressive CLL. Furthermore, we were interested in determining if the expression profile of *SOX11* gene had clinical significance. Thus, the analysis of clinical evolution showed shorter TFS and OS in *SOX11* positive patients compared to negative ones, which is in agreement to those observed by different authors in MCL patients showing an adverse outcome in *SOX11* positive cases [31–33].

Different studies have shown that *SOX* genes, including *SOX11*, may enhance Wnt (wingless-type MMTV integration site family) signaling pathway [34, 35] that operates by establishing the key downstream effector β-catenin in the cytoplasm [36]. Wnt pathway is aberrantly activated in CLL, contributing substantially to the anti-apoptotic and mitogenic characteristics of leukemic cells [37], and also active Wnt/β-catenin signaling is observed in the pre-leukemic state of monoclonal B cell lymphocytosis [38]. Furthermore, angiogenesis deregulation has also been associated

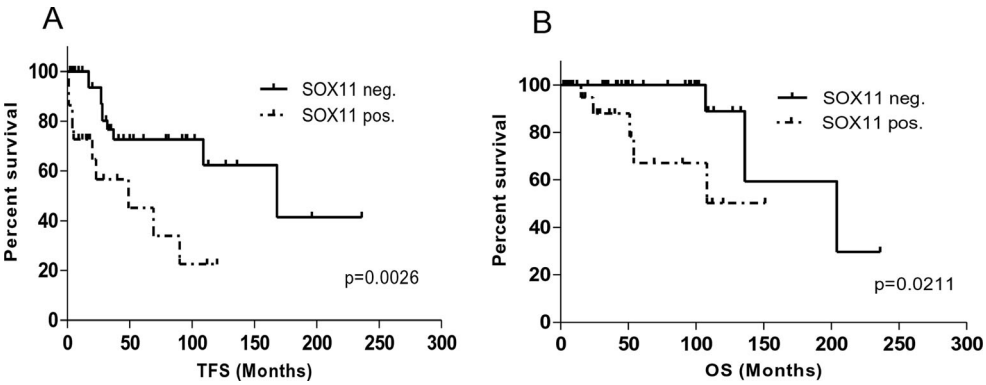

Fig. 4 Kaplan–Meier analysis of CLL patients stratified by *SOX11* expression. **a** treatment free survival (*TFS*) (p=0.0026); **b** overall survival (*OS*) p=0.0211)

with the development and clinical progression of lymphoid neoplasias, including CLL [39–42]. Recently, it has been shown that SOX11 upregulation promotes angiogenesis inducing the expression of several pro-angiogenic factors [43]. Thus, it is logical to hypothesize that *SOX11* could play a role in the progression and/or pathogenesis of CLL, mainly regulating genes involved in cell proliferation programs, apoptosis, and angiogenesis.

In conclusion, to the best of our knowledge, these findings showed for the first time an association between *SOX11* expression, UM-IGHV status, and deletion 11q/17p, as well as a positive correlation with shorter TFS and OS, adding new biological information that could contribute to a better understanding of CLL. However, more studies in a large number of patients are needed to define the role of *SOX11* expression in the pathogenesis and development of this disease.

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Conflicts of interest None

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