

Comparison of Surface CD52 and PD-L1 Expression in Pre- and Post-treatment Chronic Lymphocytic Leukemia (CLL) Patients

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ABSTRACT

Monoclonal antibodies targeting CD52 (alemtuzumab) and immune checkpoint inhibitors targeting programmed death-ligand 1 (PD-L1, also known as CD274) have shown therapeutic potential in chronic lymphocytic leukemia (CLL). This study compared surface expression of CD52 and PD-L1 between treated and untreated patients with CLL.

A total of 67 patients were divided into 2 groups: a treatment group (n=23) comprising patients currently receiving therapy or in remission, and a follow-up group (n=44) consisting of untreated patients under regular monitoring. After obtaining ethical approval, peripheral blood samples were collected and analyzed by flow cytometry using monoclonal antibodies against CD5, CD19, CD23, CD52, and PD-L1.

No statistically significant differences were observed in CD19+, CD23+, or PD-L1 expression between the 2 groups. However, CD52+ expression was significantly higher in the treatment group (43.77% vs 25.46%). The treatment group also showed significantly higher CD5+ expression (35.05% vs 18.93%), while the CD23+CD52+ population was significantly higher in the follow-up group (43.30% vs 20.72%).

Higher levels of CD5+ and CD52+ may be associated with treatment requirement in patients with CLL. These findings are hypothesis-generating and require prospective validation in cohorts with survival endpoints. We do not claim prognostic equivalence to established markers such as *TP53* disruption.

Keywords: B-Cell; B7-H1 antigen; CD5 antigens; CD52 antigen; Chronic; Flow cytometry; Leukemia; Lymphocytic

INTRODUCTION

The accumulation of monoclonal B-lymphocytes,

which have matured morphologic features but immature immunologic function in blood, bone marrow, and lymphatic tissues, leads to chronic lymphocytic

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leukemia (CLL), the most common type of leukemia in Western countries.¹ Although it is more prevalent in individuals with a mean age of 70 years, it can be seen in the third decade of life, noting that as age increases, the risk of CLL increases. While men have a slightly higher chance for CLL, the female population, on the other hand, is more susceptible to the aggressive type.^{2,3} Most patients are asymptomatic, but those meeting International Workshop on Chronic Lymphocytic Leukemia (iwCLL) criteria need treatment.⁴ Several factors may affect choosing the proper therapeutic strategy, including age, staging, toxicity profile, mutation profile, quality of life, and underlying diseases.⁵ Chemotherapeutic strategies, including various types of drugs such as fludarabine, chlorambucil, bendamustine, and cyclophosphamide, and afterward combinations with immunotherapeutic agents that act as CD20 monoclonal antibodies (eg, rituximab), have been the only options for patients with CLL for decades.⁶⁻¹⁰ Following numerous successful results from Bruton tyrosine kinase inhibitors (BTKi) and B-cell lymphoma-2 inhibitor (BCL2i), these agents are preferred as first-line treatment and are well tolerated compared with older treatments.^{6,11-13}

Since the 1970s, abnormal antigen expression has become significant for diagnosing CLL.¹⁴⁻¹⁶ In the late 1980s, the French-American-British (FAB) classification system introduced specific criteria to distinguish CLL from other lymphoproliferative diseases. This system recommended using flow cytometry or immunofluorescence to identify CLL by demonstrating strong expression of CD19 and CD5. Additionally, it indicated that CLL cells would exhibit weak expression of immunoglobulin and CD22/FMC7, while also lacking CD10 expression.¹⁷ Further investigations showed that markers such as CD38, CD49d, CD20, and CD23 are associated with disease progression and overall survival; high expression of CD38 and CD49d often indicates a more aggressive form of CLL; and CD20 and CD23 are used to help diagnose and monitor CLL, with their expression levels providing additional prognostic information.¹⁸

Cytogenetic and molecular genetic abnormalities, such as deletions on chromosomes 11q and 17p, *TP53* mutations, and the immunoglobulin heavy chain variable gene (*IGHV*) mutation status, are crucial for estimating prognosis and treatment responses.^{19,20}

Although current prognostic tools such as the CLL International Prognostic Index (CLL-IPI) seem to be

effective, studies show the need to reevaluate and potentially update these tools. This is important due to their limitations, such as not accounting for changes in prognosis and failing to adequately reflect outcomes with updated treatments.²¹⁻²⁴

Improving patient management and treatment strategies necessitates the identification of reliable biomarkers that can accurately predict prognosis, disease progression, and treatment responses.

The CD52 molecule is a low-weight glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressing on nearly all hematopoietic cells, from stem cells to mature lymphocytes and neoplastic generations of B cells.^{25,26} Efforts to obtain a humanized antibody against this antigen led to the generation of CAMPATH-1H (alemtuzumab), which leads to cell death through complement-mediated cell lysis and cell-mediated cytotoxicity in CD52-expressing cell lines.^{27,28}

Studies revealed a prognostic and predictive value of soluble CD52 (sCD52) in patients with CLL.^{29,30} Also, another study showed an increased level of surface CD52 in the residual population of CLL cells after therapy.³¹

Conversely, the programmed cell death protein 1 (PD-1; CD279)/programmed death-ligand 1 (PD-L1; CD274) pathway controls the induction and maintenance of immune tolerance within the tumor microenvironment. The activity of PD-1 and its ligands PD-L1 or programmed death-ligand 2 (PD-L2) are responsible for T-cell activation, proliferation, and secretion of cytotoxic reagents in cancer to attenuate antitumor immune responses.³² Dysregulations in this pathway have been known to have a key role in hematologic malignancies and immunologic disorders, considering them as high-value markers for the treatment and prognosis of cancers.³²⁻³⁵

Regarding the significance of the present study, it is important to note that CLL appears homogeneous at first glance, meaning that its clinical symptoms and routine laboratory findings are almost similar. However, in practice, it is a heterogeneous disease, such that patients can be classified into low-risk and high-risk groups, with rapid treatment being considered for high-risk groups and delayed treatments for low-risk groups. This highlights the necessity of proper patient classification. By accurately classifying patients, high-risk patients receive prompt treatment, with closer attention to their clinical symptoms, and novel therapies, such as immunotherapy, can be considered for them. In the

present study, we first categorize patients with CLL into several groups and then evaluate the expression of CD52 and PD-L1 markers in these different groups, assessing the expression differences in the patients.

MATERIALS AND METHODS

Patients

A total of 67 patients with a confirmed diagnosis of CLL according to the 2018 International Workshop on CLL (iwCLL) criteria were enrolled in this study. Patients were categorized into 2 main groups based on their treatment status at the time of sampling.

The treatment group (n=23) included patients who were either actively receiving therapy at the time of blood sampling or had completed therapy and were in follow-up with residual disease or complete remission. Within this group, we further defined 2 subgroups based on posttreatment status:

Complete remission (CR): Patients with no clinical evidence of disease, defined as absence of lymphadenopathy (palpable or imaging-detected), absence of splenomegaly, normal complete blood count (hemoglobin ≥ 11 g/dL in women, ≥ 12 g/dL in men; platelets $\geq 100 \times 10^3/\mu\text{L}$; absolute lymphocyte count $< 5 \times 10^3/\mu\text{L}$). Complete remission was documented in 12 patients (52.2%). These response definitions are based on the 2018 iwCLL criteria.⁴

Residual disease (RD): Patients with persistent disease after therapy, defined as presence of at least one of the following: palpable or imaging-detectable lymphadenopathy in any region, residual splenomegaly, absolute lymphocyte count $> 5 \times 10^3/\mu\text{L}$, persistent anemia (hemoglobin below normal limits), or thrombocytopenia (platelets $< 100 \times 10^3/\mu\text{L}$). Particular emphasis was placed on splenomegaly or absolute lymphocytosis $> 5 \times 10^3/\mu\text{L}$ as markers of significant residual disease. Residual disease was documented in 11 patients (47.8%), all of whom had evidence of residual disease without meeting criteria for CR.

Among the treatment group, the distribution of treatment regimens was as follows: ibrutinib monotherapy (16 patients, 69.6%), bendamustine plus rituximab (BR) (5 patients, 21.7%), and venetoclax plus rituximab (2 patients, 8.7%).

The follow-up group (n=44) consisted of treatment-naïve patients meeting iwCLL 2018 criteria for active monitoring only, who had never received any disease-directed therapy.

The median time from diagnosis to sampling was 14 months (interquartile range [IQR], 8–24 months) in the treatment group and 6 months (IQR, 3–12 months) in the follow-up group.

Regarding the Rai and Binet staging systems, in contemporary clinical practice, these systems are primarily used for educational purposes and have limited direct impact on treatment decisions, consistent with recent studies demonstrating that traditional prognostic models require reassessment in the era of targeted therapies.^{21–24} In routine clinical practice in Iran, Rai or Binet stage is often not explicitly documented in patient medical records. Where sufficient data were present, we were able to retrospectively assign stages, but this did not affect our primary outcomes.

Flow Cytometry

Peripheral blood samples were collected from patients with CLL. Then, 100 μL of fresh whole blood or cell suspension was added to 3 separate test tubes. The first tube served as an isotype control, with no reagents added. In the second tube, titrated antibodies for CD5 and CD19 were added, while the third tube received titrated antibodies for CD52, CD23, and PD-L1. Following the addition of antibodies, the tubes were incubated at room temperature for 15 minutes. For blood samples, 1 mL of red blood cell lysis solution (Arya Tashkhis Pars, Iran) was added, followed by an additional 10-to-15-minute incubation at room temperature. If using a cell suspension, the lysis step was omitted; instead, 1 mL of phosphate-buffered saline (PBS; Iran Biotech, Iran) was added, the sample was centrifuged, the supernatant was discarded, and the pellet was resuspended in 500 μL of PBS. The antibodies used included CD5–fluorescein isothiocyanate (FITC; BioLegend, San Diego, CA, USA; Cat. No. 364021; 2.5 μL), CD19–allophycocyanin (APC; BioLegend, San Diego, CA, USA; Cat. No. 302211; 2.5 μL), PD-L1–peridinin chlorophyll protein–cyanine 5.5 (PerCP-Cy5.5; BioLegend, San Diego, CA, USA; Cat. No. 329737; 2.5 μL), CD23–phycoerythrin (PE; BioLegend, San Diego, CA, USA; Cat. No. 338507; 2.5 μL), and CD52-FITC (QuantoBio, Beijing, China; Ref. A6752; 5 μL).

Propidium iodide (PI) is incompatible with the PE and PerCP conjugates used in this panel; therefore, viability was assessed using scatter characteristics. In separate validation tubes, 7-aminoactinomycin D (7-AAD; BioLegend, San Diego, CA, USA) staining

confirmed that scatter-based gating accurately excluded dead cells.

Gating strategy: Lymphocytes were identified by forward scatter (FSC) and side scatter (SSC), followed by doublet exclusion using forward scatter area (FSC-A) versus forward scatter height (FSC-H). The CD19⁺ B-cell population was then gated, and expression of CD5, CD23, CD52, and PD-L1 was analyzed within this gate. Fluorescence-minus-one (FMO) controls were used for each fluorochrome (FITC, PE, PerCP-Cy5.5, APC) to define positivity thresholds. Compensation was performed using single-stained UltraComp eBeads (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). All percentages reported for CD5, CD52, CD23, and PD-L1 expression represent the proportion of positive cells within the gated CD19⁺ B-lymphocyte population. Full-resolution original flow cytometry standard (FCS) data files and complete gating plots for all patients are available from the corresponding author upon reasonable request.

The samples were read using the BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). Initial parameters were set using the unstained tube before reading the tubes containing antibodies.

Statistical Analysis

Data normality was assessed using the Shapiro-Wilk test. An independent samples *t* test was applied for normally distributed variables, while the Mann-Whitney *U* test was used for non-normally distributed variables. A 2-sided $p < 0.05$ was considered statistically significant.

Post hoc power analysis revealed that with the available sample sizes ($n=44$ vs $n=23$) and an effect size (Cohen *d*) of 0.65 for CD52 expression, the achieved power was 0.72. Therefore, nonsignificant results, particularly for PD-L1, should be interpreted with caution and may be related to insufficient statistical power.

Multivariate analysis adjusting for molecular and cytogenetic prognostic markers (*TP53*, *IGHV*, and fluorescence in situ hybridization [FISH]) was not performed because these data were not uniformly available for all patients. In Iran, insurance systems do not cover these tests, and only approximately 45% of our cohort had such data available. Including only patients with available molecular data would have introduced selection bias and compromised cohort homogeneity.

Therefore, univariate comparisons represent the extent of our current analysis, and this limitation is explicitly acknowledged.

Statistical analyses were performed using STATA software, version 17 (StataCorp, College Station, TX, USA).

RESULTS

A total of 67 patients with CLL were included in the final analysis, of whom 44 (65.7%) were categorized into the follow-up group and 23 (34.3%) into the treatment group. Baseline demographic, hematological, and immunophenotypic characteristics of the study population are summarized in Tables 1 and 2.

Baseline Characteristics

The mean age of patients in the follow-up and treatment groups was 63.3 ± 13.2 years and 62.6 ± 12.6 years, respectively, with no statistically significant difference between the 2 groups ($p=0.84$). Male predominance was observed in both groups, with no significant difference in sex distribution ($p=0.08$).

At the time of diagnosis, patients in the treatment group exhibited significantly higher leukocyte counts compared with the follow-up group ($50.0 \pm 35.0 \times 10^3/\mu\text{L}$ vs $25.9 \pm 13.0 \times 10^3/\mu\text{L}$, $p < 0.001$). Hemoglobin levels were slightly lower in the treatment group (13.0 ± 2.2 g/dL vs 14.0 ± 1.3 g/dL, $p=0.02$), although this difference was not considered clinically significant. Platelet counts and lymphocyte percentages were also significantly different between the 2 groups ($p < 0.05$).

Comparison of complete blood counts (CBC) at the time of study demonstrated a significant difference in leukocyte counts and lymphocyte percentages ($p < 0.05$), whereas hemoglobin levels and platelet counts showed no significant differences between the groups (Table 1).

CD52 and PD-L1 Expression in CLL

Table 1. Characteristics of CLL patients.

Variable	Follow-up (n=44)	Treatment (n=23)	<i>p</i>
Age, y (SD)	63.3 (13.2)	62.6 (12.6)	0.84
Sex			0.08
Male, No. (%)	36 (81.8)	14 (60.9)	
Female, No. (%)	8 (18.2)	9 (39.1)	
Baseline CBC			
Leukocyte count, $\times 10^3/\mu\text{L}$ (SD)	25.9 (13.0)	50.0 (35.0)	0.0002
Hemoglobin, g/dL (SD)	14.0 (1.3)	13.0 (2.2)	0.02
Platelet count, $\times 10^3/\mu\text{L}$ (SD)	203.3 (69.8)	162.9 (50.0)	0.01
Lymphocytes, % (SD)	74.8 (11.1)	80.6 (8.8)	0.03
CBC at the research phase			
Leukocyte count, $\times 10^3/\mu\text{L}$ (SD)	32.7 (23.0)	27.3 (36.7)	.001 ^a
Hemoglobin, g/dL (SD)	13.7 (0.9)	13.4 (1.9)	0.39
Platelet count, $\times 10^3/\mu\text{L}$ (SD)	175.2 (64.4)	157.1 (48.5)	0.24
Lymphocytes, % (SD)	73.9 (9.6)	56.7 (17.3)	<.0001 ^a

Using Mann-Whitney test. CBC: complete blood count; CLL: chronic lymphocytic leukemia; SD: standard deviation.

Table 2. Comparison of biomarkers expression.

Variable	Study group		<i>p</i>
	Follow-up (n=44)	Treatment (n=23)	
CD19 ⁺ , % (SD)	15.36 (21.60)	3.61 (4.66)	0.086
CD5 ⁺ , % (SD)	18.93 (17.76)	35.05 (25.04)	0.014 ^a
CD19 ⁺ CD5 ⁺ , % (SD)	57.39 (27.97)	51.92 (31.30)	0.63 ^a
CD23 ⁺ , % (SD)	15.79 (16.83)	10.39 (13.93)	0.12
CD52 ⁺ , % (SD)	25.46 (20.36)	43.77 (31.16)	0.039 ^a
CD23 ⁺ CD52 ⁺ , % (SD)	43.30 (23.60)	20.72 (26.70)	0.003 ^a
PD-L1 ⁺ , % (SD)	0.36 (0.38)	0.63 (0.71)	0.17
CD52 ⁺ PD-L1 ⁺ , % (SD)	0.96 (1.09)	0.74 (0.88)	0.49

Using Mann-Whitney test. All percentages are calculated within the CD19⁺ B-cell gate. CD: cluster of differentiation; PD-L1: programmed death-ligand 1; SD: standard deviation.

Immunophenotypic Findings

Comparative analysis of surface biomarker expression between the treatment and follow-up groups was performed using flow cytometry. The gating strategy used for flow cytometry analysis is shown in Figure 1A, and quantitative comparisons of marker expression between groups are presented in Figure 1B and Table 2.

The results demonstrated significant differences in the expression of CD5, CD52, and the combined marker CD23⁺CD52⁺ between the 2 groups.

The treatment group exhibited significantly higher

expression levels of CD5⁺ cells (35.05%±25.04% vs 18.93%±17.76%, *p*=0.014) and CD52⁺ cells (43.77%±31.16% vs 25.46%±20.36%, *p*=0.039) compared with the follow-up group. CD52⁺ expression showed a mean difference of 18.31% (95% confidence interval, 1.02% to 35.60%). Furthermore, the proportion of CD23⁺CD52⁺ cells was significantly higher in the follow-up group compared with the treatment group (43.30%±23.60% vs 20.72%±26.70%, *p*=0.003).

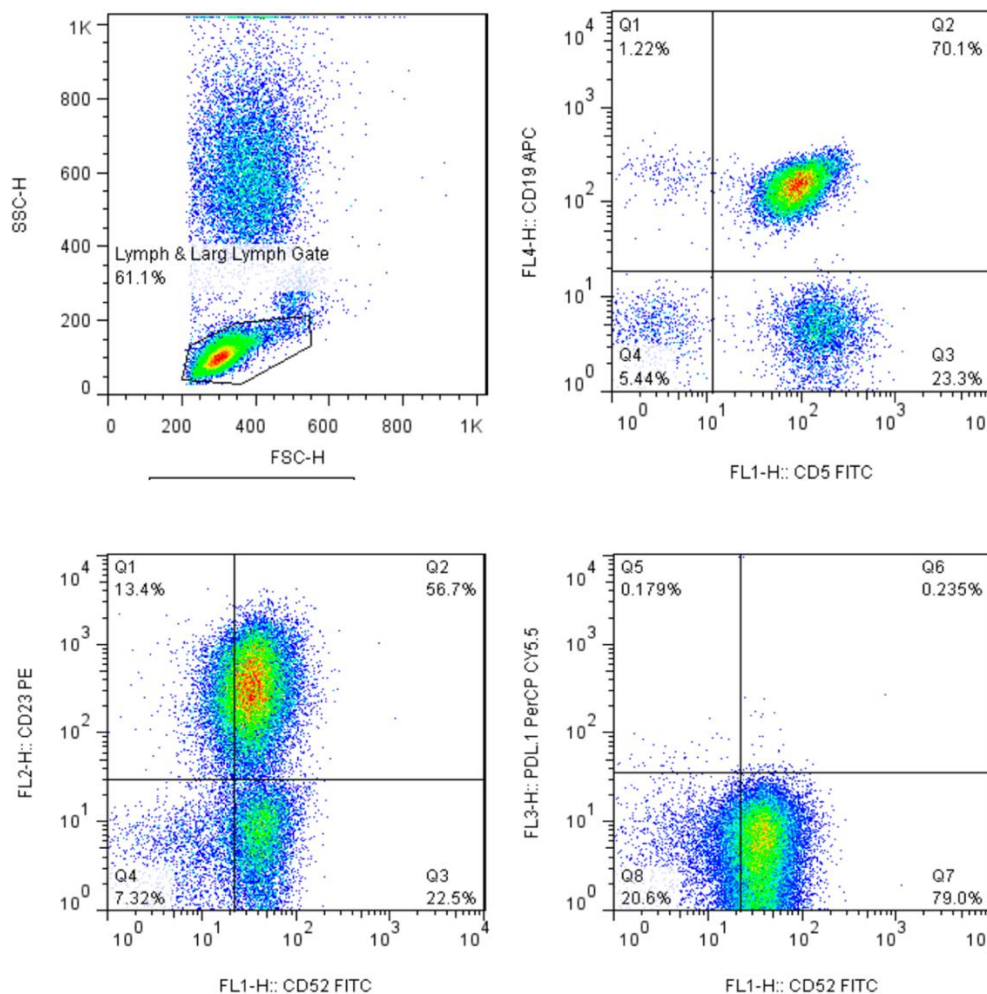
Although the expression of PD-L1⁺ cells was higher in the treatment group than in the follow-up group (0.63%±0.71% vs 0.36%±0.38%), this difference did

not reach statistical significance ($p=0.17$). PD-L1 expression levels in both groups were extremely low (<1% of CD19⁺ cells), with mean values of 0.36% in the follow-up group and 0.63% in the treatment group. Given that these values approach background fluorescence thresholds (as determined by FMO controls), the biological and clinical relevance of such minimal expression differences remains questionable and should be interpreted with extreme caution. No

statistically significant differences were observed between the 2 groups in the expression of CD19⁺ cells, CD23⁺ cells, or CD19⁺CD5⁺ populations ($p>0.05$ for all comparisons).

The results showed higher expression of CD5⁺, CD52⁺, and PD-L1 in the treatment group, whereas CD23⁺CD52⁺ expression was higher in the follow-up group. With the exception of PD-L1, these differences were statistically significant (Table 2, Figure 1A and 1B).

A



B

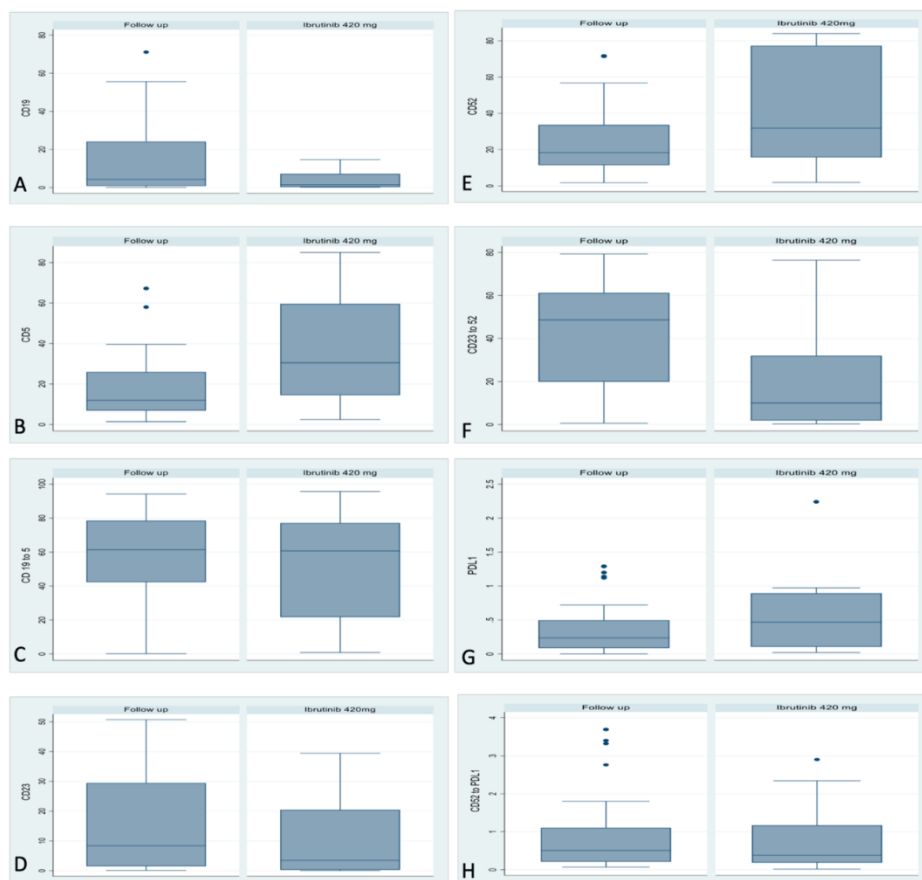


Figure 1. Representative flow cytometry plots and marker expression in patients with chronic lymphocytic leukemia (CLL). A. Representative flow cytometry plots from a patient with CLL. Lymphocytes were identified based on forward scatter height (FSC-H) and side scatter height (SSC-H) characteristics (upper left panel). CD5-FITC versus CD19-APC expression is shown in the upper right panel, CD52-FITC versus CD23-PE expression in the lower left panel, and CD52-FITC versus PD-L1-PerCP-Cy5.5 expression in the lower right panel. B. Quantitative comparison of immunophenotypic marker expression between the follow-up (n=44) and treatment (n=23) groups. Detailed quantitative comparisons are presented in Table 2. APC: allophycocyanin; CD: cluster of differentiation; FITC: fluorescein isothiocyanate; FSC-H: forward scatter height; PD-L1: programmed death-ligand 1; PE: phycoerythrin; PerCP-Cy5.5: peridinin chlorophyll protein–cyanine 5.5; SSC-H: side scatter height.

DISCUSSION

CLL is a hematological malignancy with heterogeneous clinical features.³⁶ While most patients experience lifelong treatment-free survival,³⁷ others develop progressive disease requiring treatment.³⁸ In our study, 34.3% of patients were in the treatment group and 65.7% were in the follow-up group.

The iwCLL guideline states that a rise in leukocyte count indicates disease progression only when lymphocyte count rises >50% in <2 months or

lymphocyte doubling time is <6 months.⁴ Thus, the observed differences alone do not necessarily indicate treatment requirement.

Our results showed significantly higher CD5+ and CD52+ expression in the treatment group, while CD23+CD52+ expression was significantly higher in the follow-up group, consistent with Pevna et al.⁴⁰ PD-L1 expression was numerically higher in the treatment group, but this difference did not reach statistical significance ($p=0.17$).

One possible explanation for increased CD52 expression in the treatment group is that increased CD52 expression may contribute to treatment resistance. Alternatively, tumor cell lysis during treatment may release intact CD52 antigen that attaches to residual cells.⁴¹ A previous study on ibrutinib in mantle cell lymphoma showed that high CD19⁺CD5⁺ levels may result from cell release from lymph nodes into the bloodstream,⁴² a hypothesis that could similarly apply to CD52 in our treatment group.

Higher levels of sCD52 have been associated with more aggressive disease, indicating its value in staging and monitoring.^{43–45} Interestingly, a decrease in sCD52 values during ibrutinib treatment has been reported,⁴⁶ which may explain increased surface CD52.

Immune checkpoint inhibitors have revolutionized treatment in Hodgkin lymphoma (response rates >80%) and certain non-Hodgkin lymphomas.^{50–52} Given these observations; we investigated whether checkpoint inhibition may also have therapeutic relevance in CLL. Our findings of uniformly low PD-L1 expression (<1%) in Iranian patients with CLL provide important negative findings, suggesting that unselected patients with CLL are unlikely to respond to anti-PD-1 monotherapy. This is consistent with clinical trial response rates (<15%) for nivolumab and pembrolizumab in CLL.^{53,54} Combination therapeutic strategies remain an active area of investigation.^{55–57}

Our study provides the first PD-L1 and CD52 expression data in Iranian patients with CLL. For PD-L1, the low expression (<1%) indicates that anti-PD-1 monotherapy is unlikely to be effective in unselected patients, although combination strategies merit further investigation. For CD52, significantly higher expression in the treatment group (43.77% vs 25.46%, $p=0.039$) suggests potential prognostic and predictive relevance for alemtuzumab-based therapy.

Among all prognostic markers studied in CLL over 3 decades, only *TP53* disruption has consistently demonstrated a decisive impact on patient outcomes, while markers such as *IGHV*, CD38, and ZAP-70 have shown variable results.³⁹ Thus, the identification of novel prognostic biomarkers remains an active area of investigation. Our finding of significantly higher CD52 expression in the treatment group (absolute difference 18.31%, $p=0.039$) suggests an association with treatment requirement and warrants validation in prospective studies.

Before the advent of BTKis, CLL treatment was

highly heterogeneous, including fludarabine, cyclophosphamide, and rituximab (FCR), BR, chlorambucil-based protocols, and even single-agent rituximab or corticosteroids. In Iran, the high cost of ibrutinib has limited its accessibility, leading many patients to receive older regimens such as BR. We excluded patients with highly heterogeneous histories to minimize confounding, and a sensitivity analysis excluding non-ibrutinib patients ($n=7$) did not alter the results for CD52.

Over the past 2 decades, flow cytometry on peripheral blood has become the gold standard for CLL diagnosis.⁴ According to the 2018 iwCLL guidelines, CLL diagnosis is confirmed by immunophenotyping of circulating B-lymphocytes without bone marrow aspiration or lymph node biopsy.⁴ All established prognostic tests in CLL are routinely performed on peripheral blood samples.^{19–24} Invasive procedures for research purposes would pose ethical challenges and were not approved by our ethics committee.

Several studies have evaluated PD-L1 in CLL progression and survival. Li et al. and Gamaleldin et al. reported higher PD-L1 levels in patients with poor prognosis.^{50,51} However, Akkus et al. reported lower expression with no association with prognosis.⁵³ Through STAT3 inhibition, ibrutinib may negatively impact PD-L1 expression.^{55,56} Kondo et al. showed a decrease in PD-L1 levels during ibrutinib treatment.⁵⁷ Our nonsignificant finding may reflect this effect, but our small sample size limits interpretation.

Unlike solid tumors, anti-PD-1 agents in CLL have shown modest activity (<15% response rates). This may partly be attributable to low PD-L1 expression, the immunosuppressive CLL microenvironment, and the efficacy of BTK inhibitors that already modulate this pathway.

We recognize that established prognostic markers (*TP53* mutation status, *IGHV* mutational status, and FISH-detected cytogenetic abnormalities including del(13q), trisomy 12, del(11q), and del(17p)) are essential for risk stratification. Their absence in our cohort is primarily due to lack of insurance coverage in Iran. Future prospective studies integrating immunophenotyping with molecular profiling are warranted.

This study has several limitations. First, the sample size, particularly in the treatment group ($n=23$), is modest and limits statistical power; among approximately 150 eligible patients over 5 years, only 67

(44.7%) consented to participate, reflecting real-world recruitment challenges in outpatient CLL research. Second, all analyses were performed on peripheral blood rather than lymph node or bone marrow compartments; however, this approach represents the current standard of care according to iwCLL guidelines and was required by the ethics committee to avoid invasive procedures. Third, molecular and cytogenetic data (*TP53*, *IGHV*, FISH) were not uniformly available due to lack of insurance coverage in Iran, as including only patients with such data would have introduced selection bias; similarly, Rai/Binet staging was not systematically documented, consistent with contemporary practice where treatment decisions are based on clinical parameters rather than stage category. Fourth, the cross-sectional design precludes assessment of longitudinal changes or association with survival outcomes such as time to first treatment or progression-free survival, and prospective studies are needed to validate these hypothesis-generating findings. Despite these limitations, this study provides the first PD-L1 and CD52 expression data in Iranian patients with CLL, thereby establishing a baseline for future research.

This study demonstrates significant differences in CD5 and CD52 expression between follow-up and treatment groups of patients with CLL, with higher levels of both markers in the treatment group suggesting associations with treatment requirement. The authors do not claim that CD52 or CD5 are validated prognostic biomarkers equivalent to *TP53* or 17p deletion; rather, these findings are hypothesis-generating and represent a preliminary step toward identifying potentially useful prognostic markers. The search for novel prognostic biomarkers in CLL remains active because, aside from *TP53* disruption, few markers have demonstrated consistent and decisive clinical utility, and even biomarkers with modest effects, when reproducible and biologically plausible, can contribute to risk stratification. The finding of significantly higher CD52 expression in treated patients warrants validation in prospective cohorts measuring time to first treatment, progression-free survival, and overall survival, as only such studies can determine whether CD52 provides independent prognostic information beyond *TP53* and other established markers. From a clinical perspective, this study provides the first PD-L1 and CD52 expression data in Iranian patients with CLL. The uniformly low PD-L1 expression (below 1%) indicates that anti-PD-1 monotherapy is unlikely to be effective in unselected

patients with CLL, thereby adding important negative data to the global literature. For CD52, higher expression in treated patients suggests potential utility as a biomarker for treatment requirement, though this requires prospective validation. Collectively, these data establish a reference baseline for future studies investigating immunotherapy biomarkers in Iranian patients with CLL.

STATEMENT OF ETHICS

This study was approved by the Ethics Committee of Iran University of Medical Sciences (approval code: IR.IUMS.REC.1403.237). Written informed consent was obtained from all participants.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The Data Availability section has been completed as requested

AI ASSISTANCE DISCLOSURE

During the preparation of this work, the authors used ChatGPT (OpenAI) for language editing and grammatical refinement. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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