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Clinical significance of LAIR1 (CD305) as assessed by flow cytometry in a prospective series of patients with chronic lymphocytic leukemia

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Running heads: LAIR1 (CD305) and chronic lymphocytic leukemia

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Abstract

Most patients affected by chronic lymphocytic leukemia are diagnosed by flow cytometry. Several immunophenotypic markers have been identified as significant and independent prognostic variables, especially from retrospective cohorts. However, while attractive because inexpensive and feasible in most laboratories, only few were validated by independent series.

The expression of LAIR1 (also known as LAIR-1 or CD305), an inhibitor of B-cell receptor-mediated signaling, has been reported to lack in high-risk chronic lymphocytic leukemia. However, its correlation with biological variables and its prognostic significance remain unknown.

We investigated 311 consecutive patients, prospectively enrolled since 2007. Methods for studying patients were standardized and included clinical assessment, immunophenotype, fluorescence in situ hybridization, and immunoglobulin heavy chain variable region genes status. Overall, 22.1% of patients had Binet B or C stage, 38.5% were unmutated, 15.1% had high risk cytogenetic abnormalities, 23.4% were CD38+, 37.8% CD49d+, and 59.8% LAIR1+. Expression of LAIR1 was inversely related to CD38 (p=0.0005), but was not associated to CD49d expression (p=0.96). A significantly lower expression of LAIR1 was observed in patients with B or C Binet stage (p=0.023), and in the presence of high risk cytogenetic abnormalities (p=0.048) or unmutated immunoglobulin heavy chain variable region (p<0.0001). At univariate analysis LAIR1+ was significantly associated with longer time to first treatment (p=0.0002). This favorable effect of LAIR1+ was confirmed by multivariate analysis (Hazard Ratio=2.1, p=0.03 for LAIR1).

Our results support LAIR1 expression as a reliable and inexpensive marker to independently predict time to first treatment in newly diagnosed unselected patients with chronic lymphocytic leukemia.
Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with highly variable clinical course. Some patients have a life expectancy which resembles the age-matched general population, while others progress and need treatment within few months from diagnosis. Several clinical and biological variables, some of which validated in prospective studies\(^1\) have been reported to predict outcome of CLL patients when assessed at CLL presentation. Among them, the old fashioned but still widely used clinical staging initially proposed by RAI and/or Binet,\(^2\,^3\) or the more demanding mutational status of the variable region of the heavy-chain locus of the immunoglobulin genes (IGHV) and fluorescent in situ hybridization (FISH) represent the hallmark to discriminate patients with aggressive or indolent clinical course.\(^4\,^5\) Although the latter methods have been standardized, tests are still expensive and cannot be provided by all laboratories. For this reason, the search of new cytofluorimetric markers is still of great interest, especially after CD38 and ZAP-70 have manifested major limitation, the first for its low prognostic power, the second for its well recognized technical problems.\(^1\,^6\,^7\) Recently, CD49d and other markers, such as CD25, CD26 and CD69 have been advocated as being predictive and reliable in identifying patients with peculiar molecular characteristics of the disease and different prognosis.\(^8\,-\,^{12}\)

LAIR1 (Leukocyte-Associated Immunoglobulin-like Receptor-1), also known as CD305, is a transmembrane glycoprotein acting as inhibitory receptor, which is expressed by most immune cells. The known LAIR1 ligands are the extracellular matrix collagen and C1q, the first component of the complement.\(^13\,\,^{14}\) LAIR1 expression varies during B-cell differentiation and has been recently demonstrated in patients with CLL.\(^15\,\,^{16}\) In B-cells, the in-vivo role of LAIR1 consists in inhibiting B-cell receptor (BCR)-mediated signaling\(^15\) and in controlling kinase pathways involved in cell proliferation.\(^13\) Recently, two studies performed in CLL patients showed that LAIR1 is more expressed in early than in advanced CLL stages\(^17\) and that its expression is lower in high risk CLL patients.\(^16\)

The association of LAIR1 expression with commonly recognized clinical and biological variables, and its prognostic role in patients with CLL is still unknown. With this study we analyzed LAIR1 expression in a prospective cohort of 311 unselected CLL patients consecutively enrolled in the
“CLL Veneto” registry, showing that the expression of this molecule has a relevant impact on disease progression.

Methods

Patients, follow-up and end-points

Patients with a new diagnosis of CLL presenting to three major Institutions of the Veneto region were enrolled in a regional prospective registry since 2007, which included biological and clinical investigations (CLL Veneto project). Three hundred and eleven patients affected by CLL were enrolled in the present study from the Hematology Departments of Vicenza, Verona and Padova. The registry and this study have been approved by the Institutional Review Boards of each participating Institution and all patients signed informed consent before any study procedure. All patients met the International Workshop CLL (IWCLL) criteria for the diagnosis of CLL and were treated following standard criteria for treatment onset in CLL.18

All samples for immunophenotypic, cytogenetic and molecular analysis were obtained from peripheral blood or bone marrow specimens at patient presentation and therefore before the administration of any cytotoxic treatment.

Patients were prospectively accrued between February 2007 and April 2013. Median follow-up from CLL diagnosis was 32 months (range 1-75.1). Time to first treatment (TTFT) was chosen as main parameter to ascertain the tumor aggressiveness. As a matter of fact, 15 of the 311 patients died so far, which prevented the use of overall survival as study end-point. Furthermore, TTFT was a reliable endpoint to assess tumor progression because reasons and timing for initiating cytotoxic treatment were standardized among centers by the “CLL Veneto” project, resembling what previously reported by international guidelines.18

Immunophenotypic analysis

Anticoagulated peripheral blood samples were used for immunophenotypic analysis. After collection, the samples were analyzed within 24 hours at the flow-cytometry (FC) labs of the three
hematologic Centers. Immunophenotyping was performed according to well-established techniques,\textsuperscript{19} and briefly described in Supplementary Methods. Besides standard immunophenotypic markers required for CLL diagnosis,\textsuperscript{18} all patients were analyzed for the expression of CD38 (HB7 clone), CD49d (9F10 clone), CD305 (DX26 clone). The cut-off value for LAIR1 positivity was 30\%, as reported in Supplementary Figure 1 and methods.

Fluorescence in situ hybridization and IGHV mutation analysis

Cytogenetic abnormalities involving deletions in chromosomes 11q23, 13q14, 17p12, and trisomy 12 were evaluated by interphase fluorescence in situ hybridization (FISH) as previously described.\textsuperscript{20, 21} Complex karyotype was defined as the presence of 3 or more abnormalities. Total RNA was obtained from peripheral blood specimens and the IGHV mutation analysis was performed as previously described.\textsuperscript{20, 21}

Statistical methods

Relative frequencies or mean/median values and their standard deviation, as well as range, for each categorical or continuous variable under study, the differences between groups, the correlations between variables, and the survival statistics were calculated using the SPSS 20 software (IBM Corp, Armonk NY USA) as described in supplementary methods.

Results

Clinical and biological features according to LAIR1 expression

Overall, LAIR1 was positive in 186 (59.8\%) of the 311 CLL patients. The LAIR1 positive (LAIR1\textsuperscript{+}) and negative (LAIR1\textsuperscript{-}) patients were associated with significant different distribution of some clinical and biological features, as reported in Table 1 and Figure 1. In particular, LAIR1\textsuperscript{+} patients presented with significantly lower clinical stage, had inferior rate of IGHV-unmutated cases, and more favorable cytogenetic lesions as compared to LAIR1\textsuperscript{-} patients. Moreover, a positive direct antiglobulin test (DAT) was detected more frequently in LAIR1\textsuperscript{-} patients, which was consistent with
the finding of a significant higher occurrence of autoimmune hemolytic anemia (AHA) at CLL presentation in LAIR1- patients.

**LAIR1 expression and other immunophenotypic prognostic markers**

Overall, CD38 and CD49d were positive in 23.4% and in 37.8% of patients, respectively (Table 1). When we analyzed the different expression of CD38 and CD49d in LAIR1+ and LAIR1- groups, we observed a significant association between LAIR1+ and CD38- patients (p=0.019), while no significant difference in CD49d expression was observed between the two groups (p=0.13), although CD49d was slightly less expressed in LAIR1+ patients. Similarly, when immunophenotypic expression was computed as continuous variable, the expression of LAIR1 was inversely related to CD38 but not to CD49d expression (p=0.0005 and p=0.96, respectively), as shown in Figure 1. Conversely, CD49d expression was strongly associated with CD38 expression (p<0.0001; Figure 1C). The analysis of median fluorescence intensity (MFI) of LAIR1, CD38, and CD49d confirmed the strong relation between CD38 and CD49d (p<0.0001), while no relation emerged between LAIR1 and CD38 or CD49d using MFI values (Supplementary Figure 3).

Nineteen patients were analyzed for LAIR1 expression over time. As shown in supplementary figure 2, no significant variation (p=0.64 by Wilcoxon test) was observed both among initially positive or negative cases. Median time from the diagnosis to second analysis was 48 months (9-71) with half of the patients that had received immunochemotherapy during this period.

**Prognostic relevance of LAIR1 expression and univariate analysis**

A significant lower fraction of LAIR1+ patients initiated cytotoxic treatment during follow-up (42 of 183, 22.9%) compared to LAIR1- patients (53 of 124, 42.7%, p<0.001). This translated in a significant difference in terms of TTFT according to LAIR1 expression (p=0.0002), as shown in Figure 2. Univariate analysis also identified high expression of CD38 (p=0.00003) and of CD49d (p=0.00002), unmutated IGHV (p<0.00001), high-risk cytogenetic lesions, defined as del17p, del11q or complex karyotype (p=0.0003), and Binet stage (p<0.00001) as significantly predictors of a shorter TTFT, as shown in Figure 2.
Multivariate analysis

Since the three phenotypic markers showed significant correlations in their expression and were all predictive for TTFT, LAIR1, CD38, and CD49d were included in a Cox’s proportional hazard regression model to test their strength as independent prognostic factors in terms of TTFT in our cohort of CLL patients. As shown in table 2A, LAIR1⁺ (HR 2.269, p=0.002) and CD49d⁺ (HR 2.232, p=0.008) maintained an independent significant association with shorter TTFT, while CD38 did not. The multivariate analysis was then extended to other clinical and biological significant variables in univariate analysis. As shown in table 2B, LAIR1 expression was again significantly associated with TTFT (HR 2.047, p=0.037) together with IGHV status (HR 2.881, p=0.011) and Binet stage (HR 6.457, p<0.0001), while CD49d expression lost its predictive value (HR 1.668, p=0.178).

In order to visualize their additive prognostic value in terms of TTFT, LAIR1 expression, IGHV status and Binet stage were then computed together in a Kaplan-Meyer curve, as shown in Figure 3.

Discussion

With the present study we report for the first time that LAIR1 expression, although related to commonly recognized risk factors, has a significant and independent impact on time to tumor progression in patients with CLL, supporting LAIR1 as an easily applicable and inexpensive marker to predict TTFT in patients presenting with CLL.

Importantly, our results were obtained from a prospective cohort of newly diagnosed unselected patients with CLL that were standardized in terms of phenotypical, biological and cytogenetic characterization, as part of the “CLL Veneto” project. The short follow-up of recruited patients prevented us to investigate the role of LAIR1 expression in terms of survival. However, when addressing the clinical behavior and aggressiveness of the disease, TTFT is a reliable prognostic marker strictly related to tumor progression, especially in our prospective cohort where criteria for treatment initiation were standardized between centers before patients’ enrollment. A merit of our study is that our population reflects real-life unselected patients with CLL that routinely present to our Institutions, making our results readily useful to the clinician.
Immunophenotypic analysis was performed by multicolor flow cytometry evaluating the percentage of CD19+CD5+ cells expressing LAIR1, CD38 or CD49d. We observed a significant inverse correlation between the expression of LAIR1 and CD38 and we confirmed the strong correlation of expression between CD38 and CD49d already observed by others.\textsuperscript{22} Further specific studies will need to confirm these findings and analyze cell biology according to single molecules pattern of expression. Furthermore, consistently with our findings, Poggi et al have recently observed that the expression of LAIR1 is higher in patients with low risk CLL.\textsuperscript{16} In our series, LAIR1 expression allowed us to discriminate different set of patients with significant biological and clinical differences, confirming that LAIR1\textsuperscript{−} patients were associated with worse clinical stage at diagnosis and adverse biological factors, like unmutated IGHV status, CD38\textsuperscript{+}, or high risk cytogenetic lesions.\textsuperscript{16,17}

In last years several studies have focused on the identification of biological markers that could be easily applicable in order to foresee the prognosis of CLL patients. Many new immunophenotypic markers have been proposed for identifying high risk patients,\textsuperscript{11} in addition to historical ones, such as CD38 and ZAP-70. Indeed, the prognostic power of CD38 expression has been questioned by several studies.\textsuperscript{1,6} On the other hand, technical problems have been raised by many experts for the study of ZAP-70 expression.\textsuperscript{1,7} Our previous results confirmed these technical issues showing high discordance in the quantification of ZAP-70 expression either by using different monoclonal antibodies or different approaches for the analysis.\textsuperscript{23} For these reasons we did not consider ZAP-70 expression in our study. Recently, CD49d has been advocated as independent prognostic marker from retrospective cohort.\textsuperscript{8-10} However, The expression of LAIR1 in our series seemed to overcome the prognostic power of CD49d, at least in terms of TTFT. In line with our findings, Del Poeta et al. recently reported a reduced prognostic power of CD49d when all significant prognostic factors for CLL were included in the multivariate analysis.\textsuperscript{12}

The cut-off for positivity of LAIR1 was set at 30%. This was assessed considering the distribution of positive cells frequencies in our cohort of patients, as previously done by Damle et al. for CD38\textsuperscript{4} (Supplementary Methods and Figure 1). Differently from others, this unsupervised procedure did not consider any clinical parameter to set the cut-off, obviating selection bias.\textsuperscript{8,9,12}
Interestingly, LAIR1 expression was related to the occurrence of autoimmune phenomena. Both DAT and AHA were more frequently observed in LAIR1− patients, with a quite high percentage of AHA occurrence at CLL presentation for LAIR1− (7.2%). Since it is well known that both DAT and AHA are associated with IGHV status in patients with CLL, our findings might be a consequence of the association between unmutated IGHV and lower LAIR expression, or, more intriguingly, might reflect the biological activity of LAIR1 and its role in switching-off B-cells and their potential antibody-producing activity. A larger number of patients and specific studies will be needed to address this point.

The independent prognostic power of LAIR1 expression may be connected to its peculiar biological characteristics and function. Its activity on BCR activation pathway makes LAIR1 very attractive, since drugs targeting essential components of the BCR signaling (i.e. Bruton tyrosine kinase inhibitors) have recently shown impressive activity in patients with CLL. LAIR1, now designed as CD305, is an inhibitory receptor expressed on almost all hematopoietic cells, particularly on immune system cells. After the binding of its known ligands, LAIR1 inhibits the activation of immune cells using two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) located in the cytoplasmic tail of the receptor. LAIR1 is expressed during B cell ontogenesis, but is lost on a subset of memory B cells, in all germinal center B cells, in plasmablast, and in plasma cells. From a functional point of view, LAIR1 cross-linking results in inhibition of Ca2+ mobilization induced by BCR-triggering. On the other hand, a prolonged BCR- or CD40-stimulation induces a downregulation of LAIR1 on naive B cells in vitro, suggesting an inhibitory role for LAIR1 on BCR signaling, as for other B cell inhibitory receptors, like CD22, FcgRIIb. Poggi A et al demonstrated the role of LAIR1 in modulation of BCR signaling pathways implied in the CLL-cell activation, with its inhibitor capacity that was completely lost or significantly reduced when CLL-cells did not express LAIR1. Furthermore, in vitro studies confirmed that collagen produced by lymph nodes-derived mesenchymal stromal cells was able to inhibit B cell functions through LAIR1 engagement. Altogether these data strengthen and increase the appeal of LAIR1 active role in the pathophysiology of CLL with special emphasis on microenvironmental interactions inducing...
BCR activation.\textsuperscript{25,27} The relationship between LAIR1 expression and therapy with BCR signaling inhibitors certainly deserves investigation in the future.

The information about LAIR1 expression and function on neoplastic B cells are scanty so far, but for several reasons CLL represents the right setting for characterizing this molecule. Our data show an independent role of LAIR1 in predicting TTFT of patients with CLL, while longer follow-up is needed to establish its predictivity in terms of survival. Its role in-vivo in these patients is also substantiated by the association between its low expression on B-cell surface and aggressive clinical presentation of the disease, which is consistent among different studies. Finally, the influence of LAIR1 on B-cells activation pathways requires further studies that will establish the role of this molecule in the context of the new target therapies.

\textbf{Authorship and Disclosures}

OP and CV were the principal investigators and take primary responsibility for the paper; SFO, SFI, EA, FM, RP, LT, AA recruited the patients; EF, IG, EB, EN, MF, EA, MAM, FA, LB performed the laboratory work; OP, EF, CV performed the statistical analysis; AA, MTS, GS, GP, FR revised the paper and co-ordinated the research; OP, EF, IG, and CV wrote the paper. The authors declare no potential conflicts of interest.
References


### Tables

#### Table 1. Clinical and biological characteristics of 311 patients with chronic lymphocytic leukemia at disease presentation, then divided according to LAIR1 expression.

<table>
<thead>
<tr>
<th></th>
<th>All pts (n. 311)</th>
<th>LAIR1⁺ (n. 186)</th>
<th>LAIR1⁻ (n. 125)</th>
<th>p. value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age, years (range)</td>
<td>66 (30.6-90)</td>
<td>66 (36-90)</td>
<td>67 (30.6-87)</td>
<td>0.59§</td>
</tr>
<tr>
<td>Female gender</td>
<td>121/311 (38.9%)</td>
<td>69/186 (37.1%)</td>
<td>52/125 (41.6%)</td>
<td>0.48</td>
</tr>
<tr>
<td>Median lymphocyte count, x10³/mm (range)</td>
<td>9.7 (2.1-656)</td>
<td>9.4 (2.1-270)</td>
<td>11 (2.1-656)</td>
<td>0.13*</td>
</tr>
<tr>
<td>AHA</td>
<td>11/311 (35.4%)</td>
<td>2/186 (1.1%)</td>
<td>9/125 (7.2%)</td>
<td><strong>0.008</strong></td>
</tr>
<tr>
<td>ITP</td>
<td>7/311 (2.2%)</td>
<td>5/186 (2.7%)</td>
<td>2/125 (1.6%)</td>
<td>0.70</td>
</tr>
<tr>
<td>DAT</td>
<td>13/145 (8.9%)</td>
<td>3/86 (3.5%)</td>
<td>10/59 (16.9)</td>
<td><strong>0.007</strong></td>
</tr>
<tr>
<td>BINET stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>233/299 (77.9%)</td>
<td>147/178 (82.6%)</td>
<td>86/121 (71.1%)</td>
<td><strong>0.023</strong></td>
</tr>
<tr>
<td>B</td>
<td>47/299 (15.7%)</td>
<td>23/178 (12.9%)</td>
<td>24/121 (19.8%)</td>
<td>0.145</td>
</tr>
<tr>
<td>C</td>
<td>19/299 (6.4%)</td>
<td>8/178 (4.5%)</td>
<td>11/121 (9.1%)</td>
<td>0.146</td>
</tr>
<tr>
<td>FISH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>77/211 (36.5%)</td>
<td>39/124 (31.4%)</td>
<td>38/87 (43.7%)</td>
<td>0.081</td>
</tr>
<tr>
<td>del13q</td>
<td>84/211 (39.8%)</td>
<td>57/124 (46.0%)</td>
<td>27/87 (31.0%)</td>
<td>0.033</td>
</tr>
<tr>
<td>12+</td>
<td>18/211 (8.5%)</td>
<td>14/124 (11.3%)</td>
<td>4/87 (4.6%)</td>
<td>0.131</td>
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<tr>
<td>del11q</td>
<td>17/211 (8.1%)</td>
<td>10/124 (8.1%)</td>
<td>7/87 (8.0%)</td>
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<tr>
<td>del17p</td>
<td>9/211 (4.3%)</td>
<td>1/124 (0.8%)</td>
<td>8/87 (9.2%)</td>
<td>0.004</td>
</tr>
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<td>3 or more alterations</td>
<td>6/211 (2.8%)</td>
<td>3/124 (2.4%)</td>
<td>3/87 (3.5%)</td>
<td>0.692</td>
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<tr>
<td>IGHV mutational status</td>
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<td>Unmutated IGHV</td>
<td>77/200 (38.5%)</td>
<td>34/126 (27.0%)</td>
<td>43/74 (58.1%)</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td>Immunophenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD38</td>
<td>64/274 (23.4%)</td>
<td>30/165 (18.2%)</td>
<td>34/109 (31.2%)</td>
<td>0.019</td>
</tr>
<tr>
<td>------------</td>
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</tr>
<tr>
<td>CD49d</td>
<td>90/238 (37.8%)</td>
<td>51/150 (34.7%)</td>
<td>39/88 (44.3%)</td>
<td>0.129</td>
</tr>
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**Treatment, Survival and Follow-up**

<table>
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<tr>
<th></th>
<th>Had cytotoxic treatment</th>
<th>Median TTFT, months (range)</th>
<th>Median OS, months (range)</th>
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<tr>
<td></td>
<td>95/307 (30.9%)</td>
<td>23.5 (0-75)</td>
<td>30.7 (0.5-75.1)</td>
</tr>
<tr>
<td></td>
<td>42/183 (22.9%)</td>
<td>25.1 (0-75.1)</td>
<td>29.5 (0.5-75.1)</td>
</tr>
<tr>
<td></td>
<td>53/124 (42.7%)</td>
<td>18.9 (0-74.7)</td>
<td>33.9 (1-75)</td>
</tr>
</tbody>
</table>

**Abbreviations:** AHA: autoimmune hemolytic anemia; ITP: Immune thrombocytopenic purpura; DAT: direct antiglobulin test; FISH: fluorescence in situ hybridisation; del13q: deletion in chromosome 13q14; del11q: deletion in chromosome 11q23; del17p: deletion in chromosome 17p12; +12: trisomy 12; IGHV: immunoglobulin heavy chain variable region genes; TTFT: Time to First Treatment; OS: Overall Survival. Statistical tests: °the differences between the categorical variables were computed by Fisher exact test; § t-test; * Mann-Whitney U test.
Table 2. Multivariate Cox’s regression analysis for time to first treatment (TTFT).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HR (CI 95%)</th>
<th>p. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAIR1^-</td>
<td>2.269 (1.355-3.800)</td>
<td>0.002</td>
</tr>
<tr>
<td>CD49d^+</td>
<td>2.232 (1.234-4.037)</td>
<td>0.008</td>
</tr>
<tr>
<td>CD38^-</td>
<td>1.646 (0.909-2.981)</td>
<td>0.100</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HR (CI 95%)</th>
<th>p. value</th>
</tr>
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<tbody>
<tr>
<td>LAIR1^-</td>
<td>2.077 (1.057-4.081)</td>
<td>0.034</td>
</tr>
<tr>
<td>CD49d^+</td>
<td>1.575 (0.771-3.128)</td>
<td>0.213</td>
</tr>
<tr>
<td>unmutated IGHV status</td>
<td>2.675 (1.232-5.804)</td>
<td>0.0013</td>
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<td>high risk FISH (del11q/del17p/complex)</td>
<td>2.041 (0.943-4.416)</td>
<td>0.070</td>
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<tr>
<td>Binet B or C</td>
<td>6.285 (3.071-12.816)</td>
<td>&lt;0.0001</td>
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</tbody>
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Abbreviations: HR: Hazard Ratio; CI: Confidence Interval; IGHV: immunoglobulin heavy chain variable region genes; FISH: fluorescence in situ hybridisation; del11q: deletion in chromosome 11q23; del17p: deletion in chromosome 17p12;
Figure Legends

Figure 1: Correlation between LAIR1 and other immunophenotypic prognostic markers.
Scatter plots for percentage of positive cells for LAIR1 and CD38 (A), LAIR1 and CD49d (B), and for CD49d and CD38 (C). The two-tail Spearman test for nonparametric data was performed. P value <0.05 was considered associated with a statistical significant correlation.

Figure 2: Time to first treatment (TTFT) according to identified prognostic variables.
Kaplan Meier plot for TTFT according to: phenotypical expression of LAIR1 (A), CD49d (B), and CD38 (C); IGHV status (D); cytogenetic lesions (E); and Binet Stage (F). Log-rank test was performed to compare the curves. P values <0.05 were considered statistically significant.

Figure 3: Time to first treatment (TTFT) curves based on the combination of LAIR1, IGHV status and Binet stage.
Kaplan Meier plot for TTFT showing the additive prognostic value of LAIR1, unmutated IGHV status, and B or C Binet stages. Attributing to each variable a score of 1, the curves represent the sum of adverse variables (0, 1, 2, or 3). Log-rank test was performed to compare different curves. P values were as follows: p=0.471 between 0 and 1; p<0.0001 between 1 and 2; p=0.06 between 2 and 3.
Figure 2

A) LAIR-1 expression

B) CD49d expression

C) CD38 expression

D) IGHV mutational status

E) Cytogenetic Lesions

F) Binet stage
Figure 3
Supplementary

Supplementary Methods

Immunophenotypic analysis

CD38 (HB7 clone), CD49d (9F10 clone), CD305 (DX26 clone) monoclonal antibodies (mAb) were combined with CD19 and CD5 to perform the analysis of expression on CD19+/CD5+ CLL cells (Supplementary figure 1A-F). The three mAb were PE-conjugate and purchased from BD Biosciences (Milan, Italy). After the staining and red blood cell lysis (ammonium chloride solution), the samples were washed twice and then acquired with FACSCanto I cytometers. The data were analyzed by DIVA (BD Bioscience) or FlowJo (Tree Star, Inc. Ashland, OR, USA) softwares. The expression data were reported as percentage of CD19+/CD5+ CLL cells. The threshold of positivity was set at over 30% for CD38 and CD49d, as reported in the literature. Regarding the LAIR1 expression, the cut-off at 30% was empirically chosen by observing the distribution of positive cells frequencies in our cohort of patients (Supplementary Fig. 1G). This cut-off was subsequently validated by computing time-dependent ROC curve and by calculating the Youden Index (YI= sensitivity + specificity - 1) for each cut-off value in the ROC curve (Supplementary Fig. 1H-I). As shown in fig. 1I, the highest YI value was obtained for a cut-off of LAIR1 positivity at 31%. All these analysis and graphics were performed by using R software and the “survivalROC” package.

Statistical methods

The Kolmogorov-Smirnov and the Shapiro-Wilk tests were used to verify for the normal distribution of each continuous variable. The differences between the continuous variables were computed by t-test or Mann-Whitney-Wilcoxon test as appropriate. The differences between categorical variables were computed by Fisher exact test. Spearman test was used to analyze the
relationships between immunophenotypical variables. Time to first treatment (TTFT) was calculated from the time of diagnosis to the time of first cytotoxic treatment received by the patient. Curves for TTFT curves were constructed with the method of Kaplan and Meier using SPSS, and the comparison between curves was performed using the log-rank test. $P < 0.05$ was considered associated with statistical significance. Multivariate analysis was performed with SPSS according to the Cox’s model.
Supplementary figure 1: Flow cytometry analysis.

CLL cells were selected by drawing a gate around CD19+/CD5+ cells (A); the percentage of positive cells was recorded by setting the control markers on internal negative control cells (B-F). Representative cases for CD49d (B), CD38 (C), and LAIR1 (D-F) are shown. The distribution of LAIR1+ cells frequencies in our cohort of CLL patients was constructed to set the cut-off of positivity for LAIR1 (chosen cut-off at 30% as shown by the dashed line (G). Time-dependent ROC curve for different cut-off values of LAIR1 positivity computed by survivalROC package in R software (H). Youden Index values computed for each cut-off value of the ROC curve. Dashed line shows the empirically chosen cut-off for LAIR1 positivity (I).
Supplementary Figure 1

A) CD5 vs CD19
B) CD49d vs CD19
C) CD38 vs CD19

D) LAIR1 vs CD19
E) LAIR1 vs CD19
F) LAIR1 vs CD19

G) % LAIR1-pos cells vs LAIR1

H) Sensitivity vs 1-Specificity
I) Youden Index vs % LAIR1-pos-cells cut-off values
Supplementary figure 2: LAIR1 expression over time.

Each horizontal line corresponds to a single patient. The left initial LAIR1 value refers to the diagnostic sample. Solid line: patients followed-up that received no treatment. Dashed line: patients treated with immunochemotherapy during observation time. Median time of observation (x-axis) was 48 months (range 9-71). P-value was calculated with the Wilcoxon test.
Supplementary figure 3: Correlation between MFI of LAIR1 and other immunophenotypic markers.

Scatter plots of MFI values (log-scale) for LAIR-1 and CD38 (A), LAIR1 and CD49d (B), and for CD49d and CD38 (C).
Supplementary References: