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Prognostic Value of the Expression of Receptor Tyrosine Kinase-Like Orphan Receptor 1 (ROR-1) in Chronic Lymphocytic Leukemia

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ABSTRACT

Background: The transmembrane receptor tyrosine kinase-like orphan receptor 1 (ROR1) has acted on the causation and sustentation of mature B-cell lymphomagenesis for chronic lymphocytic leukemia (CLL) cells. The study attempted to show whether there is a relationship between the level of ROR1 surface expression in CLL cells and disease findings.

Materials and Methods: The level of ROR1 cell surface expression was determined in accordance with the flow cytometric analysis of CLL patients at the first diagnosis time. Two groups were formed according to the high and low ROR1 levels. The cut-off point for the ROR1 level was calculated for advanced-stage disease using receiver operating characteristic (ROC) curves. A two-sided p-value <0,05 was considered statistically significant.

Results: 108 CLL cases with a median age of 60 were enrolled. The median percentage of ROR1 cell surface marker positivity in the CD5/CD19 positive leukemic cell was 62%. The CLL cases with high ROR1 levels have thrombocytopenia (p=0.042), anemia (p=0.028), and high beta-2 microglobulin value ≥3 mg/dL (p=0.002) and the need for first-line treatment (p=0.043).

Conclusion: The poor prognostic parameters such as splenomegaly, anemia, higher beta-2 microglobulin levels, intermediate/advanced RAİ stage disease, and need for first-line treatment had associated high-level ROR 1 expression of our CLL patients. It needs to be investigated for its effect on predicting disease burden and aggressiveness with more comprehensive studies on ROR1 expression levels in CLL cases.

Keywords: The transmembrane receptor tyrosine kinase-like orphan receptor 1 (ROR1); Chronic lymphocytic leukemia (CLL)

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by a progressive accumulation of monoclonal B lymphocytes in peripheral blood, bone marrow and nodal/extranodal lymphoid tissue[1]. Asymptomatic and early-stage CLL patients do not require any therapy at the time of diagnosis, and their median survival is usually longer than ten

years[1]. Receptor tyrosine kinase-like orphan receptor (ROR) 1 is a transmembrane glycoprotein from the receptor tyrosine kinase family (receptor tyrosine kinase (RTK))[2]. An immunoglobulin (IgG) like domain, a protein kinase domain, a frizzled protein domain and a Kringle domain together form the structure of human ROR1 protein [2]. The studies show that ROR1, a receptor in the Wnt5a pathway, is involved in the survival, proliferation and migration of CLL cells [3]. Thus, the ROR1 receptor appears to be an option for targeted therapy in CLL.

ROR1 is essential in developing normal fetal and embryonic tissue, but most mature tissue doesn't contain ROR1 protein[4]. ROR1 is reported to be highly expressed in some hematological malignancies and solid tumors such as CLL, acute lymphocytic leukemia, and lung cancer [5]. ROR1 has a role in inducing B-cell lymphomagenesis. ROR 1 expression is not present in the early stages of normal B cell development; however, it is expressed at high levels in the intermediate/late stages of development, while downregulated again in normal/mature B cells. On the other hand, ROR 1 expression has been shown in both intermediate and mature B-cell malignancies. This is known to contribute to the survival of malignant mature B-cells. High ROR1 expression in blood samples of CLL cases has been suggested to be associated with increased disease progression and shortened survival [6].

In previously published studies, the presence of ROR1 expression was observed in all CLL cases and independent prognostic factors such as ZAP-70 and immunoglobulin heavy-chain variable region mutation status[5]. However, there is limited data on whether the ROR1 expression level affects the prognosis of CLL patients. Here, we aimed to investigate the expression levels of ROR1 by flow cytometric analysis in CLL patients and the relationship between the ROR1 expression levels with the clinical and laboratory findings.

MTERIALS AND METHODS

Patients

The data of 108 CLL patients between 2010-2019 were retrospectively reviewed. The 90 CLL patients with ROR1 expression were included in the study. The diagnosis of CLL was based on the presence of >5.0 x 10^9/L lymphocytes in peripheral blood and analysis of immunophenotyping (CD51/CD191/CD231/ IgM1) by flow cytometry [1]. The time from diagnosis to disease progression or death was considered as progression-free survival. This study was performed according to the Helsinki Declaration (version Fortaleza, Brazil, October 2013) and was approved by the local Ethics Committee. All participants have provided written consent. Based on the peripheral blood flow cytometry results during diagnosis, two different patient groups were formed according to high (HiROR1) and low (LoROR1) expression levels of ROR1. CLL cases separated into HiROR1 and LoROR1 groups were compared using laboratory findings and clinical parameters such as the need for first-line treatment during a mean follow-up of 19 months.

Flow cytometry

Blood / bone marrow samples were taken into 4 mL K3 EDTA tubes (BD Vacutainer®, CA) for analysis on flow cytometric analysis. We used a stain-lyse-and-then-wash direct immunofluorescence method for monoclonal antibodies (Beckman Coulter,USA) on labelling of the cell surface markers of the specimen. CD45/CD5/CD10/CD19/CD23,CD19/CD103/CD22/CD11c/CD25/CD5/CD20/sIgκ/sIgʎ/CD45,CD19/CD3/CD79b/CD22/CD19/CD43/CD200/CD38/ROR1/CD81 and ZAP-70 expression was determined by using monoclonal antibodies containing fluorescein isothiocyanate, phycoerythrin, phycoerythrin-txasred, phycoerythrin cyanin5, allophycocyanin in all patients. The device calibration was maded daily by using an calibration check bead (Flow-Check, BC, USA). The cell count was checked for correctness and certainty by using international quality controls acquired from the United Kingdom National External Quality Assessment Scheme (UK NEQASLI, Sheffield, UK) (z score range -2.0- 2.0). Briefly, CD19+ B cells were selected (at least 2000 events according to the threshold of the isotype control) from the data file using conventional gating strategies (forward and side scatter and the pattern of CD19 expression). Genetically tests were detected by FISH evaluation for del(11q), del(13q) and del (17p) on the diagnosis time of the disease.

Statistical analysis

Statistical analyzes were performed using SPSS (Statistical Package for Social Sciences) version 17.0 for windows. Differences between demographic, clinical and laboratory variables were compared using the chi-square test, Student's t-test, or the Mann-Whitney U test. Statistical comparisons were made by the Log-rank test. The measurement on the cut-off level of ROR 1 for advanced stage disease of CLL was estimated by receiver operating characteristic (ROC) curves. A two-sided p-value <0.05 was considered statistically significant. Cox Proportional Hazards Model was used for multivariate analysis. Kaplan–Meier's curves were used for survival analysis. A two-sided p-value <0.05 was considered statistically significant.

RESULTS

The clinical and laboratory data of 108 CLL patients with a median age of 60 (37-83) years and a Male/Female ratio of 53/43(52.2%/44.8%) were evaluated. The mean follow-up time was 50.5 (25-158) months. Three patients (3.3%) died during follow-up. The number of cases with RAI stage 0-1, stage 2, stage 3-4 were 44 (40.7%), 41 (39.8%), and 23 (21.2%), respectively. The clinical and laboratory features of cases are shown in Table 1 and Table 2. ROC was used to obtain cut-off levels of ROR1 positivity for RAI stages (early versus intermediate+high). The median percentage of ROR1 cell surface marker positivity in the $CD5^+/CD19^+$ leukemic cells was 62% (Range: 42%-96%) in the peripheral blood samples using flow-cytometry. It was measured at a cut-off level for ROR1 percentage as 60% among intermediate + high risk (RAI stage 2+3+4) CLL cases (p: 0.032). We calculated the cut level with the highest precision compared to other cutting levels. The area value under the ROC curve for the 60% cut-off point determined for ROR1 was 0.73. The sensitivity and specificity rates were 92.9% and 62.5%, respectively (Figure 1). There were 60 (66.7%) cases in the HiRO R1 group. The disease-related factors of the HiROR1 group and LoROR1 group patients were compared with each other (Table 1).

HiROR1 positivity was associated with the presence of splenomegaly (p=0.011), anemia (p=0.002) and high beta-2 microglobulin level (≥3 mg/dL), and higher risk of the requirement for the first-line treatment (p=0.029). 23 out of 24 patients who required first line therapy during the follow-up period were in the HiROR1 group.

Median leukocyte count was 19835 $x10^6$ cells/L (min 4870-max 174700), median lymphocyte count was 17840 $x10^6$ cells/L (min 4580-max 129100), and median LDH value was 207 g /L (min 108 -max 601).

The leukocyte, lymphocyte and LDH values in the HiROR1 group and LoROR1 group were compared, both leukocyte (p = 0.022) and lymphocyte (p = 0.004) counts were significantly higher in the HiROR1 group, and there was no statistically significant difference in LDH value (p = 0.238).

The treatment-free survival of 24 patients who received first-line treatment was between 6 and 104.5 months, and the median survival without treatment was found to be 30.1 months. Ten patients received first-line therapy at the time of diagnosis. The median progression-free survival time of 24 patients receiving first-line therapy was 34.3 months (minimum 10-106.2 months).

The clinical and prognostic factors affecting progression-free survival are shown in Table 3. The median PFS was found to be shorter in elderly patients who need first-time treatment at the time of dignosis in the presence of HiROR1 expression, anemia, thrombocytopenia, advanced-stage disease, and increased beta-2 microglobulin level. Figure 2 shows PFS in cases according to the expression of HiROR1 or LoROR.

Cox regression analysis (Table 4) showed that old age, hemoglobin <11 g/dL, thrombocytopenia, advanced-stage disease and first-line choice therapy at diagnosis are independent risk factors correlated with progression-free survival. HiROR1 and increased beta-2 microglobulin were not among the independent risk factors for PFS, according to the Cox regression analysis.

Sixty-three patients had genetic examination results. Twenty-seven patients had no genetic data.

Of whom, 33 (52.4%) patients had no genetic features for del 17p, del 11q or del 13q mutations, nine (14.3%) had 17 p deletions, three (4.8%) had 11 q deletions, and two (19%) had 13 q deletions.

All nine patients with 17 p deletion were in the HiROR1 group. Eighteen (54.5%) of 33 patients without any genetic abnormalities, three with 11q positivity and nine (75%) of 13q deletion patients were in the HiROR1 group. Trisomy 12 was detected in four patients (HiROR1 in two patients), and trisomy 7 was found in two patients (HiROR1 in one patient). Genetic data of all cases with progressive disease were achieved; three patients were positive for 17 p deletion, two patients were positive for 11 q deletion, and the others had normal genetic features.

Table 1. The comparisions of HiROR1 and LoROR1 patients

Clinical findings of patients	108 (%)	HiROR1 Group (n:74, 31.5%)	LoROR1 Group (n:34, 68.5%)	P
≤ 65 years	34 (31.5%)	27 (36.5%)	16 (47.1%)	0.397
>65 years old	74 (68.5%)	47 (63.5%)	18 (52.9%)	
Male	60 (55.6%)	42 (56.8%)	18 (47.1%)	0.835
Female	48 (44.4%)	32 (43.2%)	16 (52.9%)	
Early stage (0,1)	44 (31.5%)	41 (29.6%)	3 (1.9%)	0.005*
High+İntermediate stage (2,3,4)	64 (68.5%)	43 (49.1%)	18 (19.4%)	
B symptoms	22 (20.6%)	15 (14.7%)	7 (5.9%)	0.398
Thrombocytopenia	29 (26.9%)	24 (82.8%)	5 (17.2%)	0.042*
Anemia (Hgb<11 g/dL)	41 (38%)	33 (80.5 %)	3 (19.5 %)	0.028*
Splenomegaly	27 (30%)	16 (59.3%)	11 (40.7 %)	0.242
Hepatomegaly	33 (36.7%)	24 (72.7 %)	9 (27.3 %)	0.702
Leukocyte count $x10^9$ / L (mean±S.D)	31.3 (± 30.27)	36.0 (± 35.12)	21.90 (± 5.55)	0.004*
Lymphocyte $x10^9$ / L (mean±S.D)	24.87 (± 24.36)	29.32 (±28.48)	15.98 (±6.91)	0.022*
LDH g /L (mean±S.D)	241 (±96.9)	248 (±104.17)	225 (± 80,05)	0.238
Beta-2 microglobulin ≥3,5 g /L	22 (20.4%)	21 (95.5 %)	1 (4.5 %)	0.002*
First line therapy at initial diagnosis	10 (11.1%)	9 (90%)	1 (10%)	0.043*
First line therapy during follow up	24 (26.6%)	23 (95.8%)	1 (4.2%)	0.001*
CD38 positivity	36 (33.3%)	22 (61.1%)	14 (39.9%)	0.690

Table 2. Clinical and laboratory features at the diagnosis time of patients with need to first line therapy

Progressive disease	24 (22.2%)
Age	
≤ 65 years	7 (29.2%)
>65 years	17 (70.8%)
Gender	
Male	15 (62.5%)
Female	9 (37.5%)
Rai Stage	
Early stage	13 (54.2%)
Advanced stage (2-3-4)	11 (45.8%)
B symptoms	12 (50%)
Anemia (Hgb<11 g/dL)	14 (58.3%)
Leukocyte count $x10^9$ / L (mean±S.sp)	16.03 (±6.17)
Lymphocyte $x10^9$ / L (mean±S.sp)	13.45 (±5.90)
LDH g /L (mean±S.sp)	379.8 (±139)
Splenomegaly	19 (79.2%)
Hepatomegaly	7 (29.1%)
First line therapy at initial diagnosis	9 (37.5%)
HiROR1	23 (95.8%)
CD38 positivity	12 (50%)

Table 3: Descriptive values and comparison results for progression free survival

	Time to treatment free survival (Months) Mean ± SE* (Kaplan–Meier survival analysis)	**P (Log rank test)**
Age	104.6± 11.5	**0,046**
<65	81.02±7.5	
≥65		
Hemoglobin level (g/dL)		
<11	75.54±8.9	**0.029**
≥11	106.7±8.5	
Thrombocytopenia		
No	108.92±7.24	**0.012**
Yes	82.66±12.90	
RAI Stage		
Early (0,1,2)	124.5±5.4	**0.001**
Advanced (3,4)	49.9±10.1	
Splenomegaly		
Yes	63.8±8.6	0.44
No		
Beta-2 microglobulin level		**0.017**
<3,5	87.3±10.5	
≥3,5	53.5±10.9	
ROR 1 expression		
HiROR1	93.6±7.8	**0.027**
LoROR1	126.8±8.7	
Need to first line treatment on diagnosis		
Yes	38.4±14.4	**0.001**
No	98.7±6.6	

The p values < 0.05 is statistically significant and it is written by bold color.
*Kaplan–Meier survival analysis results
**Log-Rank test result

Table 4: Hazard ratio of the affecting the progression free survival

	RR (Relative Risk)	**95% CI**	**P (Cox's proportional hazard regression)**
Age	7.37	2.94-18.49	**0.004**
<65			
≥65			
Hemoglobin level (g/dL)	8.44	2.42-29.31	**0.001**
<11			
≥11			
Thrombocytopenia	14.92	0.87-7.11	**0.007**
No			
Yes			
RAI Stage	10.57	3.39-32.93	**0.009**
Early (0,1,2)			
Advanced (3,4)			
Beta-2 microglobulin level	2.007	0.875-7.11	**0.023**
<3,5			
≥3,5			
Need to first line treatment at diagnosis		0.25-3.31	**0.001**
Yes	9.25		
No			
ROR 1 expression		0.63-6.31	0.082
HiROR1	2.49		
LoROR1			

The p values < 0.005 is statistically significant and it is written by bold color.
*Cox's proportional hazard regression result

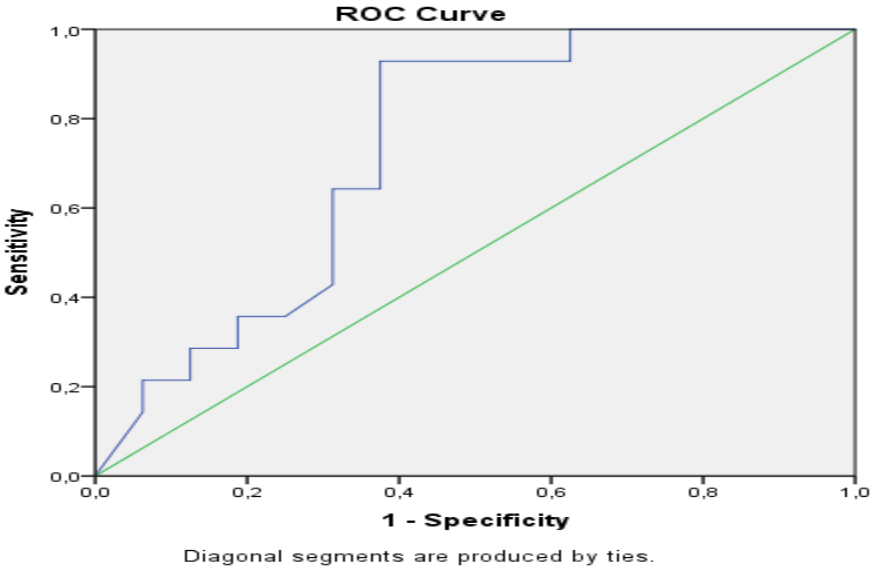

Figure 1. The receiver-operating characteristic curve (ROC) for the association of advanced stage RAI and expression of ROR 1

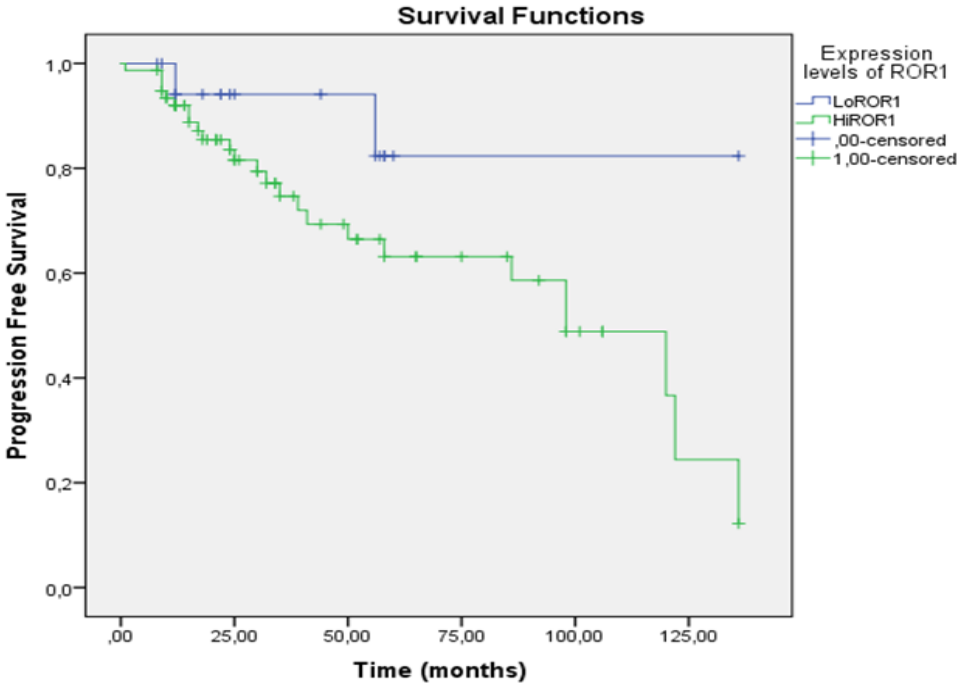

Figure 2: Progression free survival curves according to expression of ROR1

DİSCUSSİON

The relationship between ROR1 expression levels measured by flow cytometry at the time of diagnosis of 108 CLL patients and clinical/laboratory parameters of CLL patients were compared. In the current study, anemia, old age, thrombocytopenia, the elevation of β-2 microglobulin levels, and the requirement of first-line therapy during follow-up period were observed in patients with HiROR1 levels. The approach to managing CLL patients has been changing rapidly with the introduction of novel agents and new prognostic markers.The prognostic parameters of CLL patients can be explained in the three main categories: genetic abnormalities such as 17p, 13q, mutation of immunoglobulin heavy chain variable (IGHV) abnormalities, immunophenotypic and biochemical parameters such as CD38 and ZAP 70, serum thymidine kinase, β-2 microglobulin and patient-related factors such as gender, performance status, and age.

ROR1 is a type-1 tyrosine kinase-like orphan receptor normally expressed in embryogenesis, and it is not expected to be expressed in normal adult tissues. However, the leukemic cells of CLL patients have been shown to represent ROR1 on their cell surface[7]. In this study, we demonstrated in a cohort of CLL patients using flow cytometry that the level of ROR1 positivity in CLL cells ranged from 42% to 96%. In published data, the ROR1 positivity of CLL cells in a mouse model was shown to range from 72 to 99% by flow cytometry[8]. In an animal model of de novo B-cell leukemia using B6 transgenic mice generated for human ROR1, ROR1 expression was shown to be related to aggressive disease and enlarged spleen[9]. The presence of progressive lymphadenopathy/splenomegaly, the lymphocyte doubling time of less than three months, disease-associated anemia (hemoglobin level <10 g/dL), and thrombocytopenia have been associated with progressive disease for CLL patients. In a study that included 20 CLL patients, a high level of ROR1 expression was associated with progressive disease. While eight of the ten CLL patients with progressive disease required CLL therapy, none of the patients with non- progressive disease required treatment [8].

Our cut-off level is 60% for ROR 1 positivity based on the intermediate-high risk stage. There is no standardized level of ROR1 expression by flow cytometry on the CLL cell surface. In the literature, various studies reported different cut-off values. Thus, there is a lack of knowledge about the association between ROR1 expression levels and clinical/laboratory findings. One study showed that the range of ROR1 expression on the CLL cells was between 36% and 92%, and the level of expression did not differ according to the mutation status of IgVH or the progression status of the disease[10]. In a recent study, investigators defined the mean fluorescence intensity (MFI) of CD19 cells for ROR1 monoclonal antibody as 32% and tried to associate the level of ROR1 with treatment-free survival. They demonstrated that treatment-free survival and overall survival was shortened in high-level ROR1 subgroup[6].

In our study, all cases with 17p deletion and deletion 11q were in the HiROR group. We wanted to report this result even though the number of our patients was limited. In a study on 137 previously untreated CLL patients, the presence of Wnt-5a expression encoding ligand for the ROR1 surface receptor on the CLL cell surface was observed. In addition, it was defined that 17 p deletion was higher in patients with Wnt-5a positivity [11]. In another study, the patients diagnosed with CLL (n=680) were divided into two groups according to the high and low expression of ROR1 expression and compared in terms of prognostic characteristics of CLL[6]. Initially, poor cytogenetic abnormalities such as deletion 17p and deletion 11q were higher in the cases with high ROR1 expression. However, when the additional analysis was performed according to the status of IGVH mutation, like previous studies, these poor cytogenetic properties were shown to be significantly less frequent in mutated IGVH, even with high ROR1 expression [6].

Our current study showed that cases with HiROR1 had increased leukocyte and lymphocyte counts. In the previous survey, anemia, thrombocytopenia and shortening of lymphocyte doubling time were accepted as the predictor for progressive disease in CLL cases, and the higher expression of ROR1 was associated with progressive disease. The ROR1 contributes to the surveillance of CLL cells in vitro conditions, and ROR1 has also been shown to

contribute functionally to leukemogenesis [12]. In our study, the increased leukocyte and lymphocyte counts in the cases with HiROR1 can be explained by the prolonged survival of leukemia cells.

Anemia, thrombocytopenia and the requirement for first-line treatment are already associated with advanced stage. But, it is noteworthy that in our study, the high level of ROR1 expression was relaed to high β-2 microglobulin levels. β-2 microglobulin is an essential parameter of the CLL International Prognostic Score (CLL-IPI). β-2 microglobulin levels in patients with CLL correlate with tumor burden and stage. High levels of β-2 microglobulin are related to poor prognosis in CLL patients [13]. It is known that some exogenous cytokines regulate β-2 microglobulin. It is unclear which CLL patients may have high levels of cytokines [14]. However, it was known that IL6 inhibits apoptosis in CLL cells, and it could be secreted from the vascular endothelium[15]. Another study demonstrated that Stat3 phosphorylation could be able to induce by IL6, causing higher ROR1 protein levels[14-16]. Although the number of patients is limited, more extensive studies, including cytokine levels, are required to explain the relationship between high β-2 microglobulin levels and HiROR1.

CONCLUSION

Findings of the study showed that being over 65 years of age, having a hgb level below 11 gr/dL, the presence of thrombocytopenia, β-2 microglobulin level higher than 3.5, being in the HiROR1 group shortened PFS with univariate survival analysis. Anemia and thrombocytopenia are also poor prognostic factors in RAI and Binet staging system, among the commonly used CLL prognostic scoring systems [17].

According to our study, PFS is shortened by old age (7.37 times), anemia (8.4 times), thrombocytopenia (14.9 times), increased β-2 microglobulin (2 times), taking a first line treatment at the diagnosis time (9.25 times). Other comorbidities and a decrease in treatment tolerance deteriorate prognosis with increasing age. In line with the German CLL Study Group (GCLLSG) prognostic score, we also showed that advanced age and high β-2 microglobulin levels shortened progression-free survival [17]. Cox regression analysis revealed that being over 65 years of age, having hemoglobin below 11, the diagnosis of thrombocytopenia, the advanced stage of disease, and the administration of first-line therapy are independent risk factors for PFS, but being in the HiROR1 group was not considered as an independent risk factor.

Moreover, the lack of access to the ROR1 negative CLL case group is the limitation of this study design. In this study, HiROR1 expression was associated with unfavorable prognostic parameters such as anemia and thrombocytopenia, which are observed in the advanced stage of disease. However, it is noteworthy that β-2 microglobulin level and the requirement for first-line treatment at the follow-up were associated with higher ROR1 expression, raising the question of whether disease progression is faster in patients with HiROR1. More comprehensive studies with larger sample sizes are recommended.

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Statement of Ethics

This study was performed according to the Helsinki Declaration (version Fortaleza, Brazil, October 2013) and was approved by the local Ethics Committee. All participants provided written informed consent.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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