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Original Article

Prognostic Significance of Flow Cytometric Immunophenotyping in Acute Myeloid Leukemia

Brian A. Webber, Melissa M. Cushing and Shiyong Li

Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA, USA

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Abstract: The prognostic significance of flow cytometric immunophenotyping (FCI) in acute myeloid leukemia (AML) has been controversial. In this study, we re-investigated the possible role of FCI in the prediction of AML relapse following standard chemotherapy. A total of 209 AML cases with follow-up information were analyzed. Among those, 78 cases were in remission (M:F=44/34; mean age of 48.9 years) and 131 had relapse (M:F=71/60; mean age of 51.3 years). The expression of CD34, HLA-DR or a combination of both was significantly different between the remission and relapse groups for all AML as well as AML without t(15;17). None of the pammyeloid markers or their combinations analyzed was found to correlate with treatment outcomes. Complex cytogenetic abnormalities were more likely associated with relapse group than with remission group, but were not statistically significant after excluding AML with t(15;17). In conclusion, FCI is useful in predicting treatment outcome and disease relapse in AML.
Key Words: Flow cytometric immunophenotyping, acute myeloid leukemia, acute promyelocytic leukemia, chromosome translocation, cytogenetics, prognosis

Introduction

Flow cytometric immunophenotyping (FCI) has a well established role as a diagnostic modality in acute leukemias, particularly as a tool for assigning lineage and facilitating further pathologic classifications [1-14]. However, cytogenetic evaluation constitutes the predominant method for assessing prognosis in acute leukemias [15-18]. Although several authors have investigated the prognostic implications of immunophenotype in acute myeloid leukemia (AML) [17, 19-27], no clear consensus has emerged regarding the role of FCI in predicting treatment response, relapse, or overall survival.

While several studies have failed to demonstrate any significant association between FCI and prognosis, others have reported significant correlation between several immunophenotypic markers and clinical outcomes, albeit without universal reproducibility [2, 28-37]. Immunophenotypic markers that, in various studies, have been implicated as predictive of adverse outcomes include CD7, CD9, CD11b, CD13, CD14, CD33, CD34, CD56, and terminal deoxynucleotidyl transferase (TdT) [35, 38-44]. In addition, co-expression of CD34 and HLA-DR has been shown to be an independent predictor of failure to achieve complete remission (CR) [19, 39, 45-49]. Another study ascribed a more favorable prognosis to cases in which myeloblasts demonstrate a panmyeloid phenotype, co-expressing myeloperoxidase (MPO), CD13, CD33, CDw65 and CD117 [22].

In this report, we retrospectively analyze FCI and cytogenetic findings in 209 cases of AML with or without relapse. Our aim was to determine whether any independent correlation exists between immunophenotype and probability of disease relapse.

Materials and Methods

Case Selection

AML cases diagnosed at Emory University Hospital between August 1997 and March

2003 were retrieved from our pathology electronic database. Cases meeting World Health Organization criteria for AML with available immunophenotyping results and appropriate follow-up information were included and corresponding cytogenetic results were obtained. All AML subtypes were included in the sample population, without exclusion of secondary AML or subtypes with specific cytogenetic abnormalities. Outcomes were defined as complete remission (CR, no evidence of disease at least 10 weeks after induction therapy) or relapse (persistent disease or recurrent disease 10 weeks or greater following induction chemotherapy). Any recurrence of disease occurring before October 2003 was recorded, along with the immunophenotypic and cytogenetic profiles at relapse.

Flow Cytometric Immunophenotyping

Flow cytometric immunophenotyping was performed on bone marrow aspirate or peripheral blood samples collected in RPMI 1640 culture medium. Specimen processing was performed according to a routine red cell lysis protocol. Single cell suspensions were stained with various 4 fluorochrome-conjugated antibody combinations and analyzed in reference to isotype-matched fluorochrome-conjugated control antibodies. Samples were stained with monoclonal antibodies for the following antigens: CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD19, CD20, CD22, CD25, CD33, CD34, CD36, CD38, CD45, CD56, CD103, CD117 and HLA-DR (Becton Dickinson Biosciences, San Diego, CA). Although intracytoplasmic staining on permeabilized samples for myeloperoxidase (MPO) and terminal deoxynucleotidyl transferase (TdT) was performed on selected samples when deemed necessary for clinical classification purposes at the time of diagnosis, these results were not included in the analysis due to insufficient numbers. Samples were acquired on a dual-laser FACSCalibur flow cytometer (Becton Dickinson Biosciences) and subsequently analyzed using the CellQuest computer software program (Becton Dickinson Biosciences). Myeloblast immunophenotype was determined with an antigen defined as positive when at least 20% of the myeloblasts expressed the marker at a fluorescence intensity above cutoffs established using the corresponding isotype-matched control antibody.

Cytogenetic Studies

All cytogenetic studies were performed at Emory Medical Laboratories according to standard protocols with results reported in accordance with the International System for Human Cytogenetic Nomenclature (ISCN) guidelines. Karyotype and/or fluorescence in situ hybridization results at initial presentation and at relapse were retrieved and recorded along with the corresponding FCI results.

Statistical Analysis

Bivariate and multivariate analyses were performed on the total sample, both including and excluding acute promyelocytic leukemia (APL) cases, for correlation between immunophenotype and remission status with endpoints defined as maintained CR or relapse. Multivariate analysis was adjusted for impact of age (less than or greater than 60 years old) and cytogenetic prognostication categories (favorable, intermediate, or unfavorable prognoses). Odds ratios (with corresponding 95% confidence intervals) for relapse were calculated with results less than 1 indicating less likelihood of relapse and results greater than 1 indicating greater likelihood of relapse. Significant results were defined as those with $p<0.05$.

Results

Clinicopathologic Features

A total of 209 AML cases were retrieved. 78 of the patients were in CR and 131 patients had documented persistent disease or disease relapse. The cohort included 26 cases of APL with documented t(15;17) and all statistical analyses were performed in tandem on the entire patient sample and on the sample excluding these APL cases. Clinical and biological characteristics of the patients are presented in **Table 1**.

Immunophenotypic Findings

Frequencies of expression for 24 surface antigens in AML patients, both including (n=209) and excluding (n=183) APL cases, are presented in **Table 2**. Frequencies of selected combinations of antigen expression and/or cytogenetic findings are also shown.

Table 1 Patient characteristics by outcome for both total sample (n=209) and AML only (n=183)

Patient characteristics	Total AML sample (n=209)		AML excluding APL (n=183)	
	Remission n (%)	Relapse n (%)	Remission n (%)	Relapse n (%)
Age				
<60	58 (27.75)	78 (37.32)	41 (22.40)	76 (41.53)
60+	20 (9.57)	53 (25.36)	13 (7.10)	53 (28.96)
Sex				
Female	34 (16.27)	60 (28.71)	22 (12.02)	58 (31.69)
Male	44 (21.05)	71 (33.97)	32 (17.49)	71 (38.80)
Diagnosis				
AML excluding APL	54 (25.84)	129 (61.72)	54 (29.51)	129 (70.49)
APL with t(15;17)	24 (11.48)	2 (0.96)	0 (0.00)	0 (0.00)
Cytogenetics				
Good	31 (15.58)	18 (9.05)	7 (4.05)	16 (9.25)
t(8;21)	1	8	1	8
t(15;17)	24	2	0	0
inv6	6	8	6	8
Intermediate	30 (15.08)	75 (37.69)	30 (17.34)	75 (43.35)
no abnormality	26	53	26	53
+8	1	7	1	7
other	3	16	3	16
Poor	14 (7.04)	31 (15.58)	14 (8.09)	31 (17.92)
11q23	4	10	4	10
del(5q)/-5	2	9	2	9
-7	2	12	2	12
abn(3q)	1	5	1	5
t(9;22)	2	2	2	2
complex	1	14	1	14

The most frequently expressed antigen was the non-specific lymphoid progenitor marker CD38 (92% of all cases).This was followed by the myeloid lineage markers CD13 (91%), CD33 (87%), and CD117 (80%). Also demonstrating relatively high prevalence rates were the hematopoietic progenitor cell markers CD34 (71%) and HLA-DR (79%). Monocytic markers were moderately frequent: including CD4 (63%), CD11b (41%), CD11c (43%), CD14 (16%), and CD36 (34%). CD7, a T cell antigen known to show aberrant expression in a subset of AML cases, was positive in 28% of all cases. Of note, another T cell antigen, CD2, was expressed in a considerable number of cases (18%). B cell markers CD10, CD19, and CD22 were present in 13%, 8%, and 2% of all cases, respectively.

Not surprisingly, CD34 and HLA-DR were expressed in a higher percentage of cases when APL patients were excluded from the group. This increase was seen for these markers when assessed individually (CD34+ in 71% of total sample and 77% of AML excluding APL sample; HLA-DR in 79% and 90%, respectively) as well as when both markers were co-expressed (59% and 67%, respectively).

Other combinations of co-expressed antigens that were chosen partially based on results from previous studies were analyzed. Of note, co-expression of all 3 pan-myeloid markers included in the panel was present in 65% of all cases. Cases showing strong evidence of monocytic differentiation (that is, expressing at

Table 2 Prevalence of antigen markers for both total sample and AML excluding APL

Antigen markers	Total AML sample (n=209)		AML excluding APL (n=183)	
	n	Percent	n	Percent
Single markers				
CD2+	38	18.18	34	18.58
CD4+	131	62.68	121	66.12
CD5+	6	2.87	6	3.28
CD7+	58	27.75	58	31.69
CD8+	0	0.00	0	0.00
CD10+	28	13.40	28	15.30
C11B+	86	41.15	83	45.36
C11C+	89	42.58	89	48.63
CD13+	190	90.91	165	90.16
CD14+	34	16.27	34	18.58
CD15+	139	66.51	123	67.21
CD16+	3	1.44	3	1.64
CD19+	17	8.13	16	8.74
CD20+	0	0.00	0	0.00
CD22+	5	2.39	5	2.73
CD23+	7	3.35	7	3.83
CD25+	22	10.53	22	12.02
CD33+	181	86.60	156	85.25
CD34+	149	71.29	140	76.50
CD36+	71	33.97	68	37.16
CD38+	192	91.87	170	92.90
CD56+	41	19.62	39	21.31
CD117+	167	79.90	146	79.78
HLA-DR+	157	79.29	155	90.12
Combination markers				
CD34+/HLA-DR+	124	59.33	123	67.21
CD13+/CD33+/CD117+	136	65.07	116	63.39
CD34+/HLA-DR+/CD7+	46	22.01	46	25.14
CD34+/HLA-DR+/CD56+	20	9.57	20	10.93
CD34+/CD7+	51	24.40	51	27.87
CD34+/CD56+	26	12.44	25	13.66
CD11B+/CD11C+/CD36+/ CD14+ (must express 3 of 4)	50	23.92	50	27.32
	(n=199)		(n=173)	
CD56+ with t(15;17)	2	1.01	0	0.00
CD56+ with t(8;21)	2	1.01	2	1.16
CD56+ with inv(16)	2	1.01	2	1.16

least 3 of CD11b, CD11c, CD36, and CD14) comprised 24% of all cases. Only 2 cases of AML with t(8;21) demonstrated CD56 expression. Similarly, CD56 expression was only seen in 6 of the cases with favorable cytogenetic findings.

Cytogenetic Features

Cytogenetic findings were available for 199 of 209 cases. Of the translocations that have been associated with a favorable prognosis—namely t(8;21), inv(16), and t(15;17)—31 of 49 patients (63%) were in remission at the completion of the follow-up period, while 18 (37%) had relapsed. Of note, only t(15;17) was significantly associated with maintained remission status ($p<0.05$). Of the 45 patients with unfavorable cytogenetic findings–that is, del(5q)/-5, -7, abnormal 3q, abnormal 11q23, or t(9;22)— 31 (69%) had relapsed and 14 (31%) were still in remission at the completion of the study. Of 105 patients in the intermediate cytogenetics category—including

Table 3 Antigen marker as a predictor of relapse for total AML

Antigen Markers	Total AML sample (n=209)	
	OR 95%CI[a]	AOR 95%CI[b]
CD15 -	Referent	Referent
CD15+	0.46 (0.24, 0.86)**	0.58 (0.30, 1.14) ns
CD33-	Referent	Referent
CD33+	0.32 (0.12, 0.86)**	0.41 (0.14, 1.18) ns
CD34-	Referent	Referent
CD34+	4.24 (2.25, 7.98)**	4.08 (2.06, 8.04)**
HLA-DR-	Referent	Referent
HLA-DR+	8.85 (4.04, 19.35)**	6.99 (2.92, 16.71)**
CD34-/HLA-DR-	Referent	Referent
CD34+/HLA-DR+	8.05 (4.27, 15.19)**	7.05 (3.58, 13.87)**

[a]Unadjusted Odds Ratio and 95% Confidence Intervals; [b]Adjusted Odds Ratio and 95% Confidence Intervals – adjusted for age (less than 60 years or 60+ years) and cytogenetics (good, intermediate or poor); **statistically significant at $p<0.05$; ns: statistically not significant.

trisomy 8, normal karyotype, or cytogenetic abnormalities other than the above--75 (71%) had relapsed and 30 (29%) were in remission.

Correlation of Immunophenotype with Clinical Outcomes

In the entire sample, 78 (37%) patients were in CR and 131 (63%) had relapsed. When APL cases were excluded from the analysis, 54 (30%) maintained CR status, while 129 (70%) had relapsed. Analysis of the individual markers in the entire sample demonstrated significantly increased likelihood of relapse ($p<0.05$) in cases expressing CD34 (OR=4.24; 95% CI=2.25-7.98) and HLA-DR (OR=8.85; 95% CI=4.04-19.35). Co-expression of CD34 and HLA-DR was also significantly associated with relapse (OR=8.05; 95% CI=4.27-15.19). Alternatively, CD15 (OR=0.46; 95% CI=0.24-0.86) and CD33 (OR=0.32; 95% CI=0.12-0.86) correlated with maintenance of CR (**Table 3**).

When APL cases were excluded from the analysis sample, cases showing CD34 (OR=3.10; 95% CI=1.52-6.32) or HLA-DR (OR=3.05; 95% CI=1.09-8.49) expression, in addition to those co-expressing CD34 and HLA-DR (OR=5.25; 95% CI=2.64-10.41), remained significantly associated with relapse in the bivariate analysis. Also, CD13 expression in the APL-excluded group correlated with relapse (OR=2.88; 95% CI=1.02-8.19). CD2 (OR=0.39; 95% CI=0.18-0.83), CD10 (OR=0.42; 95% CI=0.18-0.95), CD11b (OR=0.50; 95% CI=0.26-0.95), CD14 (OR=0.45; 95%CI=0.21-0.97), CD15 (OR=0.36; 95% CI=0.17-0.78), and CD36 (OR=0.47; 95% CI=0.24-0.98) all correlated with maintenance of CR. In addition, cases expressing 3 of 4 of the monocytic markers CD11b, CD11c, CD36, and CD14 were also associated with CR (OR=0.46; 95% CI=0.23-0.91) (**Table 4**).

In the multivariate analysis, with adjustment for age and cytogenetic results, CD34 (OR=4.08; 95% CI=2.06-8.04) and HLA-DR (OR=6.99; 95% CI=2.92-16.71) expression, as well as co-expression of these markers (OR=7.05; 95% CI=3.58-13.87), remained significantly associated with increased relapse rate when all cases were included in the analysis. Importantly, these continued to show significance when APL cases were excluded. The multivariate analysis in the APL-excluded group also showed continued correlation with relapse for CD13 (OR=2.88; 95% CI=1.02-8.19) and with CR for CD2 (OR=0.40; 95% CI=0.17-0.94), CD10 (OR=0.37; 95% CI=0.15-0.90), CD15 (OR=0.42; 95% CI=0.19-0.94), and CD36 (OR=0.48; 95% CI=0.24-0.95) (**Table 5**).

Using multivariate logistic regression analysis, co-expression of CD34 and HLA-DR was the only immunophenotype finding that continued to show prognostic significance. This was true both when APLs were included (OR=6.79; 95%

Table 4 Antigen marker as a predictor of relapse for AML excluding APL

Antigen Markers	AML excluding APL sample (n=183)	
	OR 95%CI[a]	AOR 95%CI[b]
CD2 -	Referent	Referent
CD2+	0.39 (0.18, 0.83)**	0.40 (0.17, 0.94)**
CD10 -	Referent	Referent
CD10+	0.42 (0.18, 0.95)**	0.37 (0.15, 0.90)**
CD11b -	Referent	Referent
CD11b+	0.50 (0.26, 0.95)**	0.55 (0.28, 1.11) ns
CD13 -	Referent	Referent
CD13+	3.44 (1.28, 9.27)**	2.88 (1.02, 8.19)**
CD14 -	Referent	Referent
CD14+	0.45 (0.21, 0.97)**	0.47 (0.20, 1.11) ns
CD15 -	Referent	Referent
CD15+	0.36 (0.17, 0.78)**	0.42 (0.19, 0.94) **
CD34-	Referent	Referent
CD34+	3.10 (1.52, 6.32)**	3.06 (1.43, 6.55)**
CD36-	Referent	Referent
CD36+	0.47 (0.24, 0.89)**	0.48 (0.24, 0.95)**
HLA-DR-	Referent	Referent
HLA-DR+	3.05 (1.09, 8.49)**	3.23 (1.09, 9.60)**
CD34-/HLA-DR-	Referent	Referent
CD34+/HLA-DR+	5.25 (2.64, 10.41)**	5.21 (2.51, 10.84)**
CD11B+/CD11C+/CD36+/ CD14+ (less than 3 of 4)	Referent	Referent
CD11B+/CD11C+/CD36+/ CD14+ (at least 3 of 4)	0.46 (0.23, 0.91)**	0.49 (0.24, 1.02)

[a]Unadjusted Odds Ratio and 95% Confidence Intervals; [b]Adjusted Odds Ratio and 95% Confidence Intervals – adjusted for age (less than 60 years or 60+ years) and cytogenetics (good, intermediate or poor); **statistically significant at $p<0.05$; ns: statistically not significant.

CI=3.43-13.47) and when they were excluded (OR=4.41; 95% CI=2.06-9.44) from the test population. In the former analysis, intermediate (OR=3.40; 95% CI=1.52-7.60) and unfavorable (OR=3.01; 95%CI=1.15-7.85) cytogenetic features were also associated with an increased relapse rate, as was age greater than 60 years old in the latter. In both analyses, however, co-expression of CD34 and HLA-DR was the strongest and independent predictor of relapse (**Table 6**).

Discussion

Previous studies have addressed whether immunophenotype has predictive value with respect to clinical outcomes in AML, often achieving statistical significance for specific antigen markers or combinations thereof. However, the findings of these various investigations are conflicting, and no consensus has emerged regarding which, if any, markers hold prognostic significance [17-49]. We retrospectively studied FCI, in conjunction with age and cytogenetic features, as a means of predicting disease relapse in AML patients over approximately 6 years at a

single institution. In order to allow comparison with these other studies, some of which excluded APL cases from their test populations, we performed all statistical analyses twice: once including all AML cases (n=209) and the other including only those cases failing to demonstrate t(15;17) (n=183).

Our findings support previous reports ascribing poor prognosis to AML cases with myeloblasts expressing the hematopoietic progenitor cell markers CD34 and HLA-DR [19; 39, 45-49]. Although we identified several antigenic markers associated with prognostic outcomes on initial bivariate analysis, multivariate analysis confirmed only independent CD34 and HLA-DR expression, along with CD34/HLA-DR co-expression, as having significant predictive value in the all inclusive population. While inclusion of cases demonstrating the prognostically favorable t(15;17), almost by definition negative for CD34 and HLA-DR, clearly confounds these results, these markers remained significantly associated with increased relapse risk even when APL cases were excluded from the test population.

In fact, excluding the t(15;17) cases yielded additional significant associations on simple multivariate analysis with CD2, CD10, CD15, and CD36 correlating with CR and CD13 correlating with relapse. While these additional findings admittedly simply add to the complicated landscape of potentially prognostic markers already described, application of the more stringent multivariate logistic regression model of analysis again revealed only CD34 and HLA-DR co-expression to be significant among the immunophenotypic markers. In fact, co-expression of these markers proved more predictive of poor outcome than advanced age and absence of favorable cytogenetic features.

Given the reportedly favorable prognosis ascribed to cases with a panmyeloid phenotype [22], we investigated and failed to demonstrate any prognostic implications for cases co-expressing the myeloid-exclusive markers present in our panel--namely CD13, CD33, and CD117. Unfortunately, we were unable to implement the more stringent panmyeloid criteria established by Legrand *et al* (requiring co-expression of CD13, CD33, CDw65, CD117, and MPO) due to insufficient numbers of cases evaluated for MPO and exclusion of CDw65 from routine testing at our institution. Similarly, the reported poor prognosis associated with CD56 expression in AML could not be adequately assessed because of limited numbers of these cases present in our sample population.

In conclusion, the results of this study support the findings of previous investigations in ascribing poor prognostic implications to AML cases with myeloblasts co-expressing the hematopoietic progenitor markers CD34 and

Table 5 Multivariate logistic regression results – predictors of relapse for total AML

Antigen markers/variables	Total AML sample (n=209)	
	Adjusted OR	95%CI
Age less than 60	Referent	Referent
Age 60 or older	2.03	(0.96, 4.28) ns
Good cytogenetics	Referent	Referent
Intermediate cytogenetics	3.40	(1.52, 7.60)**
Poor cytogenetics	3.01	(1.15, 7.85) **
CD15 -	Referent	Referent
CD15+	0.63	(0.29, 1.37) ns
CD33-	Referent	Referent
CD33+	0.62	(0.18, 2.06) ns
CD34-/HLA-DR-	Referent	Referent
CD34+/HLA-DR+ (Pos)	6.79	(3.43, 13.47)**

OR: odds ratio; CI: confidence interval; **statistically significant at $p<0.05$; ns: statistically not significant.

Table 6 Multivariate logistic regression results – predictors of relapse for AML excluding APL

Antigen markers/variables	AML excluding APL sample (n=183)	
	Adjusted OR	95%CI
Age less than 60	Referent	Referent
Age 60 or older	2.28	(1.01, 5.15)**
Good cytogenetics	Referent	Referent
Intermediate cytogenetics	1.44	(0.48, 4.34) ns
Poor cytogenetics	1.26	(0.37, 4.27) ns
CD13 -	Referent	Referent
CD13+	2.21	(0.72, 6.78) ns
CD15 -	Referent	Referent
CD15+	0.56	(0.22, 1.40) ns
CD34-/HLA-DR-	Referent	Referent
CD34+/HLA-DR+	4.41	(2.06, 9.44)**
CD11B+/CD11C+/CD36+/ CD14+ (less than 3 of 4)	Referent	Referent
CD11B+/CD11C+/CD36+/ CD14+ (at least 3 of 4)	0.83	(0.35, 1.96) ns

OR: odds ratio; CI: confidence interval; **statistically significant at p<0.05; ns: statistically not significant.

HLA-DR. Since almost all AML cases undergo immunophenotyping and given the relative speed and availability of testing, any significant prognostic information to be gleaned from flow cytometric analysis, in conjunction with cytogenetic and other clinical findings, may be helpful in influencing treatment strategies.

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Please address all correspondences to Shiyong Li, MD, PhD, Department of Pathology and Laboratory Medicine, Emory University Hospital F143D, 1364 Clifton Road NE, Atlanta, GA 30322. Tel: 404-712-5456; Fax: 404-712-4140; Email: sli2@emory.edu

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