CD49d Expression in Chronic Lymphocytic Leukemia (CLL) and Small Lymphocytic Lymphoma: A Reference Laboratory Perspective

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Introduction

CD49d expression is an independent predictor of disease progression in chronic lymphocytic leukemia based on academic studies[1]. However, its performance in a reference laboratory setting has not been described. Reference laboratories have unique constraints due to specimen transportation and the processing of large numbers of specimens. We sought to test whether such a setting could reproduce similar results. In addition, we hypothesize that measuring the expression of CD49d on CLL/SLL cells could replace the problematic ZAP-70 assay. This study compares CD49d expression to other prognostic indicators including ZAP-70 and Cytogenetics/FISH, which is considered a gold standard for prognosis.

Methods

- Forty-four consecutive cases of CLL/SLL were submitted to the NeoGenomics Laboratories in Aliso Viejo, CA, and evaluated for CD49d and ZAP-70 expression. Sixteen cases had concurrent Cytogenetics/FISH performed. FISH probes included: 6q- [SEC63 (6q21), MYB (6q23)], ATM (11q22.3), p53 (17p13.1), Trisomy 12 (Cen 12), 13q-/-13 (13q14, 13q34), and CCND1/IgH t(11;14).
- Antibodies used for immunophenotyping are shown in Table 1. The first five were purchased from Beckman Coulter, Miami, FL.
  - CD3-ECD
  - CD19-PC-Cy5.5
  - CD5-FITC
  - CD45-Krome Or
  - CD49d-APC
  - ZAP-70-PE (Cell Signaling Technology, Beverly, MA, clone 136F12, rabbit IgG monoclonal)

Table 1: Panel configuration

Tube	FITC	PE	ECD	PC5.5	PE-Cy7	APC	APC-A700	APC-A750	Pac Blue	KrO
ZAP-70	CD5	cZAP-70	3	19	-	49d	-	-	-	45

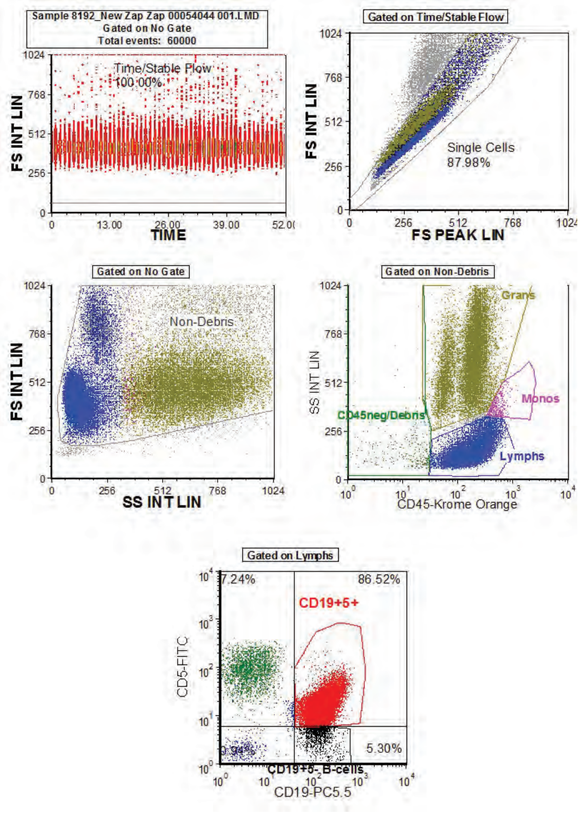
- One hundred microliters (µl) of an adjusted cell suspension containing 3–5 x 10^5 cells was aliquoted into each tube.
- A cocktail consisting of CD3, CD5, CD19, CD45 and CD49d antibodies was added to the cells and incubated for 30 min. to label cell surface markers.
- The cells were washed with filtered phosphate-buffered saline (PBS) containing 0.1% (w/v) NaN_3 (PBS/azide), pH 7.4.
- The cell pellet was fixed by adding 0.1 mL solution A (Caltag, Fix and Perm kit) with vortexing. After 15 min. at 4°C in the dark, the cells were washed twice with PBS/azide.
- Then 0.1 mL of permeabilizing solution (solution B from Caltag, Fix and Perm kit) was added and the pellet gently resuspended. ZAP-70 antibody was then added to the tube and incubated for 30 min. in the dark.
- For washing, the cells were suspended in 3 mL of PBS/azide, centrifuged for 5 min. at 350xg and the supernatant decanted. This washing procedure was repeated.
- After resuspending the cell pellet in 0.5 mL of PBS/azide, 60,000 cells were acquired on a Beckman Coulter Navios flow cytometer.
- Listmode data was analyzed with FCS Express v4 software. The expression of ZAP-70 in CD19+5+ monoclonal CLL cells was measured in comparison to positive ZAP-70 staining in CD3+ T-cells. CD49d expression was measured using negative granulocyte fluorescence to adjust the cursor.

Some cases had concurrent cytogenetic and/or FISH testing performed. Patients with normal cytogenetics/FISH or with the 13q deletion were categorized as good prognosis. Abnormal cytogenetics or FISH results showing 17p deletion, 6q deletion, 11q deletion, trisomy 12, 11/14 translocation or complex karyotype were categorized as poor prognosis.

Results

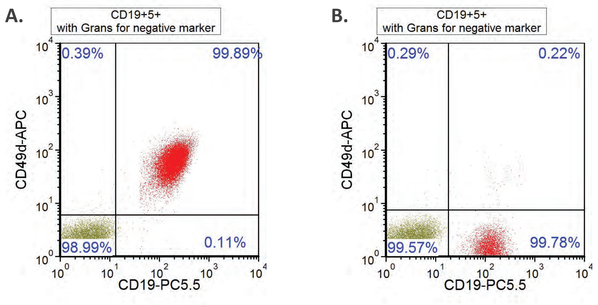
The cascade gating strategy for analysis of the flow cytometry data is shown in Figure 1. Starting from the upper left, then upper right, then middle-right, then left and finally bottom, each plot is gated on the region in the previous plot.

Figure 1. Sequential gating strategy



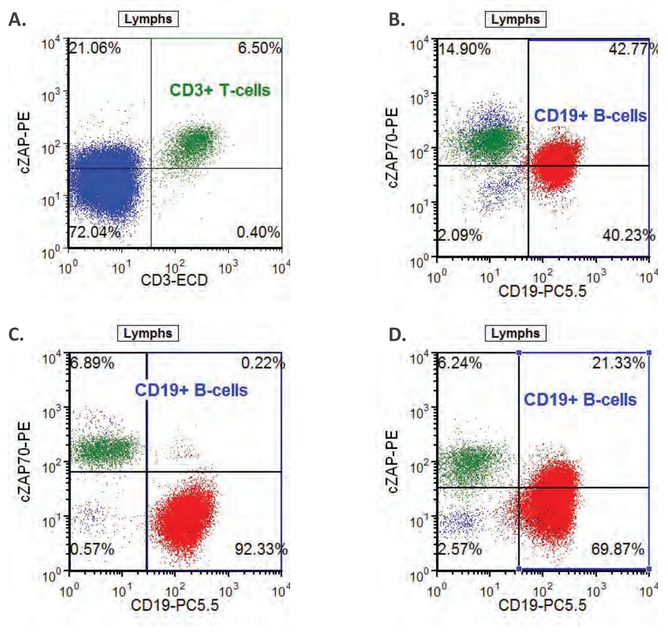
Plots showing CD49d expression on positive and negative cases are shown in Figure 2. CD49d expression for CD19+5+ lymphocytes were measured in comparison to negative granulocytes to determine the cut-off for negative staining. CD49d expression was considered positive when the MFI was greater than one log above the MFI for the negative granulocytes.

Figure 2. CD49d expression on positive and negative cases



Plots showing ZAP-70 expression on positive (panel B), negative (panel C) and indeterminate (panel D) cases are shown in Figure 3. ZAP-70 expression for CD19+5+ lymphocytes were measured in comparison to positive CD3+ lymphocytes (panel A).

Figure 3. ZAP-70 expression in positive, negative and indeterminate cases



Both percent positive staining and the MFI ratio of CD3+ T-cells divided by CD19+5+ B-cells were measured. Either greater than 20% staining or an MFI ratio <3.0 was considered elevated ZAP-70 expression in CD19+5+ B-cells.

Nineteen of the 44 cases (43%) had elevated CD49d and 12 (27%) had elevated ZAP-70 (Table 2). There was no correlation between these two variables.

Table 2: Elevations of ZAP-70 and CD49d expression are not correlated

	CD49d >20%	CD49d <20%	
Zap-70 >20%	6	6	P=0.735
Zap-70 <20%	13	19	

Since the percentage of ZAP-70 expression has been shown to be unreliable[2] we also evaluated the ratio of the MFI for T-cell ZAP-70 to the MFI for CD19+5+ B-cell ZAP-70. There was still no correlation with CD49d expression.

ZAP-70 and CD49d expression were evaluated in comparison to Cytogenetics/FISH testing. When analyzing these results, normal cytogenetics/FISH results and 13q deletion were considered good prognostic indicators. Other genetic changes were considered a bad prognosis. Fisher's test of ZAP-70 percent and ratio showed no correlation with Cytogenetics/FISH results (Table 3).

Table 3: Lack of correlation between Cytogenetics/FISH and ZAP-70

	ZAP:MFI ratio >3.3	ZAP:MFI ratio <3.0	
FISH/Cyto poor	7	1	P=0.42
FISH/Cyto good	11	0	

However, CD49d expression did correlate with FISH/Cytogenetics (Table 4) as previously reported. Fisher's exact test for the 2x2 table gave a P value of 0.035.

Table 4: CD49d expression is correlated with Cytogenetics/FISH prognosis

	CD49d >20%	CD49d <20%	
FISH/Cyto poor	8	1	P=0.035
FISH/Cyto good	2	5	

Conclusions

CD49d expression can easily and objectively be measured for CLL patients in a reference laboratory setting even with sample delays in testing. There is a significant correlation between CD49d results and parallel Cytogenetic/FISH testing. CD49d expression did not correlate with ZAP-70 expression. Our results are consistent with previous studies of CD49d expression in an academic setting[1, 3]. In spite of the constraints found in a reference lab setting, measurement of CD49d by flow cytometry can be used to provide valuable information on CLL prognosis. Variables such as delays in testing, bulk processing, and gating do not impact CD49d results. Due to the lack of reproducibility and short sample stability issues with ZAP-70, a superior and more reproducible alternative is CD49d. We conclude that CD49d is a more robust flow cytometry test which can replace the outdated and problematic ZAP-70 assay.

References

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