SUPPLEMENTARY APPENDIX

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

Omar Perbellini,[1] Erika Falisi,[2] Ilaria Giaretta,[2] Elisa Boscaro,[3] Elisabetta Novella,[2] Monica Facco,[3] Stefania Fortuna,[4] Silvia Finotto,[2] Eliana Amati,[1] Francesco Maniscalco,[1] Anna Montaldi,[5] Alberta Alghisi,[5] Fiorenza Aprili,[6] Laura Bonaldi,[7] Rossella Paolini,[8] Maria Teresa Scupoli,[1] Livio Trentin,[3] Achille Ambrosetti,[1] Gianpietro Semenzato,[3] Giovanni Pizzolo,[1] Francesco Rodeghiero,[2] and Carlo Visco[2]

[1]Section of Hematology, Department of Medicine, University of Verona; [2]Department of Hematology and Cell Therapy, San Bortolo Hospital, Vicenza; [3]Department of Medicine, Hematology and Clinical Immunology Branch, University of Padua; [4]Department of Medicine, Azienda ULSS N°4 Alto Vicentino, Santorso; [5]Department of Immunohematology, Genetics and Transfusional Medicine, San Bortolo Hospital, Vicenza; [6]Laboratory of Cytogenetics, Department of Pathology and Diagnostics, Azienda Ospedaliera Universitaria Integrata Verona; [7]Istituto Oncologico Veneto-IRCCS, Padua; and [8]Department of Oncohematology, Santa Maria della Misericordia Hospital, Rovigo, Italy

 doi:10.3324/haematol.2013.096362
Manuscript received on October 11, 2013. Manuscript accepted on January 8, 2014.
Correspondence: carlovisco@hotmail.com

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.[19] 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. [4,8] 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%. [Sup. Ref. 1] All these analysis and graphics were performed by using R software and the “survivalROC” package.

Statistical methods

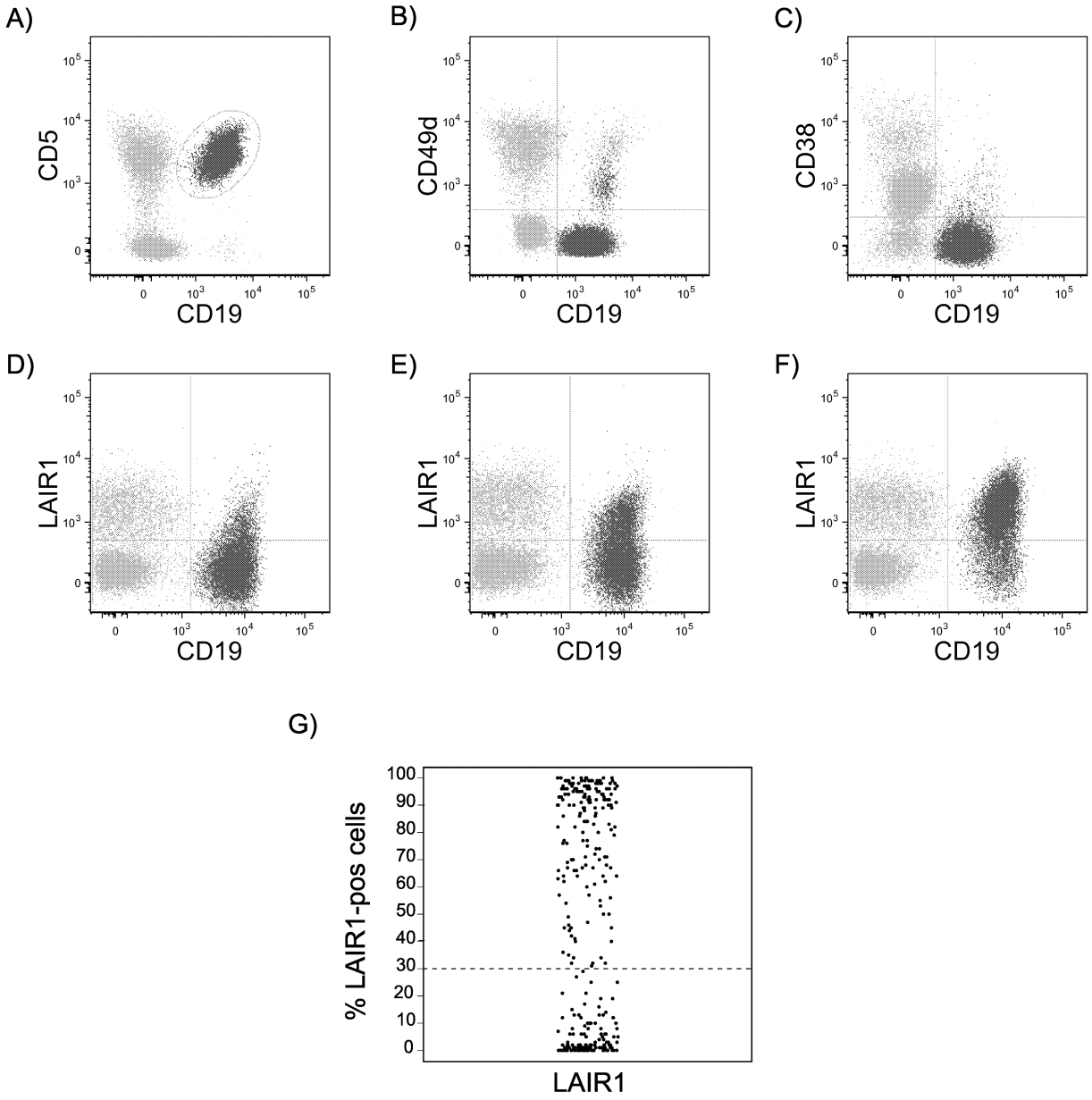
The Kolmogorov-Smirnov and the Shapiro-Wilk tests were used to verify for the normal distribution of each continuous variable. The differences between the continuous variables were computed by t-test or Mann-Whitney-Wilcoxon test as appropriate. The differences between categorical variables were computed 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

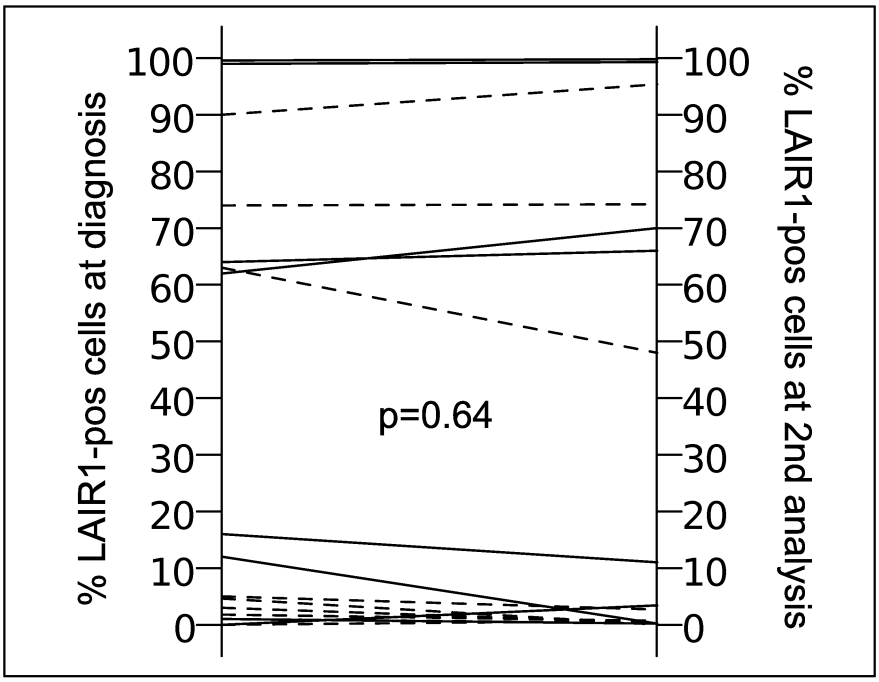


1

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.

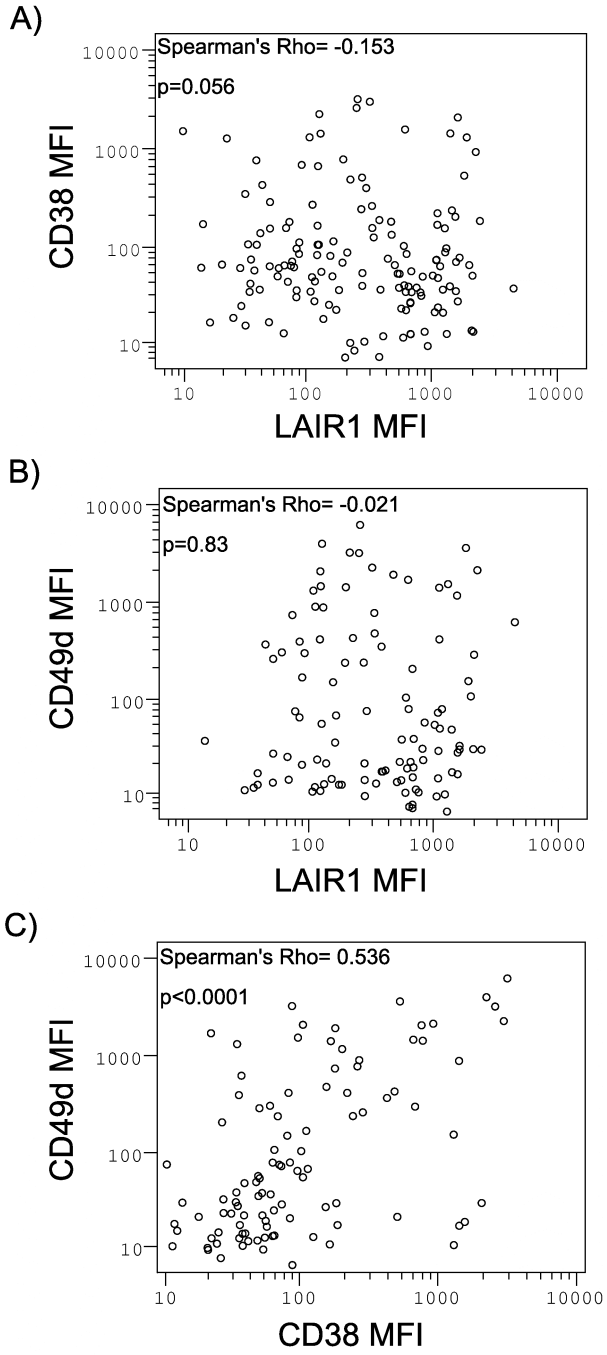
Supplementary Figure 2



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 Figure 3



Supplementary References:

1. Heagerty PJ, Lumley T, Pepe MS. Time-dependent ROC curves for censored survival data and a diagnostic marker. Biometrics. 2000;56(2):337-44.