CD49d expression is an independent risk factor of progressive disease in early stage chronic lymphocytic leukemia

Davide Rossi,[1] Antonella Zucchetto,[2] Francesca Maria Rossi,[2] Daniela Capello,[1] Michaela Cerri,[1] Clara Deambrogi,[1] Stefania Cresta,[1] Silvia Rasi,[1] Lorenzo De Paoli,[1] Chiara Lobetti Bodoni,[3] Pietro Bulian,[2] Giovanni Del Poeta,[4] Marco Ladetto,[3] Valter Gattei,[2] and Gianluca Gaidano[1]

[1]Division of Hematology, Department of Clinical and Experimental Medicine and BRMA, Amedeo Avogadro University of Eastern Piedmont and Azienda Ospedaliero-Universitaria Maggiore della Carità, Novara; [2]Clinical and Experimental Onco-Hematology Unit, Centro di Riferimento Oncologico, I.R.C.C.S., Aviano; [3]Division of Hematology, Department of Experimental Medicine and Oncology, University of Turin, Turin and [4]Department of Hematology, S. Eugenio Hospital and University of Tor Vergata, Rome, Italy

ABSTRACT

Identification of prognosticators for Binet A chronic lymphocytic leukemia is important for selecting patients with dismal prognosis. We analyzed CD49d expression in 140 consecutive Binet A chronic lymphocytic leukemia. At diagnosis, CD49d ≥30% (54/140, 38.6%) associated with proliferation markers, namely CD38 ≥30% (p=3.9×10^{-6}), LDH (p=0.007) and β2-microglobulin (p=0.020). Univariate log-rank analysis identified CD49d ≥30% as a risk factor of treatment free survival (p=8.3x10^{-5}), time to progression to a more advanced stage (p=4.7×10^{-4}), and time to lymphocyte doubling (p=0.009). Multivariate analysis selected CD49d ≥30% as an independent treatment free survival predictor after adjustment for biological (HR 2.28; 95% CI 1.71-4.45, p=0.015) and both biological and clinical variables analyzed together (HR 3.33, 95% CI 1.61-6.90, p=0.001). Within Binet A subgroups harboring favorable biological variables (IGHV homology <98%, favorable karyotype, CD38 <30%, ZAP70 <20%) or clinical variables, CD49d ≥30% consistently identified a subset of patients with short treatment free survival. Our observations indicate CD49d ≥30% as a new marker for the initial prognostic assessment of Binet A chronic lymphocytic leukemia.

Key words: chronic lymphocytic leukemia, CD49d, Binet A, prognostic factor.

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Introduction

Chronic lymphocytic leukemia (CLL) is a markedly heterogeneous disease and the behavior of patients belonging to the same clinical risk category is not uniformly predictable.[1-8] This notion is best exemplified by the variability in time to progression and survival of early stage CLL.[2,3] The identification of prognostic subgroups within Binet A CLL is currently a major challenge.[4-8] Based on available guidelines, Binet A CLL patients are not candidates for treatment.[9] However, ongoing studies have proposed starting cytoreductive therapy already in patients with Binet A CLL harboring unfavorable prognostic markers (*http://dcllsg.web.med.uni-muenchen.de/cll7/index.php*). Expanding the availability of independent outcome predictors for early stage CLL may help refine the prognostic stratification of Binet A CLL patients.

CD49d, an adhesion molecule mediating cell-to-cell and cell-to-extracellular matrix interactions, represents a novel prognostic marker for CLL.[10-15] This study aimed at verifying whether CD49d expression may contribute to further refinement of the prognostic stratification of Binet A CLL.

Design and Methods

Patients

The study was based on a consecutive series of 140 previously untreated Binet A CLL who presented for initial evaluation at the Division of Hematology of the Amedeo Avogadro University of Eastern Piedmont from June 1996 through June

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Correspondence: Davide Rossi, M.D., Division of Hematology, Department of Clinical and Experimental Medicine & BRMA, Amedeo Avogadro University of Eastern Piedmont, via Solaroli 17, 28100 Novara, Italy. E-mail: rossidav@med.unipmn.it
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2006. Median follow-up of alive patients was 54.2 months. Patients provided informed consent in accordance with local IRB requirements and the Declaration of Helsinki. CLL diagnosis was based on NCI Working Group criteria and confirmed by a flow cytometry score >3.[9,16] Clinical variables at diagnosis are reported in *Online Supplementary Table S1*. The following biological variables were analyzed on peripheral blood mononuclear cells (PBMNC) collected at diagnosis: i) IGHV gene homology to germline; ii) FISH karyotype; iii) *TP53* mutations; iv) CD49d, CD38 and ZAP70 expression. Patients were managed according to NCI Working Group guidelines.[9]

Molecular studies

In frame IGHV rearrangements were amplified and sequenced from genomic DNA.[17] Sequences were aligned to ImMunoGeneTics (IMGT) directories, and considered mutated if homology to the corresponding germline gene was <98%. Mutations of *TP53* exons 2 through 10 were analyzed by DNA direct sequencing and confirmed on both strands on independent amplimers.[18] Probes (Vysis, London, United Kingdom) used for FISH analysis were: LSI13 and LSID13S319 for del13q14; CEP12 for +12; LSIp53 for del17p13; and LSI-ATM for del11q22-q23. At least 500 interphase cells were examined. Karyotype stratification was carried out according to Döhner.[8]

Flow cytometry

Flow cytometric analysis was performed in blind without knowledge of clinical, molecular or cytogenetic data using fresh (n=16) or cryopreserved (n=124) PBMNC collected at CLL diagnosis. Expression of CD49d was analyzed by three-color immunofluorescence by combining phycoerithrin (PE)-conjugated anti-CD49d mAbs with Peridinin-Chlorophyll-Protein-Cyanine-5.5 (PerCP-Cy5.5)-conjugated anti-CD19 mAbs and fluorescein isothyocyanate-conjugated anti-CD5 mAbs.[13] Expression data were reported as percent of $CD5^+CD19^+$ CLL cells displaying specific fluorescence intensity > 98-99% of the same cell population stained with control Ig. CD38 and ZAP70 were analyzed as reported.[13,19] The 30% cut-off value for CD49d expression was selected according to the literature[13,14] Cut-off points of 30% and 20% were used to define positivity for CD38 and ZAP70 respectively.[4,6,7,13,14] Frozen samples were stained immediately after thawing. Analysis of CD38 and CD49d expression performed on paired fresh and frozen-thawed samples (n=12) led to superimposable results (*data not shown*).

Statistical analysis

Treatment free survival (TFS) was measured from diagnosis to first line treatment, death or last follow-up. All patients underwent first treatment at the time of documentation of progressive and symptomatic disease according to NCI Working Group guidelines.[9] Time to lymphocyte doubling was measured from diagnosis to lymphocyte doubling, death or last follow up. As previously reported,[6] time to progression to a more advanced stage was defined as the time elapsed from diagnosis to progression to a more advanced stage, first line treatment according to NCI Working Group guidelines, death or last follow-up. Overall survival (OS) was measured from diagnosis to last follow-up or death. Time to Richter's syndrome transformation was measured from diagnosis to transformation, death or last follow-up.

All infective episodes occurring at the time of CLL diagnosis or during follow-up were registered and graded according to NCI CTC v0.3 *(http://ctep.cancer.gov/reporting/ctc_v30.html)*. Time to first infection was measured from diagnosis to first infection, last follow-up, first line treatment or death. In patients who had experienced a first infective episode, time to recurrent infection was measured from first infection to second infection, last follow-up, first line treatment or death. Categorical variables were compared by χ^2 test and Fisher's exact test when appropriate. Continuous variables were compared by the Mann-Whitney test. Survival was analyzed by the Kaplan-Meier method using log-rank statistics to test for significant associations.[20] Multivariate Cox analysis was performed using a forward stepwise algorithm.[21] All statistical tests were two-sided. Statistical significance was defined as p-value <0.05. The analysis was performed with SPSS software v.15.0 (Chicago, IL, USA).

Results and Discussion

The study was based on 140 (73 males, 67 females) consecutive, previously untreated Binet A CLL (*Online Supplementary Table 1S*). Median age was 69 years. According to Rai, 108/140 (77.1%) patients were in stage 0. IGHV homology ≥98% occurred in 42/137 (30.7%) patients, CD38 ≥30% in 32/140 (22.9%), ZAP70 ≥20% in 38/140 (28.4%), and del17p13/del11q22-q23/+12 in 43/140 (30.7%).

Expression of CD49d ≥30% was observed in 54/140 (38.6%) Binet A CLL and associated with proliferation markers. As previously observed,[13-15] CD49d associated with expression of CD38 (p=3.9×10^{-6}), a marker of proliferating CLL cells *in vivo*.[22,23] Along with CD38 expression, CD49d also associated with other markers of rapid cell turnover, namely high LDH (p=0.007) and high β-2-microglobulin (p=0.020) (*Online Supplementary Table S2*).

Compared to previous studies investigating all CLL stages,[13,15] CD49d expression in our series was not associated (p>0.05 in all instances) with IGHV homology or ZAP70 (*Online Supplementary Table S2*). This observation may reflect a peculiar characteristic of this patient subgroup composed only of Binet A CLL. In fact, the association of CD49d with IGHV homology and ZAP70 was documented by Shanafelt *et al.*[15] in a series comprising mostly, though not exclusively, early stage CLL. Compared to our Binet A CLL cohort, however, the series by Shanafelt *et al.*[15] included many patients carrying unfavorable predictors, including higher prevalence of ZAP70 positivity (60% in the series from Shanafelt *et al.*[15] *vs.* 28% in our series) and of unmutated IGHV (44% in the series from Shanafelt *et al.*[15] *vs.* 30% in our series). Differences in the composition of the two series may be responsible for the observed dis-

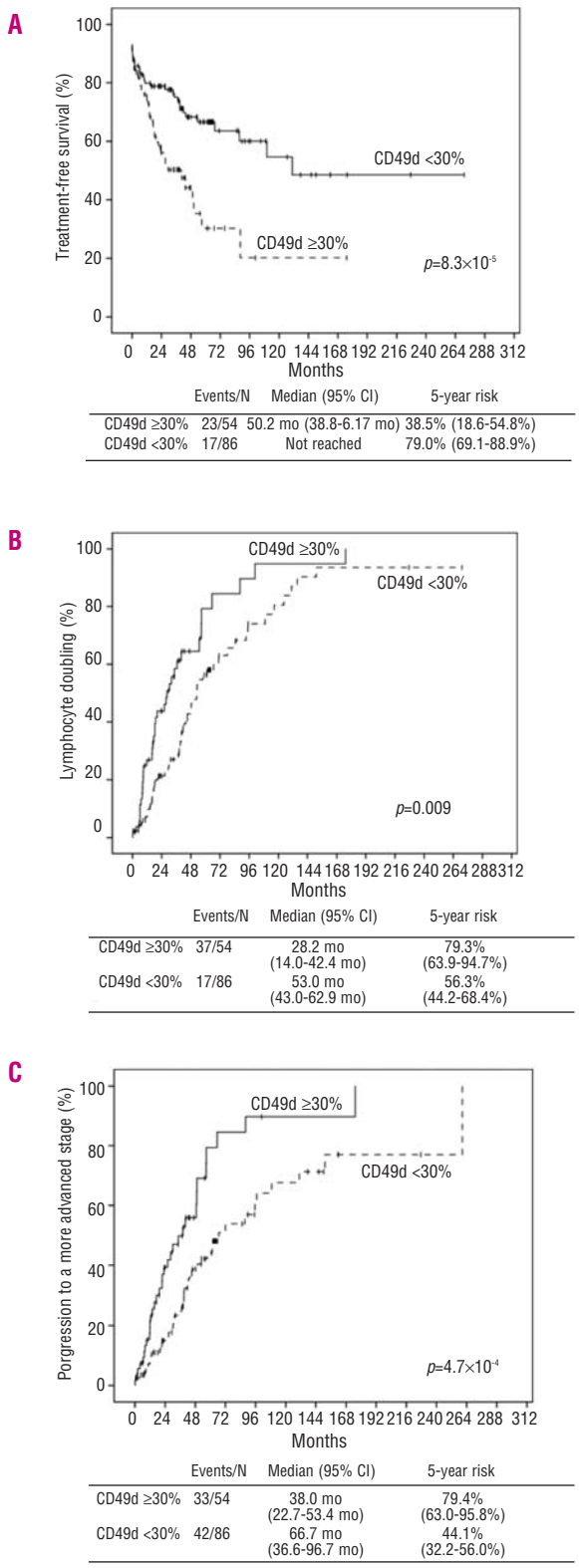

Figure 1. Survival curves for treatment free survival, time to lymphocyte doubling and time to progression to a more advanced stage according to CD49d expression. Univariate analysis identified CD49d expression ≥30% as a risk factor of short treatment free survival (A), short time to lymphocyte doubling (B), and short time to progression to a more advanced stage (C).

crepancy. In line with this hypothesis, when we analyzed all CLL stages from our institution, CD49d expression was found to associate with IGHV homology (CD49d ≥30%/IGHV homology ≥98%: 46.6% *vs.* CD49d <30%/IGHV homology ≥98%: 27.2%; p=0.008) and, at least in part, with ZAP70 expression (CD49d ≥30%/ZAP70 ≥20%: 38.2% *vs.* CD49d <30%/ZAP70≥20%: 28/103, 27.0%; p=0.098). The association between CD49d expression and markers of proliferation in Binet A CLL prompted investigations on the impact of CD49d on disease kinetics. TFS was used as the primary endpoint of progressive disease according to NCI criteria.[9] However, TFS may not capture all events of lymphocyte doubling and progression to a more advanced stage since a fraction of these events does meet NCI criteria for progressive disease requiring treatment.[9] Therefore, lymphocyte doubling and time to progression to a more advanced stage were used as additional endpoints of disease kinetics.[6]

Table 1. Multivariate analysis for TFS in Binet A CLL.[a]

	HR	*95% CI*	*p*
CD49d and biological covariates[b]			
del17p13/del11q22-q23/+12	4.89	2.45-9.73	6.1×10^{-6}
CD49d ≥30%	2.28	1.17-4.45	0.015
CD49d and clinical-biological covariates[c]			
del17p13/del11q22-q23/+12	4.07	1.96-8.45	1.5×10^{-4}
CD49d ≥30%	3.33	1.61-6.90	0.001
Lymphocytes >20×10⁹/L	3.31	1.61-6.76	0.001
Hb <13 g/dL	1.99	1.01-3.93	0.045

[a]*HR: hazard ratio; 95% CI, 95% confidence interval; p, p value calculated by Cox analysis.* [b]*Biological covariates at CLL diagnosis that entered the analysis were CD49d expression, IGHV gene homology, del17p13/del11q22-q23/+12, CD38 expression, ZAP70 expression, TP53 mutation.* [c]*Biological and clinical covariates at CLL diagnosis that entered the analysis were CD49d expression, IGHV gene homology, del17p13/del11q22-q23/+12, TP53 mutation, CD38 expression, ZAP70 expression, age, sex, ECOG PS, lymphocyte count, Hb, platelet count, lymph node involvement, splenomegaly, percentage of bone marrow lymphocytes, pattern of bone marrow involvement, and levels of LDH, β-2-microglobulin, alkaline phosphatase, and albumin.*

Univariate log-rank analysis identified CD49d expression ≥30% as a risk factor of short TFS, time to lymphocyte doubling and time to progression to a more advanced stage. Median TFS for patients with CD49d expression ≥30% was 50.2 months (5-year TFS: 38.5%), whereas median TFS for patients with CD49d expression <30% was not reached and the 5-year TFS was 79.0% (p=8.3×10^{-5}) (Figure 1A). Median time to lymphocyte doubling for patients with CD49d expression ≥30% was 28.2 months compared to 53.0 months for patients with CD49d expression <30% (p=0.009) (Figure 1B). Median time to progression to a more advanced stage for patients with CD49d expression ≥30% was 38.0 months, compared to 66.7 months for patients with CD49d expression <30% (p=4.7×10^{-4}) (Figure 1C). Other predictors of short TFS identified by univariate log-rank analysis are listed in *Online Supplementary Table S3*.

Multivariate analysis selected CD49d expression ≥30% (HR 2.28; 95% CI 1.71-4.45, p=0.015) as an independent predictor of TFS after adjustment for potentially confounding biological variables (Table 1). Also, multivariate analysis selected CD49d expression ≥30% as

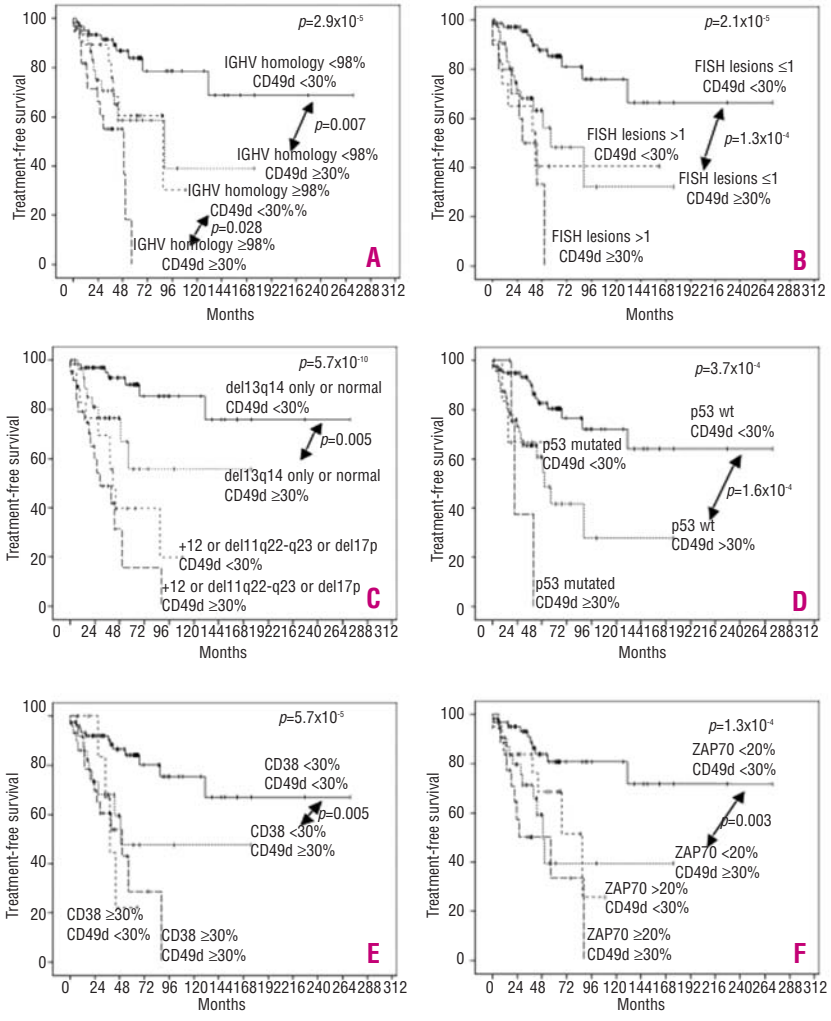

Figure 2. Survival curves for treatment free survival according to CD49d expression in combination with other biological risk factors. CD49d expression ≥30% segregated a group of CLL displaying short treatment free survival despite being characterized by IGHV homology <98% (panel A), number of FISH lesions ≤1 (panel B), normal FISH or del13q14 only (panel C), wild type *TP53* (panel D), CD38 <30% (panel E), or ZAP70 <20% (panel F). Also, the combination of CD49d expression ≥30% and IGHV homology ≥98% (panel A), identified a subset of patients that displayed the worst treatment free survival.

an independent predictor of TFS (HR 3.33, 95% CI 1.61-6.90, p=0.001) after adjustment for potentially confounding biological and clinical variables together (Table 1). At the time of the analysis, 24 patients had died and the 5-year OS was 86.6% (95% CI 79.8-93.4%). Due to the few events in the individual risk groups, CD49d expression ≥30% did not predict for OS by univariate analysis in Binet A CLL (p=0.753). Also, CD49d expression ≥30% did not represent a risk factor for transformation to Richter's syndrome (p=0.263), time to first infection (p=0.683), or time to recurrent infection (p=0.200).

Based on current guidelines, observation is the standard management of Binet A CLL.[2,3,9] However, Binet A CLL is a clinically heterogeneous disease with time to progression and survival spanning from months to normal lifespan.[2,3] On these grounds, the identification of poor risk patients within subgroups otherwise characterized by favorable predictors might be important. Our data demonstrate that CD49d expression is useful when used in combination with other prognostic markers. In fact, CD49d expression in our series identified a subgroup of Binet A CLL that displays rapid disease progression and need of treatment despite being characterized at diagnosis by favorable predictors (Figure 2, *Online Supplementary Figure S1*). This observation is reproducible in all favorable risk categories. Among patients harboring favorable biological predictors, CD49d expression ≥30% segregated CLL displaying short TFS despite being characterized by IGHV homology <98% (p=0.007), number of FISH lesions ≤1 (p=1.3×10^{-4}), normal FISH or del13q14 only (p=0.005), wild type *TP53* (p=1.6×10^{-4}), CD38 <30% (p=0.005), or ZAP70 <20% (p=0.003) (Figure 2). Also, among patients harboring favorable clinical predictors, CD49d expression ≥30% identified CLL displaying short TFS despite being characterized by age <70 year (p=0.004), Rai 0 stage (p=0.001), absence of splenomegaly (p=2.2×10^{-4}), lymphocyte count <20×10^9/L (p=0.002), Hb ≥13 g/dL (p=2.7×10^{-5}), platelets ≥150×10^9/L (p=2.1×10^{-4}), bone marrow lymphocytes ≤50% (p=0.009), nondiffuse bone marrow pattern (p=0.001), β-2-microglobulin ≤2.5 mg/L (p=0.003), LDH ≤1 × upper limit of normal (ULN) (p=0.007), and alkaline phosphatase ≤1 × ULN (p=0.011) (*Online Supplemtary Figure 1*). CD49d is also able to discriminate unfavorable risk categories among Binet A CLL carrying poor prognosticators. Indeed, patients displaying the worst TFS were identified by the combination of CD49d expression ≥30% and one of the following: IGHV homology ≥98% (p=0.028), lymphocyte count ≥20×10^9/L (p=0001), bone marrow lymphocytes

>50% (p=0.010), β2-microglobulin >2.5 mg/L (p=0.030), or LDH >1 × ULN (p=0.001) (Figure 2, *Online Supplementary Figure S1*).

The cut-off point for CD49d expression is not univocal. According to previous evidence based on log-rank and ROC analysis,[13,14] we positioned the cut-off for CD49d expression at 30%. An alternative cut-off point at 45% for CD49d expression has been proposed.[15] In our series, CD49d expression ≥30% (log rank: 15.479; p=8.3×10^{-5}) was slightly more efficient than CD49d expression ≥45% (log rank: 15.184; p=9.7×10^{-5}) in predicting TFS.

CD49d expression consistently identifies a subgroup of CLL characterized by poor outcome in all studies available today. These observations, along with the easy practicality of the methodology required for CD49d analysis,[13-15] indicate CD49d expression as a new marker that should be included in the initial prognostic assessment of CLL. The mechanisms underlying the proliferative predisposition of CLL expressing CD49d remain elusive. Defining the precise role of CD49d in CLL pathogenesis is of potential clinical interest, given the availability of a humanized anti-CD49d monoclonal antibody already available for the treatment of other diseases.[24]

Authorship and Disclosures

DR and GG designed the study, analyzed and interpreted data, performed statistical analysis and drafted the manuscript; AZ, FMR, MC, CD and CLB performed and analyzed data; SC, and SR, collected biological data; LDP collected clinical data; DC, PB, GDP, ML and VG contributed to data analysis and interpretation. The authors declare no conflict of interests.

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