Prospective cytometric study evaluating the alkaline phosphatase activity as a potential functional biomarker of primitive malignant cells in acute leukemia

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INTRODUCTION

Alkaline phosphatase (ALP) is an enzyme highly expressed in primitive stem cells (SCs), including pluripotent SCs, embryonic SCs, and embryonic germ cells. ALP activity is altered in some disease conditions, such as leukemia. We previously adapted a fluorescence microscopy based method to detect ALP+ cells, and this new flow cytometric strategy was used to study the ALP activity in human leukemia in combination with immunophenotyping (LG Rico, 2016). The aim of this study was to use this rapid quantitative assay to study clinical relevance for early detection of ALP activity in a prospective monitoring of leukemic patients at diagnosis, post-treatment follow-up, and relapses to show that changes in the ALP levels can be used to detect rare populations of highly refractory malignant cells that can be involved in disease persistence and relapses.

MATERIALS AND METHODS

A total of 25 patients diagnosed with acute myeloid leukemia (AML) were included in this study. Peripheral blood and/or marrow specimens were analyzed prospectively at diagnosis, follow-up, and/or relapses. ALP staining was combined with CD34, CD38, CD90, CD117 and CD123 immunophenotyping and no-lyse no-wash methods using the Attune™ NxT Flow Cytometer (Thermo Fisher). Vybrant™ DyeCycle™ Violet Stain (DCV) was used to discriminate nucleated cells from erythrocytes and debris. Alkaline Phosphatase Live Stain (APLS) was obtained from Thermo Fisher. Clinical data from cytomorphology analysis, immunophenotyping, karyotype and molecular biology was also used in this investigation. APLS was used as described previously (LG Rico, 2016). Statistical analysis was performed using RStudio. Two-sided Fisher's exact tests were used to test for differences between categorical variables. Two-sided Wilcoxon rank sum tests were used to compare continuous variables.

RESULTS AND DISCUSSION I

We implemented a novel multicolor panel for the screening of ALP^{high} cells in leukemia, consisting of 7-colors and 4-laser excitation. Figure 1 shows a representative acquisition protocol with the gating strategy and representative dot plots to analyze the ALP activity in combination with immunophenotyping. The no-lyse no-wash strategy allowed us preserve the ALP function and detect rare events more accurately, especially in samples from patients in treatment. The use of 4-laser excitation allowed us to minimize the color compensation.

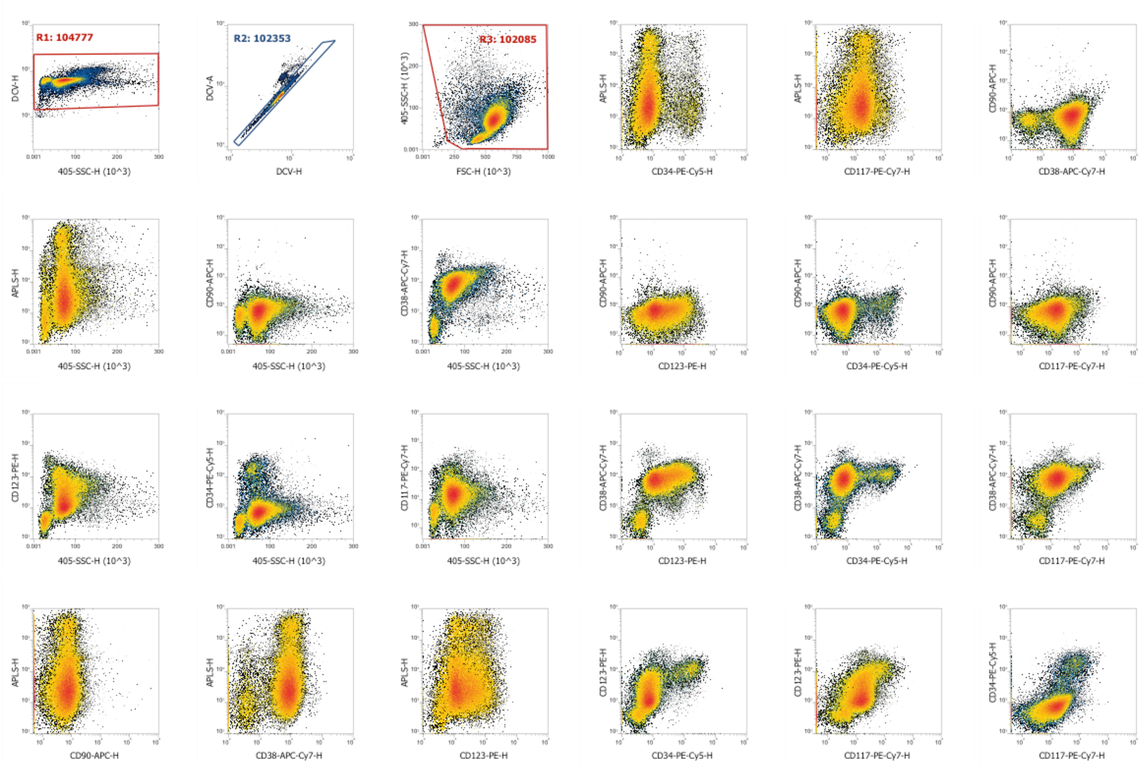

Figure 1. New cytomic panel used for the screening of ALP+ cells. Gate **R1** is used to discriminate nucleated cells from erythrocytes and debris. **R2** is used to discriminate doublets using DNA staining. **R3** discriminates leucocytes from apoptotic cells and debris. Dot-plots show ALP activity in combination with APC-CD90, APC-Cy7-CD38, PE-CD123, PE-Cy5-CD34, and PE-Cy7-CD117. Samples were acquired using Attune™ NxT Flow Cytometer (Thermo Fisher). Gating was sequential for all other plots. The statistics in the region represents count of the gate.

RESULTS AND DISCUSSION II

ALP activity was studied in 25 AML patients at diagnosis (from a total of 34) and were classified in two groups according to the numbers of leukemic blasts expressing ≥ 10% of ALP+ cells and less than 10% of ALP+ cells. Differences between the two groups were analyzed. Table 1 shows AML patient characteristics between APLS^{high} ≥ 10% (n=17) and ALP^{high} < 10% (n=8) groups. From those patients with more than 10% of ALP+ leukemic cells at diagnosis, 16 relapsed (94.1%), showing significant differences when compared to the ALP^{high} <10% group (P= 0.001; 95% CI = 0.00-0.36), giving support to the hypothesis that ALP activity at diagnosis may predict relapses and disease persistence. Four patients within the ALP^{high} < 10% group had CD34-/CD123+/CD117+ leukemic cells with significant differences (P= 0.023; 95% CI = 1.02-829.85) when compared with the group of more than 10% of ALP positive cells, indicating that phenotype may be associated with better outcome than the CD34+ phenotype. No differences were found when comparing risk cytogenetic classification between both groups.

	Patients (n=25)	APLS^{high} ≥ 10% (n=17)	APLS^{high} < 10% (n=8)	p-value (CI=95%)
Cytogenetic Risk Groups				
• ***Favorable***	3 (12%)	2 (11.8%)	1 (12.5%)	1.000
• ***Intermediate***	20 (80%)	14 (82.3%)	6 (75%)	1.000
• ***Unfavorable***	2 (8%)	1 (5.9%)	1 (12.5%)	1.000
Leukemic Blast Markers				
• **CD34+/CD123+/CD117+**	17 (68%)	14 (82.3%)	3 (37.5%)	0.061
• **CD34+/CD123-/CD117+**	1 (4%)	0 (0%)	1 (12.5%)	0.320
• **CD34-/CD123+/CD117+**	5 (20%)	1 (5.9%)	4 (50%)	**0.023***
• **CD34-/CD123+/CD117-**	2 (8%)	2 (11.8%)	0 (0%)	1.000
Complete Response (CR)	15 (60%)	8 (47%)	7 (87.5%)	0.087
Time to CR	3.7 [1 – 18]	5.2 [1 – 18]	2 [1 – 5]	0.256
Relapse	18 (72%)	16 (94.1%)	2 (25%)	**0.001****
Time to Relapse	5.7 [1 – 29]	5.9 [1 – 29]	4	0.716
Exitus	9 (36%)	8 (47%)	1 (12.5%)	0.182
Overall Survival (months)	9.1 [2 – 15]	8.7 [2 – 15]	12	0.696

Table 1. Comparison of patient characteristics and outcomes between APLS^{high} ≥ 10% and ALP^{high} < 10% AML groups. Risk cytogenetics, blast markers, complete response, time to achieve complete response, relapse, time to relapse, exitus and overall survival were compared. P-value < 0.05 was considered as significant.

RESULTS AND DISCUSSION IV

Figure 2 shows the cytometric study of ALP levels in combination with immunophenotyping performed in a patient from the ALP^{high} ≥ 10% group. ALP^{high} cells at diagnosis were 37.64% of the CD123+ population and 49.11% of the CD34+ cells. After the first induction, the same patient had more than 40% of CD34+ blasts with 20% of ALP^{high} cells. Finally, after the third induction, the patient had 17% of CD34+ blasts with more than 20% of ALP^{high} cells. ALP cells were systematically detected in this representative patient at a levels greater than 20%. Overall, patients with increased numbers of ALP^{high} cells eventually relapsed and/or showed worse outcome than those ones with undetectable or low numbers of ALP^{high} leukemic cells.

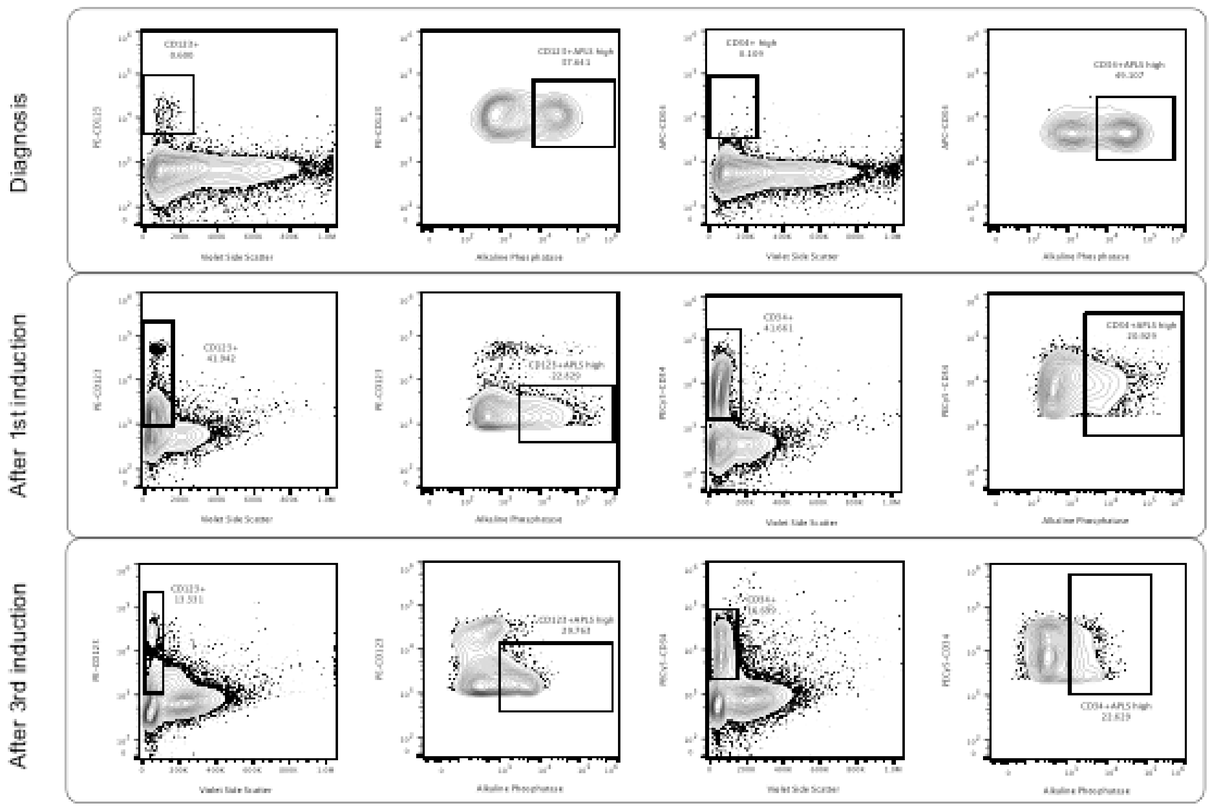

Figure 2. Representative flow cytometric study of the alkaline phosphatase activity in a patient of the ALP^{high} ≥ 10% group. Alkaline phosphatase positive cells are represented in combination with CD34 and CD123 staining at diagnosis (upper row), after first induction (middle row) and after third induction (lower row). The statistics in the region represents percentage of the gate.

CONCLUSIONS

Our results suggest that ALP^{high} leukemic cells appear to sustain leukemogenesis over time and may be related to relapses and therapy resistance.

This new cytomic functional approach may help to better identify leukemic cells that remain elusive using current optimized multicolor panels for leukemia immunophenotyping.

Increased numbers of primitive ALP^{high} leukemic cells appear to be a good predictor of relapse and treatment refractoriness.

Further experiments will be needed, such as cell sorting, serial transplantation assays and genome-wide transcriptome studies to enlighten the role of primitive subsets of ALP^{high} cells in blood cancer.

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