Detection of minimal residual disease in unselected patients with acute myeloid leukemia using multiparameter flow cytometry to define leukemia-associated immunophenotypes and determine their frequencies in normal bone marrow

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Background and Objectives. Detection of minimal residual disease (MRD) by multiparameter flow cytometry is an emerging prognostic factor in patients with acute myeloid leukemia (AML). The present analysis aimed at improving the applicability of this approach to more patients with AML.

Design and Methods. Bone marrow samples from unselected patients with AML at diagnosis and from healthy volunteers were immunophenotyped applying triple-stainings of 31 antigens. Leukemia-associated immunophenotypes were defined by gating on populations displaying an aberrant or infrequent immunophenotype and by applying Boolean algebra. The combination of gates obtained was applied to list mode data files containing measurements of normal bone marrow samples. Dilution experiments of AML samples in normal bone marrow were performed to test the linearity of measurements.

Results. At least one aberrant/infrequent immunophenotype was identified (median, 2; range, 1-5) in all 68 analyzed AML patients. The median frequencies of cells displaying an aberrant/infrequent immunophenotype within normal bone marrow ranged from 0.00% to 1.20% (median, 0.07%). Limiting this analysis to only the most sensitive aberrant/infrequent immunophenotype per patient resulted in frequencies of cells displaying an aberrant/infrequent immunophenotype within normal bone marrow ranging from 0.00% to 0.43% (median, 0.05%). Serial dilution experiments confirmed the linearity of measurements (R>0.90 in all cases analyzed).

Interpretation and Conclusions. The application of multiparameter flow cytometry to identify cells displaying an aberrant/infrequent immunophenotype and to quantify MRD is feasible in unselected patients with AML.

Key words: acute myeloid leukemia, minimal residual disease, leukemia-associated immunophenotypes, multiparameter flow cytometry, immunophenotyping.

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Treatment of patients with newly diagnosed acute myeloid leukemia (AML) has improved over the past decades due to the intensification of induction and post-remission chemotherapies and due to the incorporation of autologous and allogeneic transplantation procedures into the first-line management of the disease. Long-term remissions are, however, achieved in only one quarter of patients.[1,2]

The prognosis of patients with AML can be estimated based on several patient-specific and disease-related factors among which karyotype abnormalities have the most important impact.[3-6] However, only the incorporation into stratification models of treatment-dependent factors, such as the early clearance of leukemic blasts as assessed during aplasia following induction therapy,[3,7,8] and the speed of achievement of complete remission,[9-11] has improved the accuracy of the estimation of the prognosis which is quite heterogeneous when using only pre-treatment factors. The assessment of minimal residual disease (MRD) by molecular and flow cytometric methods is increasingly being used for a reliable quantification of the burden of leukemic cells persisting even after achievement of complete remission. Thus, for the subgroups of patients with leukemia-specific fusion genes such as *PML/RARα*, *CBFβ/MYH11*, or *AML1/ETO*, which comprise 15% of all AML cases,[6] the application of quantitative reverse transcriptase polymerase chain reaction (RT-PCR) methods may allow a highly sensitive and accurate quantification of leukemic cells.[12] A considerably higher proportion of AML patients, i.e. up to 80% of all cases, can be assessed for MRD and can be assigned a specific prognosis by using multiparameter flow cytometry.[13-16] The inclusion of unselected AML cases, however, has not yet been accomplished because leukemia-associated aberrant immunophenotypes have been exclusively used only if strongly disease-associated. The application of multiparameter flow cytometry-based detection of MRD for all AML patients and the expansion of its use in cases with less disease-specific aberrant immunophenotypes basically requires an estimation of the sensitivity of multiparameter flow cytometry as used for this approach. The present study aimed to define at least one aberrant immunophenotype in an unselected cohort of patients with AML and to determine its frequency in normal bone marrow samples.[17]

Design and Methods

AML samples

Fresh bone marrow samples from unselected patients with newly diagnosed and untreated AML were used. Diagnoses were made by cytomorphology, cytochemistry, cytogenetics and molecular genetics in all cases.[18-20]

Normal bone marrow samples

Normal bone marrow was obtained from healthy volunteers and from healthy subjects donating bone marrow for allogeneic transplantations.

Flow cytometry

All studies were performed on bone marrow samples. The samples were processed by a Ficoll-Hypaque gradient to isolate mononuclear cells and the erythrocytes were lyzed.[21]

Applying triple-stainings and isotype controls, monoclonal antibodies against 31 antigens were used in the following combinations designed for diagnostic purposes (conjugated with the fluorochromes FITC, PE, and PC-5, respectively): CD11b/CD117/CD34,CD14/CD13/CD4,CD15/CD34/CD33,CD34/NG2(7.1)/CD33,CD34/CD116/CD33,CD34/CD13/CD19,CD34/CD135/CD117,CD34/CD15/D33,CD34/CD19/CD13,CD34/CD2/CD33,CD34/CD56/CD33,CD36/CD235a/CD45,CD38/CD133/CD34,CD38/CD34/CD90,CD4/CD64/CD45,CD64/CD4/CD4,CD65/CD87/CD34,CD7/CD3/CD34,CD90/CD17/CD34,HLADR/CD33/CD34,MPO/LF/cCD15,TdT/cCD33/cCD45,TdT/cyCD2/cyCD3, and TdT/cyCD79a/cyCD3.

The fluorochromes within two of the combinations of antibodies (CD34/CD13/CD19, CD64/CD4/CD45) were changed to optimize their performance. All antibodies were purchased from Immunotech (Marseilles, France), except for those against CD15, CCD15 and CD64 (Medarex, Annandale, NJ, USA); CD133 (Milteny Biotech, Bergisch Gladbach, Germany); and MP0 and LF (Caltag, Burlingame, CA, USA). The respective combinations of antibodies were added to 10^6 mononuclear cells (volume, 100 mL) and incubated for ten minutes. After addition of 2 mL lysing solution (ammonium chloride-based; prepared at a local pharmaceutical institute) the samples were incubated for another 10 minutes and were then washed twice in phosphate-buffered saline (PBS) and resuspended in 0.5 mL PBS. Multiparameter flow cytometry analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San José, CA, USA). For AML samples 20,000 events were acquired, whereas for normal bone marrow samples 250,000 events were acquired which is the planned number for acquisition of MRD samples. Live-gating was not applied. Analysis of list-mode files was performed using CellQuest software (Becton Dickinson).

Gating strategy

Leukemia-associated immunophenotypes (LAIP) were defined by gating on populations displaying an aberrant expression of surface or cytoplasmic antigens and by applying Boolean algebra. An example is given in Figure 1. LAIP were grouped into 1) cross-lineage antigen expression; 2) asynchronous antigen expression; 3) antigen overexpression; and 4) lack of antigen expression. The combination of gates obtained by this strategy was applied to the list mode files containing the data from normal bone marrow samples which had been collected using the same combinations of antibodies. Some of the combinations of antibodies offered the possibility to define basically different LAIP in different patients, e.g. HLA-DR$^-$CD33$^+$CD34$^+$ and HLA-DR^{++}CD33^{++} CD34$^+$. It must be stressed that in most cases there are several leukemic populations within one AML sample and that it is not possible in these cases to include all leukemic cells into one LAIP. Therefore, the frequencies of LAIP-positive cells are lower than described in other diseases, such as acute lymphoblastic leukemia. The frequencies of cells within the normal bone marrow samples carrying the respective LAIP were determined for each individual LAIP as defined in cases with AML. In order to estimate the degree of reduction of the leukemic cell mass which is detectable by the present approach for each individual LAIP, we calculated the log difference *frequency in leukemic bone marrow/median frequency of LAIP in normal bone marrow*. For example, the log difference would have been 3.00 in a case in which LAIP-positive cells formed 50% of the leukemic bone marrow cells and a median of 0.05% of normal bone marrow cells. In case of a median frequency of 0.00% of LAIP-positive cells in normal bone marrow this frequency was set at 0.004% in order to allow the calculation of the log difference (0.004% is the highest frequency displayed as 0.00% and was chosen as the worst case possible). If more than one LAIP was defined in a single patient the most sensitive LAIP was selected, on the basis of the maximum log difference in comparison to the other LAIP in the same patient, for the subsequent evaluations.

Dilution experiments

In order to define the range in which the MRD measurements by the present approach are linear, serial dilution experiments in normal bone marrow were performed for selected AML samples. The selection was based on the availability of an AML sample and a sample of normal bone marrow on the same day. AML samples were diluted in normal bone marrow at concentrations of 10%, 1%, 0.1%, 0.01%, and 0.001%. Dilutions were performed before Ficoll-hypaque separation. The frequencies of cells carrying the respective LAIP were deter-

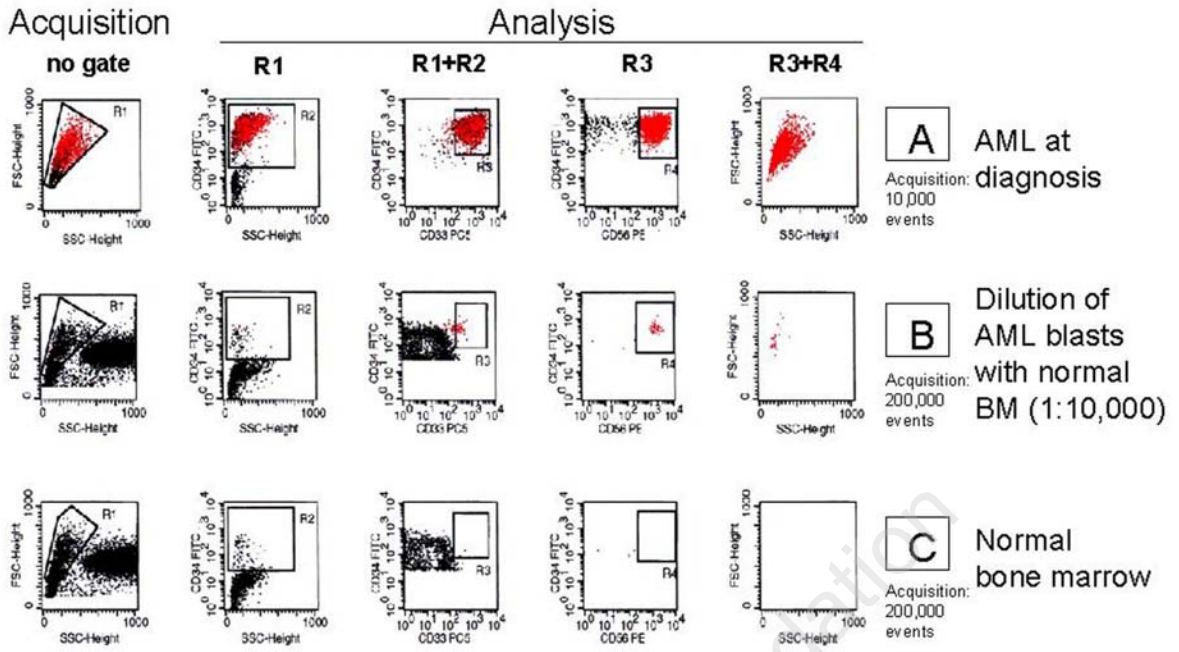

Figure 1. Gating strategy. The gating strategy used in the present study. Gates were applied on AML populations as displayed in dot plots showing distributions of light scatter properties and antigen expressions, respectively. The gates were combined using Boolean algebra. Application of the identical gating strategy is shown for the AML sample (A), for the AML sample diluted in normal bone marrow (B), and for a sample of normal bone marrow (C).

mined by the gating strategies described above. The coefficient of correlation and the *p* value were calculated by Spearman's rank correlation using SPSS 11.0.1.

Study conduct

Prior to therapy all patients gave their informed consent to participation in the current evaluation after the purpose and investigational nature of the study as well as of potential risks had been explained to them. The study design adhered to the declaration of Helsinki and was approved by the ethics committees of the participating institutions prior to its initiation.

Results

Patients and normal bone marrow

Sixty-eight patients with newly diagnosed and untreated AML were analyzed for the expression of an aberrant immunophenotype by flow cytometry, applying the panel of monoclonal antibodies described above. The data on FAB subtypes and cytogenetics are shown in Table 1. Normal bone marrow samples were obtained from 26 healthy volunteers and healthy donors.

Identification of leukemia-associated immunophenotypes

Application of the extensive panel of antibodies described above resulted in the identification of 140 leukemia-associated immunophenotypes within the 68 analyzed patients (Table 2). Only one aberrant immunophenotype was identified in 23 patients, but in the other 45 patients 2 to 5 aberrant immunophenotypes were identified (2 LAIP: n=24; 3 LAIP: n=16; 4 LAIP: n=3; 5 LAIP: n=5; median number of LAIP per patient: 2). Most of the identified aberrant immunophenotypes were defined as overexpression of antigens (n=46) while asynchronous expression of progenitor cell markers and differentiation markers was present in only 20 cases (Table 2). The number of aberrant immunophenotypes identified by the application of the various combinations of antibodies is detailed in Table 3.

Given the immunophenotypic heterogeneity of AML cells within individual cases, the gating on cells displaying an aberrant immunophenotype covers only a part of the cells. The percentage of cells within the AML samples carrying the aberrant immunophenotype under consideration was assessed in each case and found to range from 10.13% to 76.14% (median, 25.10%). If only the

Table 1. FAB subtypes and karyotypes of 68 patients with AML.

FAB subtype	N.
M1	13
M2	27
M3	6
M3v	5
M4	7
M4eo	7
M5a	2
M6	1

Karyotype	N.
normal	21
t(8;21)	5
inv(16)	7
t(15;17)	11
t(9;11)	2
complex aberrations	7
-7/7q-	2
+8 alone	4
other	9

most sensitive aberrant immunophenotype per patient was considered, i.e. the one yielding the highest log difference *frequency in leukemic bone marrow/median frequency of LAIP in normal bone marrow*, the percentage of cells in the leukemic bone marrow displaying the aberrant immunophenotype ranged from 10.13% to 76.14% (median, 25.81%). The distributions of these frequencies among the different classes of aberrant immunophenotypes as well as among the respective combinations of antibodies are shown in Table 3.

Normal bone marrow cells carrying a leukemia-associated immunophenotype

Twenty-six samples of normal bone marrow were analyzed by the panel of antibody combinations described above. The frequencies of cells within these normal bone marrow samples carrying the respective LAIP were quantified using the gating strategy described above. The median number of normal bone marrow samples analyzed for each LAIP was 24 (range, 11 to 26; total number of analyses, n=2863). The median percentage of LAIP-positive cells within the normal bone marrow samples was calculated for each of the 140 LAIP. This median percentage ranged from 0.00% to 1.20% (median, 0.07%). Restricting these analyses to the most sensitive LAIP in each patient, resulted in a range from 0.00% to 0.43% for the median percentage of LAIP-positive cells in the normal bone marrow (median, 0.05%). The distributions of these frequencies among the different classes of LAIP as well as among the respective combinations of antibodies are shown in Table 3.

Quantification of differences in cells carrying aberrant immunophenotypes between AML samples and normal bone marrow

To estimate the magnitude of the reduction in leukemic cell mass that is detectable by the present approach the log difference *frequency in leukemic bone marrow/median frequency of LAIP in normal bone marrow* was calculated for each LAIP. The median of this difference was 2.47 and its range from 0.99 to 4.23. Restricting these analyses to the most sensitive aberrant immunophenotype in each patient gave a range from 1.58 to 4.23 for the log difference *median frequency of LAIP in normal bone marrow/frequency in leukemic bone marrow* (median, 2.82). The distributions of these log differences among the different classes of LAIP as well as among the respective combinations of antibodies are shown in Table 3.

Dilution of AML samples in normal bone marrow

To assess the linearity of quantification of MRD using the present approach, serial dilution experiments were performed in four cases of AML. The aberrant immunophenotypes displayed were overexpression of NG2/7.1 (n=2), cross-lineage coexpression of CD56 together with expression of CD33 and CD34 (n=1), and lack of expression of HLA-DR with positivity for CD33 and CD34 (n=1). Dilutions covering a range from 50% to 0.01% resulted in coefficients of correlation (CC) of 1.00 ($p<0.0005$) in all four cases. Further dilutions down to 0.0001% resulted in a CC of 0.91 ($p=0.002$) in one case with overexpression of NG2/7.1 and in a CC of 1.00 ($p<0.0005$) in the other three cases.

Evaluation of MRD during CR

The impact of the log difference, determined before consolidation therapy, on event-free survival, was analyzed in 18 patients who were treated uniformly according to the German AML Cooperative Group 1999 trial and for whom data on clinical follow-up were available (Figure 2). The data indicate that patients with a log difference lower than the median value determined for these 18 patients (2.38) had a clearly although not statistically significantly worse outcome than the patients with a higher log difference.

Discussion

Precise evaluation of response to therapy and of MRD in complete remission is becoming increasingly important for the management of patients with AML.[73,9-11] Early assessment of residual blasts during aplasia following induction therapy as well as MRD levels during remission after both induction and consolidation therapy have been shown be of major

Table 2. Descriptions of LAIPs.

LAIP (FITC/PE/PC5)	n	% pos. cells in AML bone marrow	% positive cells in normal bone marrow median	min	max	no. of analyzed normal BM samples	log diff. % AML / % normal BM
CD11b+CD117+CD34-	9	12.72-42.52	0.01-0.25	0.00-0.06	0.11-0.67	21	1.96-3.41
CD11b-CD117++CD34+	1	15.55	0.33	0.01	0.81	21	1.68
CD14++CD13+CD4(+)	1	20.37	0.13	0.03	0.53	14	2.20
CD14+CD13-CD4+	1	10.89	0.07	0.00	0.48	14	2.19
CD14-CD13+CD4+	3	39.47-49.41	0.26-0.47	0.03-0.11	0.48-0.92	14	2.03-2.19
CD15+CD34++CD33+	1	19.01	0.09	0.00	0.47	11	2.32
CD34(+)CD56+CD33+	1	76.14	0.02	0.00	0.17	26	3.58
CD34++CD13+CD19-	5	21.19-53.31	0.02-0.52	0.00-0.15	0.31-1.48	11	2.01-3.03
CD34++CD15+CD33+	1	23.70	0.02	0.00	0.09	15	3.07
CD34++CD2-CD33++	1	27.43	0.04	0.00	0.14	25	2.84
CD34++CD56-CD33++	1	34.78	0.34	0.08	0.83	26	2.01
CD34+7.1+CD33+	1	27.34	0.14	0.00	0.32	11	2.29
CD34+CD116+CD33+	4	11.52-27.71	0.06-0.15	0.00-0.01	0.18-2.05	24	2.13-2.66
CD34+CD13+CD19+	7	10.23-44.27	0.00-0.12	0.00-0.02	0.00-0.74	11	2.19-3.43
CD34+CD135++CD117++	5	14.46-44.70	0.08-0.18	0.00	0.32-0.86	25	2.06-2.78
CD34+CD15+CD33+	3	10.22-16.86	0.03-0.36	0.00-0.04	0.06-0.68	15	1.68-2.67
CD34+CD19+CD13+	1	10.58	0.01	0.00	0.13	15	3.02
CD34+CD2+CD33+	8	13.85-63.45	0.04-0.35	0.00-0.07	0.27-1.18	25	2.06-2.72
CD34+CD56+CD33+	10	12.49-73.22	0.00-0.43	0.00-0.13	0.02-3.16	26	1.60-3.96
CD34-7.1+CD33+	1	10.44	0.01	0.00	0.11	11	3.02
CD34-CD135(+)CD117+	1	49.84	0.04	0.00	0.18	25	3.10
CD34-CD135+CD117+	4	14.93-44.67	0.02-0.30	0.00-0.03	0.10-1.92	25	2.10-2.94
CD34-CD15++CD33+	1	25.75	0.26	0.07	0.71	15	2.00
CD34-CD56+CD33+	3	11.15-25.99	0.05-0.13	0.01-0.03	0.68-1.63	26	1.95-2.69
CD36++CD235a++CD45(+)	2	14.62/16.20	0.04/0.12	0.01/0.02	0.21/0.61	11	2.09/2.61
CD38++CD133++CD34+	1	20.42	0.10	0.01	0.51	11	2.31
CD38++CD34++CD90-	6	12.18-62.95	0.01-0.32	0.00-0.02	0.25-1.30	12	2.28-3.80
CD38++CD34++CD90(+)	1	33.45	0.04	0.00	0.86	12	2.92
CD38+CD34++CD90-	1	36.90	0.12	0.04	0.58	12	2.49
CD38+CD34-CD90++	1	42.34	0.37	0.08	1.85	12	2.06
CD38-CD133++CD34+	1	11.52	1.19	0.20	3.52	11	0.99
CD4++CD64++CD45++	2	20.05-37.21	0.06-0.42	0.00-0.05	3.64-3.89	12	1.68-2.79
CD64++CD4++CD45++	1	23.64	0.08	0.00	0.78	14	2.47
CD64++CD4+CD45++	2	18.26/30.84	0.12/0.29	0.00/0.02	2.17/2.81	14	2.03/2.18
CD64++CD4-CD45++	1	24.92	0.05	0.02	0.23	14	2.70
CD65+CD87++CD34-	1	10.62	0.04	0.00	0.55	26	2.42
CD65+CD87++CD34+	1	25.28	0.08	0.00	0.66	26	2.50
CD65+CD87-CD34++	1	28.22	0.11	0.01	0.47	26	2.43
CD7+CD33+CD34-	1	15.60	0.09	0.02	0.36	26	2.24
CD7+CD33+CD34+	4	10.67-25.80	0.02-0.09	0.00-0.01	0.14-0.59	26	2.25-3.11
CD90(+)CD117++CD34+	1	22.04	1.20	0.20	1.95	11	1.26
HLA-DR++CD33+CD34+	3	11.88-23.47	0.07-0.21	0.00-0.02	0.40-1.68	26	1.85-2.23
HLA-DR++CD33+CD34++	1	40.94	0.03	0.00	0.79	26	3.14
HLA-DR+CD33-CD34+	2	17.61/52.55	0.05/0.28	0.01/0.12	0.37/1.04	26	2.27/2.55
HLA-DR+CD33-CD34++	1	25.87	0.03	0.00	0.56	26	2.94
HLA-DR-CD33+CD34-	7	14.72-68.42	0.00-0.26	0.00-0.06	0.06-2.22	26	2.20-4.23
HLA-DR-CD33+CD34+	11	14.51-51.18	0.00-0.08	0.00-0.02	0.03-0.95	26	2.23-4.02
HLA-DR-CD33-CD34+	1	34.30	0.01	0.00	0.06	26	3.54
MPO+LF-cCD15-	8	10.13-55.62	0.00-0.54	0.00-0.05	0.07-5.85	22	1.39-3.57
TdT+cCD33++cCD45++	4	19.75-45.80	0.01-0.07	0.00	0.35-7.06	16	2.45-3.66

prognostic relevance and to add significantly to the information available from pre-treatment parameters in these patients. As a consequence, methods for quantifying the leukemic cell mass are needed that can be applied to the total AML population under study. While PCR-based approaches may be used for about half of all cases with AML,[18] multiparameteter flow cytometry has been shown to cover up to 80%.[13-16] The present study aimed to extend the use of multiparameter flow cytometry to unselected patients with AML and to estimate the sensitivity of MRD quantification achievable by this approach.

Table 3. Frequencies of LAIP-positive cells in normal bone marrow.

	number of LAIPs	*% positive cells in AML bone marrow*	*% positive cells in normal bone marrow*			*number of analyzed normal bone marrow samples*	*log diff. % AML / % normal BM*
			median	*min*	*max*		
Total	140	25.10 (10.13-76.14)	0.07 (0.00-1.20)	0.00 (0.00-0.20)	0.50 (0.00-7.06)	24 (11-26)	2.47 (0.99-4.23)
1 LAIP only	68	25.81 (10.13-76.14)	0.05 (0.00-0.43)	0.00 (0.00-0.13)	0.32 (0.00-7.06)	25 (11-26)	2.82 (1.58-4.23)
asynchronous	20	22.37 (10.22-42.52)	0.07 (0.01-0.34)	0.01 (0.00-0.06)	0.29 (0.05-2.05)	21 (11-25)	2.45 (1.68-3.41)
cross-lineage	37	21.02 (10.23-76.14)	0.05 (0.00-0.43)	0.00 (0.00-0.13)	0.27 (0.00-3.16)	26 (11-26)	2.49 (1.60-3.96)
lack of expression	37	31.82 (10.13-68.42)	0.07 (0.00-0.54)	0.01 (0.00-0.12)	0.44 (0.03-5.85)	26 (12-26)	2.53 (1.39-4.23)
overexpression	46	25.52 (10.44-62.95)	0.09 (0.01-1.20)	0.00 (0.00-0.20)	0.69 (0.11-7.06)	14 (11-26)	2.43 (0.99-3.80)
CD11b/CD117/CD34	10	26.45 (12.72-42.52)	0.08 (0.01-0.33)	0.01 (0.00-0.06)	0.33 (0.05-0.81)	21	2.51 (1.68-3.41)
CD14/CD13/CD4	5	39.47 (10.89-49.41)	0.26 (0.07-0.47)	0.03 (0.00-0.11)	0.53 (0.48-0.92)	14	2.19 (2.03-2.20)
CD15/CD34/CD33	1	19.01	0.09	0.00	0.47	11	2.32
CD34/7.1/CD33	2	10.44; 27.34	0.01; 0.14	0.00; 0.00	0.11; 0.32	11	3.02; 2.29
CD34/CD116/CD33	4	23.47 (11.52-27.71)	0.08 (0.06-0.15)	0.01 (0.00-0.01)	0.39 (0.18-2.05)	24	2.37 (2.13-2.66)
CD34/CD13/CD19	12	34.77 (10.23-53.31)	0.04 (0.00-0.52)	0.00 (0.00-0.15)	0.29 (0.00-1.48)	11	2.91 (2.01-3.43)
CD34/CD135/CD117	10	27.75 (14.46-49.84)	0.08 (0.02-0.30)	0.00 (0.00-0.03)	0.44 (0.10-1.92)	25	2.43 (2.06-3.10)
CD34/CD15/CD33	5	16.86 (10.22-25.75)	0.06 (0.02-0.36)	0.00 (0.00-0.07)	0.09 (0.06-0.71)	15	2.23 (1.68-3.07)
CD34/CD19/CD13	1	10.58	0.01	0.00	0.13	15	3.02
CD34/CD2/CD33	9	24.78 (13.85-63.45)	0.10 (0.04-0.35)	0.01 (0.00-0.07)	0.55 (0.14-1.18)	25	2.46 (2.06-2.84)
CD34/CD56/CD33	15	22.91 (11.15-76.14)	0.02 (0.00-0.43)	0.00 (0.00-0.13)	0.21 (0.02-3.16)	26	3.10 (1.60-3.96)
CD36/CD235a/CD45	2	14.62; 16.20	0.04; 0.12	0.01; 0.02	0.21; 0.61	11	2.09; 2.61
CD38/CD133/CD34	2	11.52; 20.42	0.10; 1.19	0.01; 0.20	0.51; 3.52	11	0.99; 2.31
CD38/CD34/CD90	9	42.34 (12.18-62.95)	0.12 (0.01-0.37)	0.01 (0.00-0.08)	0.75 (0.25-1.85)	12	2.49 (1.58-3.80)
CD4/CD64/CD45	2	20.05; 37.21	0.06; 0.42	0.00; 0.05	3.64; 3.89	12	2.79; 1.68
CD64/CD4/CD45	4	24.28 (18.26-30.84)	0.10 (0.05-0.29)	0.01 (0.00-0.02)	1.48 (0.23-2.81)	14	2.33 (2.03-2.70)
CD65/CD87/CD34	3	25.28 (10.62-28.22)	0.08 (0.04-0.11)	0.00 (0.00-0.01)	0.55 (0.47-0.66)	26	2.43 (2.42-2.50)
CD7/CD33/CD34	5	18.50 (10.67-25.80)	0.06 (0.02-0.09)	0.00 (0.00-0.02)	0.26 (0.14-0.59)	26	2.35 (2.24-3.11)
CD90/CD117/CD34	1	22.04	1.20	0.20	1.95	11	1.26
HLA-DR/CD33/CD34	26	31.41 (11.88-68.42)	0.06 (0.00-0.28)	0.00 (0.00-0.12)	0.45 (0.03-2.22)	26	2.65 (1.85-4.23)
MPO/LF/cCD15	8	14.20 (10.13-55.62)	0.19 (0.00-0.54)	0.01 (0.00-0.05)	2.16 (0.07-5.85)	22	1.95 (1.39-3.57)
TdT/cCD33/cCD45	4	27.89 (19.75-45.80)	0.02 (0.01-0.07)	0.00 (0.00-0.00)	2.53 (0.35-7.06)	16	3.28 (2.45-3.66)

The present data demonstrate that at least one leukemia-associated immunophenotype could be defined in each of the 68 unselected AML patients analyzed. However, in some cases the aberrant immunophenotype identified is present in 0.5% of normal bone marrow cells: this limits the applicability of the approach in those cases to a lower sensitivity than the ones for highly aberrant immunophenotypes which may be as great as 0.01%. Furthermore, it is important to recognize that when applying this approach not all AML cells are included in the respective populations as defined by the individual aberrant immunophenotype. In fact, only those subpopulations that display a phenotype different from the one of populations in normal bone marrow are covered. As a consequence, the median number of cells displaying the aberrant immunophenoytpe within the leukemic bone marrow at diagnosis is 25.10%.

To clarify the clinical utility of this approach the log difference *frequency in leukemic bone marrow/median frequency of LAIP in normal bone marrow* was calculated (median, 2.47; range, 0.99 to 4.23). This value corresponds to the therapy-induced reduction in leukemic cell mass that can be quantified. The obtained log differences are even higher if only the most sensitive LAIP per patient are considered (median, 2.82; range, 1.58 to 4.23). This approach represents a substantial improvement since the values obtained for this parameter are clearly higher than those obtained for the standard method currently used to quantify the reduction of leukemic cell mass, i.e. cytomorphology, for which the maximum log difference is 1.30 (100% blasts in leukemic bone marrow/5% blasts in remission bone marrow) and the estimated median log difference is 1.00 (50% blasts in leukemic bone marrow/5% blasts in remission bone marrow).

The antibody combinations most frequently identifying an aberrant immunophenotype were HLA-DR/CD33/CD34 and those detecting a cross-lineage expression of lymphoid markers, as has already been demonstrated[22] (Table 3). The validity of MRD quantification using this approach was ensured by the serial dilution experiments which

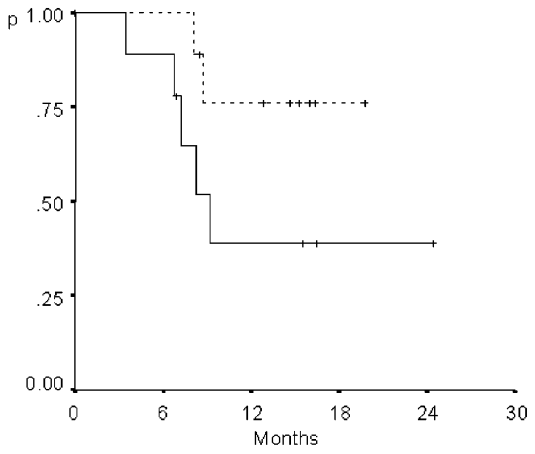

Figure 2. Impact of log difference on event-free survival The event-free survival is shown for patients separated according to the median of the log difference determined before consolidation therapy (greater than median shown as solid line (n=9), lower than median shown as dashed line (n=9); *p*=0.1009).

showed a high accuracy of the applied method for the range between 50% to 80% and 0.01% LAIP-positive cells.

These data are in line with those in previous reports indicating a high validity and accuracy of MRD quantification by multiparameter flow cytometry.[23-26] However, in contrast to the present data, previous studies focused on selected cases with only AML, i.e. those displaying an aberrant immunophenotype in the majority of cells at diagnosis, and, furthermore, excluded patients not achieving a complete remission.[13-15] Cases with infrequent immunophenotypes which were included in the present analysis were not considered in prior studies. Thus, only 54% to 60% of all cases initially immunophenotyped were included into the analyses of the prognostic significance of MRD quantification. The significance of the present results is further emphasized by the fact that the sensitivity of MRD-quantification determined for this unselected AML population (0.05%) is in the range and even substantially lower than the limits having been shown to be of major prognostic value, i.e. 0.2% to 0.5%,[14] 0.035% to 0.045%,[15] and 0.01% to 1%.[13] Indeed, the median frequencies of LAIP-positive cells in the patients in the current analyses were 0.34% during aplasia following induction therapy and 0.04% after achievement of complete remission (*data not shown*), further validating the feasibility of the present approach since these are within the range and even substantially higher than the respective frequencies determined in normal bone marrow. The present analyses are based on samples processed by Ficoll-hypaque separation and lysis. Thus, the populations in which the aberrant immunophenotypes were defined represent only the immature parts of the bone marrow samples, since the more mature cells have been eliminated. This is in contrast to other published studies and again adds value to the present results which indicate that MRD can be quantified in all patients with AML. The preliminary analyses on the prognostic impact of MRD levels determined using the present approach support this concept, although a larger number of patients must be analyzed in order to draw firm conclusions. In any case, the most effective way of cell processing for MRD analyses has not yet been determined.

In conclusion, extending multiparameter flow cytometry analyses of MRD levels from 50% to 100% of an unselected cohort of patients with AML has been shown to be feasible and results in a high sensitivity that is expected to allow valid quantification of MRD both during aplasia following induction therapy and after achievement of complete remission. It may thus represent a tool providing important prognostic information in patients with AML.

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Pre-publication Report & Outcomes of Peer Review

Contributions

WK: principal investigator; SS: contribution to conducting the work and interpreting results; CS: contribution to conducting the work and interpreting results; H-JK: contribution to conducting the work; WH: contribution to interpreting results; TH: contribution to conducting the work and interpreting results. All authors in addition contributed to the design of the study and the revision of the manuscript. The auhors thank Karin Hecht, Rita Lapping, and Eva Goecke for their excellent technical assistance. Primary responsibility for the publication and for each Table and Figure: WK.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Alberto Orfao, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Orfao and the Editors. Manuscript received December 13, 2002; accepted May 7, 2003.

In the following paragraphs, Professor Orfao summarizes the peer-review process and its outcomes.

What is already known on this topic

Previous reports have shown that most AML patients display aberrant phenotypes that can be used to follow minimal residual disease (MRD) levels after therapy. However a substantial proportion of cases (around 15-20%) remain described as showing normal phenotypes. Immunophenotypic evaluation of MRD levels at specific time points early after achieving morphologic complete remission is of great value for predicting relapse in AML.

What this study adds

In this study a strategy based on the identification of leukemia-associated phenotypes either absent or present at low frequencies in normal bone marrow is applied to extend identification of informative phenotypes to all AML patients at diagnosis. Although the follow-up data presented should be considered as preliminary, they indicate that enumeration of bone marrow cells carrying these aberrant/infrequent phenotypes after therapy also has a prognostic impact for predicting disease-free survival.