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Research Article / Araştırma Makalesi

Comparison of Flow Cytometry Results of Acute Myeloid Leukemia Patients at Diagnosis and Relapse

Akut Miyeloid Lösemili Hastaların Tanı ve Relaps Dönemindeki Akım Sitometri Sonuçlarının Karşılaştırılması

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Abstract: In most patients with acute myeloid leukemia (AML), leukemic cells become undetectable after chemotherapy. Nevertheless, leukemia may subsequently relapse due to minimal residual disease (MRD). Flow cytometric monitoring of MRD is prognostically informative. However immunophenotypic shifts at relapse is possible and may limit flow cytometric MRD-testing. Our objective was to evaluate the antigen changes in our AML patients. Patients diagnosed between September 2002 and November 2016 were analyzed retrospectively. Bone marrow samples were collected at diagnosis and relapse from 40 patients with de novo (n=34) or secondary (n=6) AML, aged 19 to 77 years. Bone marrow samples were collected into tubes containing K3EDTA. Phycoerhtyrine (PE) and fluorescein isothiocyanate (FITC) (eBioscience and BD Bioscience, San Jose, California) surface antigens were used according to the routine panel used in our laboratory. Analyses were done according to CD45 SSC gating strategy by Becton Dickinson FACSCalibur device. Overall, 34 of 40 (85%) cases showed changes (gain and/or loss of antigen) of at least one marker (n=10). Antigen changes were observed in 2 (n=7), 3 (n=6), 4 (n=6), 5 (n=4) or 6 (n=1) antigens in other patients. Antigen changes were found in 16 of 18 antigens (88.9%) totally. CD20 and CD45 were the only antigens with no change. Patients with AML demonstrate a high frequency of immunophenotypic shift at relapse. Antigen changes at relapse should be kept in mind in the minimal residual disease era.

Keywords: acute myeloid leukemia; flow cytometry; relapse; minimal residual disease

Özet: Akut miyeloid lösemi (AML)li çoğu hastada lösemik hücreler kemoterapi sonrası kaybolur. Ancak minimal kalıntı hastalık (MKH) nedeniyle lösemi nüksü gözlenebilir. MKH'nin akım sitometrik olarak takibi prognostik açıdan bilgi sağlar. Fakat relaps anında immünfenotipik kaymalar olabilir ve akım sitometri ile MKH değerlendirilmesini kısıtlayabilir. Bu çalışmada AML hastalarındaki antijen değişikliklerinin saptanması amaçlanmıştır. Çalışmada Eylül 2002 ve Kasım 2016 arasında AML tanısı alan 19-77 yaş arası geriye dönük olarak değerlendirildi. Kemik iliği örnekleri tanı ve relaps anında elde edildi. Hastaların 34'ü de novo, 6'sı sekonder AML idi. Kemik iliği örnekleri K3EDTA içeren tüplere alındı. Phycoerhtyrine (PE) ve fluorescein isothiocyanate (FITC) (eBioscience and BD Bioscience, San Jose, California) yüzey antijenleri laboratuvarımızda kullanılan rutin panele göre kullanıldı. Analizler CD45 kapılama stratejisine göre Becton Dickinson FACSCalibur cihazı ile yapıldı. Kırk hastanın 34'ünde (%85) en az 1 antijende değişiklik (antijen kazanımı ve/veya kaybı) mevcuttu (n=10). Antijen değişiklikleri 2 (n=7), 3 (n=6), 4 (n=6), 5 (n=4) ya da 6 (n=1) antijende gözlendi. Antijen değişiklikleri 18 antijenin 16'sında (%88.9) saptandı. Hiçbir hastada değişiklik gözlenmeyen antijenler sadece CD20 ve CD45'ti. AML'li hastalarda relaps sırasında immünfenotipik kayma sıklığı yüksektir. MKH değerlendirilirken relapsta gelişen antigen değişiklikleri göz önünde bulundurulmalıdır.

Anahtar Kelimeler: akut miyeloid lösemi; akım sitometri; relaps; minimal kalıntı hastalık

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1. Introduction

In most patients with acute myeloid leukemia (AML), leukemic cells become undetectable after chemotherapy. Nevertheless, leukemia may subsequently relapse due to persisting chemoresistant cells indistinguishable from normal hematopoietic progenitors by conventional morphologic analysis, i.e., minimal residual disease (MRD) (1–3). In both childhood and adult AML, MRD is a powerful and independent prognostic factor (4–13).

Flow cytometric monitoring of MRD is prognostically informative and, unlike PCR, is not limited to patients with specific genetic abnormalities (7–9,14–22). Nevertheless, standard flow cytometric monitoring of MRD has a sensitivity often not exceeding 0.1% (15,18,19) and requires considerable expertise to avoid incorrect MRD estimates.

Leukemia subclones at diagnosis may become predominant at relapse, resulting in immunophenotypic shifts (19). One limitation of flow cytometric MRD-testing is the possibility of phenotypic changes over time with gains/losses of specific abnormalities or patterns of abnormalities because of disease evolution, sub-clone selection, and/or progression through the cell cycle (23,24).

In this study, the antigen changes from 40 AML patients at diagnosis and relapse have been retrospectively analyzed.

2. Material and Methods

Patients diagnosed between September 2002 and November 2016 were included in the study. Bone marrow samples were collected at diagnosis and relapse from 40 patients with de novo (n=34) or secondary (n=6) AML, aged 19 to 77 years. Acute promyelocytic leukemia patients were not included in this study due to the different protocols used in therapy, low probability of relapse and specific genetic features. The diagnosis of AML was established according to morphology and flow cytometry. Survival time was calculated as the time between diagnosis and death or the time between diagnosis and data collection. The study was approved by local ethical committee and is in accordance with the current version of the Helsinki Declaration.

Flow Cytometric Analysis

Bone marrow samples were collected into tubes containing K3EDTA. Phycoerhtyrine (PE) and fluorescein isothiocyanate (FITC) (eBioscience and BD Bioscience, San Jose, California) surface antigens listed in Table 1 were used. Analyses were done according to CD45 SSC gating strategy by Becton Dickinson FACSCalibur device.

Table 1. Surface antigens determined by flow cytometry in bone marrow blasts

PE	FITC
CD10	CD19
CD5	CD20
CD22	CD7
CD33	Anti-HLA DR
CD13	CD15
CD34	CD14
CD64	CD3
CD16-CD56	Anti-MPO
CD79a	TdT

PE: Phycoerhtyrine, FITC: Fluorescein isothiocyanate, CD:Cluster of dirrerentiation antigen, HLA: Human leukocyte antigen, MPO: Myeloperoxidase, TdT: Terminal deoxynucleotide transferase

An antigen is supposed to be positive if it is expressed in ≥ 20% of cells, except for TdT and MPO (≥10%) and CD45 (≥90%) according to the consensus guidelines for immunologic diagnosis of acute leukemia (25).

Statistical Analysis

IBM SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Shapiro Wilk's test was used to evaluate the distribution forms of the variables. Data were summarized as

mean ± standard deviation. Wilcoxon t test was used for comparison. $p<0.05$ was considered as statistically significant.

3. Results

Patient characteristics are listed in Table 2. The amount (%) of CD14 expression was decreased at relapse (p=0.048). CD22 and TdT expressions (%) were increased at relapse (p=0.005 and p=0.005). Although number of CD45, CD19, CD10, CD5, CD33, CD7, anti HLA DR, CD15 and CD 34 positive patients increased; number of CD14 and CD13 positive patients decreased at relapse. Expression of markers in bone marrow blasts as determined by flow cytometry and positivity rates of markers in bone marrow blasts are listed on Table 3 and 4.

Overall, 34 of 40 (85%) cases showed changes (gain and/or loss of antigen) of at least one marker (n=10). Antigen changes were observed in 2 (n=7), 3 (n=6), 4 (n=6), 5 (n=4) or 6 (n=1) antigens in other patients. Antigen changes were found in 16 of 18 antigens (88.9%) totally. Antigen changes between diagnosis and relapse are listed in Table 5. Changes were all gain of antigen in 7 patients, all loss of antigen in 5 patients, both gain and loss of antigen in 11 patients.

Aberrant antigens CD20 and CD22 were not expressed in our patients. CD10 was expressed in 2.5% of patients only at relapse. CD7 was the most common aberrant antigen and more frequent at relapse. Most frequent changes at relapse were observed in CD13 and CD64 (Table 4 and 5).

Table 2. Patient characteristics

Characteristics	
Gender (Male/Female)	15/25
Age at diagnosis (years)	38.52 ±16.55
Interval between AML diagnosis and relapse (months)	14.57 ± 9.2
Interval between AML relapse and death (months)	4.4 ± 5.95
FAB classification	
• **AML M0**	8 (20%)
• **AML M1**	8 (20%)
• **AML M2**	10 (25%)
• **AML M4**	6 (15%)
• **AML M5**	7 (17.5%)
• **AML M6**	1 (2.5%)
White blood cell count at diagnosis ($x10^9$/L)	18.81 ± 31.23
White blood cell count at relapse ($x10^9$/L)	20.21 ± 27.15
Bone marrow blast count at diagnosis (%)	65.89 ± 14.43
Bone marrow blast count at relapse (%)	65.51± 20.18
Survival (months)	24.51± 25.7

AML:Acute myeloid leukemia, FAB: French American British

Table 3. Expression of markers in bone marrow blasts as determined by flow cytometry

Marker	Expression in newly diagnosed AML patients (%)	Expression in relapsed AML patients (%)	p
CD14	9.34 ± 13.47	4.59 ± 8.29	0.048
CD45	95.77 ± 6.75	96.88 ± 4.13	0.896
CD19	6.81 ± 12.78	8.61 ± 15.5	0.377
CD10	1.73 ± 2.69	2.36 ± 3.96	0.183
CD5	6.64 ± 13.06	7.3 ± 14.48	0.304
CD20	2.36 ± 2.73	2.16 ± 2.14	0.851
CD22	2.91 ± 3.28	8.19 ± 15.24	0.005
CD33	76.71 ± 31.91	82.74 ± 25.45	0.420
CD7	16.53 ± 24.71	23.65 ± 28.89	0.126
CD13	53.87 ± 33.74	56.89 ± 32.79	0.823
Anti-HLA DR	60.99 ± 32.8	72.79 ± 27.56	0.066
CD34	38.57 ± 34.3	46.76 ± 34.99	0.098

CD15	21.67 ± 23.94	23.32 ± 5.51	0.362
CD64	46.57 ± 34.78	35.46 ± 31.57	0.230
CD16-56	16.82 ± 29.44	19.08 ± 28.46	0.263
CD3	5.22 ± 7.04	4.63 ± 5.29	0.588
Anti-MPO	42.12 ± 37.07	49.77 ± 35.35	0.091
TdT	1.49 ± 2.34	3.8 ± 6.46	0.005

AML: Acute myeloid leukemia, CD:Cluster of dirrerentiation antigen, HLA: Human leukocyte antigen, MPO: Myeloperoxidase,TdT: Terminal deoxynucleotide transferase

Table 4. Positivity of markers in bone marrow blasts as determined by flow cytometry

Marker	Positivity in newly diagnosed AML patients	Positivity in relapsed AML patients
CD14	18.5% (5/27)	3.4% (1/29)
CD45	85.1% (23/27)	93.1% (27/29)
CD19	10.8% (4/37)	12.5% (5/40)
CD10	0% (0/40)	2.5% (1/40)
CD5	6.9% (2/29)	9.1% (3/33)
CD20	0% (0/35)	0% (0/36)
CD22	0% (0/38)	0% (0/39)
CD33	85% (34/40)	92.5% (37/40)
CD7	23.7% (9/38)	33.3% (13/39)
CD13	76.9% (30/39)	75% (30/40)
Anti-HLA DR	80% (32/40)	90% (36/40)
CD34	50% (20/40)	67.5% (27/40)
CD15	37.5% (12/32)	45.2% (14/31)
CD64	68.4% (26/38)	56.4% (22/39)
CD16-56	21.4% (6/28)	30% (10/30)
CD3	2.8% (1/36)	2.5% (1/40)
Anti-MPO	63.9% (23/36)	83.3% (30/36)
TdT	4.5% (1/22)	11.1% (2/18)

AML: Acute myeloid leukemia, CD:Cluster of dirrerentiation antigen, HLA: Human leukocyte antigen, MPO: Myeloperoxidase, TdT: Terminal deoxynucleotide transferase

Table 5. Antigen changes between diagnosis and relapse

Marker	No change	Loss of antigen	Gain of antigen
CD14	17/27 (63%)	8/27 (29.6%)	2/27 (7.4%)
CD45	27/27 (100%)	0/27 (0%)	0/27 (0%)
CD19	31/37 (83.8%)	3/37 (8.1%)	3/37 (8.1%)
CD10	39/40 (97.5%)	0/40 (0%)	1/40 (2.5%)
CD5	26/29 (89.7%)	1/29 (3.4%)	2/29 (6.9%)
CD20	35/35 (100%)	0/35 (0%)	0/35 (0%)
CD22	36/38 (94.7%)	0/38 (0%)	2/38 (5.3%)
CD33	36/40 (90%)	1/40 (2.5%)	3/40 (7.5%)
CD7	31/38 (81.5%)	2/38 (5.3%)	5/38 (13.2%)
CD13	27/40 (67.5%)	7/40 (17.5%)	6/40 (15%)
Anti-HLA DR	35/40 (87.5%)	1/40 (2.5%)	4/40 (10%)
CD34	34/40 (85%)	1/40 (2.5%)	5/40 (12.5%)
CD15	22/31 (71%)	3/31 (9.7%)	6/31 (19.3%)
CD64	26/38 (68.4%)	8/38 (21%)	4/38 (10.6%)
CD16-56	27/28 (96.4%)	0/28 (0%)	1/28 (3.6%)
CD3	23/26 (88.5%)	2/26 (7.7%)	1/26 (3.8%)
Anti-MPO	27/36 (75%)	2/36 (5.5%)	7/36 (19.5%)
TdT	17/18 (94.4%)	0/18 (0%)	1/18 (5.6%)

AML: Acute myeloid leukemia, CD:Cluster of dirrerentiation antigen, HLA: Human leukocyte antigen, MPO: Myeloperoxidase, TdT: Terminal deoxynucleotide transferase

4. Discussion and Conclusion

Several studies have described shifts in immunophenotype at relapse in AML patients, with different frequencies, probably related to the number of analyzed antigens and their combinations. Most of these shifts involve individual antigens, but the leukemic phenotypes generally remain unaltered during the course of the disease (26-29).

Using 9 panels of 3 antibodies Baer et al (30) examined samples at diagnosis and relapse from 136 patients with AML and showed phenotypic changes in the leukemia cells in 91% of patients. Li et al (26) demonstrated immunophenotype changes in leukemic cells at relapse, compared with diagnosis, in 11/12 (91.7%). Similarly, Voskova et al (25) found a complete change in leukemia-associated immunophenotype (LAIP)s in approximately 20% of AML's, with 80% having at least one LAIP similar to the ones present at diagnosis. Coustan-Smith et al (31) determined the prevalence of expression shifts using paired samples collected at diagnosis and relapse from AML patients for a total of 168 tests. In 146 of the 168 (86.9%) tests, at least one of the selected markers was aberrantly expressed both at diagnosis and at relapse. In an additional 13 (7.7%) tests, markers not present at diagnosis were detected at relapse. In only 9 (5.4%) tests, an aberrantly expressed marker at diagnosis reverted to normal range at relapse. In all 16 patients studied, markers aberrantly expressed at diagnosis in more than 50% of blasts remained abnormally expressed at relapse.

In our study, 34 of 40 (85%) patients showed changes (gain and/or loss of antigen) of at least in one marker (n=10). Antigen changes were observed in 2 (n=7), 3 (n=6), 4 (n=6), 5 (n=4) or 6 (n=1) antigens in other patients. Antigen changes were found in 16 of 18 antigens (88.9%) totally.

The increased expression of CD34, CD33, CD2 and CD7 and decreased expression of CD13, CD14 and CD15 were observed in AML at relapse (30,32). However, no significant differences in the frequency of single antigen expression, such as CD7, CD10, CD13, CD15, CD19, CD33, CD34, CD56, CD117 and HLA-DR, between 48 AML patients at diagnosis and at relapse were detected in another study (33).

In our study, CD22 and TdT expressions were increased and CD14 expression was decreased but the expression levels were under the positivity cut-off. The differences between expressions of other markers at diagnosis and relapse were not found statistically significant Table 3).

CD56 is one of lymphoid antigens that are frequently expressed in AML (21). Similar frequency (20% to 30%) and FAB subtype predominance (M2 and M5) of aberrant expression of CD56 in AML were observed (35,36). CD16-56 positivity was found in 21.4% of patients at diagnosis and 30% at relapse in our study (Table 4).

Li et al (26) showed that CD33, CD117 and HLA-DR were the most stable markers. However, losses and gains of antigens, such as CD11b, CD4 and CD15 were more frequently observed in AML patients at relapse.

We observed that changes were all gain of antigen in 7 patients, all loss of antigen in 5 patients, both gain and loss of antigen in 11 patients. Aberrant antigens CD20 and CD22 were not expressed in our patients. CD10 was expressed in 2.5% of patients only at relapse. CD7 was the most common aberrant antigen and more frequent at relapse.

Using multiparameter flow cytometry (MFC) appropriately requires considerable expertise and experience; analysis and data interpretation have some subjective elements and therefore potential operator-dependent biases (36,37). Since our laboratory and operator has considerable expertise and experience we suggest that the effect of sample processing and instrument settings on our results is minimal. However different immunophenotypic markers selected for analysis can make the interpretation of our results difficult.

Although randomized clinical trials evaluating the value of MRD-testing using different techniques in heterogeneous populations of persons with AML at diverse times during therapy and across different therapies are clearly needed, data from all clinical trials could potentially prove useful if carefully annotated

with details of the performance characteristics of the MRD-test used (38). Therefore, we suggest that the results of our study can add new data for standardization of MRD panels at least for routine practice.

The limitations of our study can be listed as the small number of patients, using a different monoclonal antibody panel compared with some other laboratories and the lack of cytogenetic and molecular markers from all patients. The monoclonal antibody panel was slightly different even in the same patient at relapse. However, these limitations can be encountered in routine practice and give more realistic results compared with clinical trials.

In conclusion, patients with AML demonstrated a high frequency of immunophenotypic shift at relapse. Gross cytogenetic clonal evolution may be a contributing factor but making definitive conclusions according to cytogenetic analysis is not as possible as flow cytometry due to less availability and more technical problems. Further studies are needed about the implication of our findings in the minimal residual disease era.

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