Combined analysis of bcl-2 and MDR1 proteins in 256 cases of acute myeloid leukemia

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A B S T R A C T

Background and Objectives. The objective of this study was to investigate the expression of MDR1 and bcl-2 proteins in *de novo* acute myeloid leukemia (AML).

Design and Methods. The expression of MDR1 and bcl-2 was analyzed by flow cytometry in a large series of 256 consecutive cases of AML. The results were recorded as percentage of positivity and relative mean fluorescence intensity (rMFI). To determine individual protein levels, an index which equals the product of the percentage of positive cells and rMFI was generated.

Results. Using cut-offs of ≥800 and 300 of the index value for bcl-2 and MDR1 expression, respectively, we identified 4 different classes of AML: 1) double negative; 2) single positive bcl-2$^+$/MDR1$^-$; 3) single positive bcl-2$^-$/MDR1$^+$; 4) double positive. The highest incidence of double negative cases was observed in the M2 class whereas double positive cases occurred more frequently in the M4, M5 and M6 subgroups. Seventy-eight percent and 71% of M0 and M1, respectively, showed single positive bcl-2$^+$/MDR1$^-$ expression (p = 0.00001). Twenty-eight percent of patients belonging to the double positive category achieved complete remission, whereas for double negative, single positive bcl-2$^+$MDR1$^-$ and single positive bcl-2$^-$/MDR1$^+$ category, the complete remission rate was 69%, 52% and 56%, respectively (p = 0.00038). In multivariate analysis, the double positive status independently affected frequency of complete remission (p = 0.008).

Interpretation and Conclusions. Bcl-2 is over-expressed in CD34$^+$ AML; conversely, MDR1 is over-expressed in CD34$^-$ AML. However, the combined expression of the two proteins defines a subset of AML with a very poor prognosis.

Key words: AML, bcl-2, MRD1.

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Clinical drug resistance is the primary cause of treatment failure in acute myeloid leukemia (AML) and it is generally recognized as being conferred by various mechanisms, including cellular drug resistance, leukemia regrowth and drug pharmacokinetics.[1-4] One of the mechanisms, the first to be discovered, leading to cellular drug resistance of AML is mediated by P-glycoprotein (permeability-glycoprotein) also called P-gp or MDR1 (multi drug resistance-1) protein.[5-8] However, in recent years it has become increasingly clear that P-gp/MDR1 represents only one of an expanding family of mechanisms with a potential role in cellular drug resistance.

In fact, non-P-gp mechanisms of chemoresistance have been demonstrated in AML; mechanisms associated with altered apoptotic response were among the first to be identified.[9-10] The mitochondrial-mediated pathway of apoptosis is regulated by the bcl-2 family of anti-apoptotic (bcl-2, bcl-xl, mcl-1) and pro-apoptotic proteins (bax, bad, and bak). Recently, bcl-2 overexpression has been reported to be associated with drug resistance in hematologic malignancies,[9,11] whereas high bax levels were declared to be a favorable prognosticator in AML.[12] Furthermore, evidence has been published that the bax/bcl-2 ratio may be an additional and significant prognostic parameter in AML.[10] Based on these premises, the aim of the present study was to investigate, by flow cytometry, the expression of MDR1 and bcl-2 proteins in a large series of 256 consecutive cases of AML.

Design and Methods

Patients

Starting from 1993, the immunophenotypic profiles of 256 consecutive cases of *de novo* AML were studied (Table 1); the diagnosis of AML was made according to the FAB criteria.[13,14] The expression of lymphoid markers did not necessarily rule out the diagno-

sis of AML M0, provided the cells were negative for cytoplasmic CD3, CD22, CD79a and an EGIL score[15] for biphenotypic leukemia was < 2.5. Due to its unique clinico-biological features and the specific therapy used, cases of acute promyelocytic leukemia were excluded from the analysis. Two hundred and forty out of the 256 patients were eligible for intensive chemotherapy. Patients aged 18-60 years were treated according to the EORTC/GIMEMA AML-10 and AML-12 protocols;[16] these are regimens based on three drugs associating cytosine arabinoside, etoposide and an anthracycline (daunorubicin, idarubicin or mitoxantrone) as induction therapy.

Patients with an HLA compatible sibling were given allografts, whereas the others underwent autologous stem cell transplantation. Patients older than 60 years were enrolled into the AML-13 EORTC/GIMEMA[17] randomized trial. The patients were to receive mitoxantrone, etoposide and cytosine arabinoside as induction therapy. Upon achievement of complete remission (CR) they were to be randomly assigned to an oral (idarubicin, etoposide and subcutaneous cytosine arabinoside) or intravenous (idarubicin, etoposide and cytosine arabinoside) consolidation program. CR and relapse were defined according standardized criteria.[18]

Immunophenotyping

The expression of CD34 (HPCA-2 Becton-Dickinson, Mountain View, CA, USA), CD71 (CD71 FITC, Becton Dickinson, Mountain View CA), bcl-2 (bcl-2 FITC, DAKO, Glostrup, Denmark) and MDR1 (MRK16 FITC, Immunotech, Marseille, France) antigens was investigated on fresh bone marrow (BM) samples taken at diagnosis; all samples contained at least 80% blasts. MRK16, which reacts with an extracellular epitope of the human 170-kd P-glycoprotein, CD34 and CD71 antigens were tested as described previously.[19-21] In order to assay bcl-2 expression, the cells were first incubated with CD33 (CD33 PE Becton Dickinson, Mountain View, CA, USA) or CD34 allophycocyanin-conjugated monoclonal antibodies for 15 min at room temperature in the dark. After two washings in phosphate-buffered solution (PBS), the cells were fixed and permeabilized using a commercially available kit (Fix & Perm Permeabilization kit, Caltag, Burlingame, CA, USA) and then incubated for 15 min at room temperature in the dark with 10 μL of anti-bcl-2. After two additional washings in PBS the samples were analyzed.[22]

Flow cytometry analysis

The samples were run on a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA), equipped with an argon laser emitting at 488 nm. A minimum of 10,000 events, within a side scatter versus forward light scatter blast gate, were acquired in a list mode file format and analyzed with CELLQuest software. For each marker under study, the results were recorded as percentage of positivity and relative mean fluorescence intensity (rMFI). The percentage of positivity was determined by comparing the fluorescence distribution histogram of positively stained cells to that of cells stained with the appropriate isotype control; rMFI was calculated as the ratio of sample mean channel/control mean channel.[10] Finally, to determine individual protein levels, we designed an index for each antigen. This index equals the product of the percentage of positive cells and rMFI. The threshold above which AML cases were qualified as having an *apoptotic-related* phenotype was established at 800 for the bcl-2 index; BM samples from 11 normal donors were also used as controls. In these samples, the bcl-2 index was evaluated both on purified $CD34^+$ cells and differentiated myeloid cells, its median value being 370 (±63) and 150 (±17), respectively. Similarly, a MDR1 index value ≥ 300 distinguished AML cases with an *MDR1-related* phenotype. The threshold of 800 and 300 for the bcl-2 and MDR1 index, respectively, was arbitrary; it was identified after testing several cut-off points and

Table 1. Clinical characteristics of the series of patients.

	Age ≤ 60 yrs	*Age > 60 yrs*	*Total*
N. (%)	117 (46%)	139 (54%)	256
Treated with chemotherapy (n.)	117	123	240
Age (years)			
Median	44	69	62.5
Range	(18-60)	(61-84)	18-84
Sex n, (%)			
Male	62 (53)	78 (56)	140 (55)
Female	55 (47)	61 (44)	116 (45)
WBC ($\times 10^9$/L)			
Median	24.2	13.5	16.7
Range	1.2 - 390	0.7-256	0.7 – 390
FAB n, (%)			
M0	11 (9)	17 (13)	28 (11)
M1	29 (25)	26 (19)	55 (22)
M2	22 (19)	38 (27)	60 (23)
M4	20 (17)	24 (17)	44 (17)
M5	31 (27)	27 (19)	58 (23)
M6	4 (3)	7 (5)	11 (4)
Cytogenetics# n, (%)			
Good	15 (15)	0 (0)	15 (8)
Intermediate	51 (51)	51(59)	102 (55)
Poor	34 (34)	36 (41)	70 (37)
Total, n.	100	87	187

#available in 187 of 256 patients.

Table 2. The distribution of Bcl-2, MDR1, CD34 and CD71 expression within the different FAB classes. The values given are those of the index, which equals the product of percentage of positive cells and relative mean fluorescence intensity.

	M0	*M1*	*M2*	*M4*	*M5*	*M6*	*P*
Bcl-2 index							
median	1732	1437	459	852	874	1606	0.00001
range	241-10025	146-7604	1-5207	3-8920	2-4603	290-3616	
MDR1 index							
median	23	98.5	114	397	851	60	0.0033
range	1-1035	1-4939	1-2133	3-3920	6-4975	14-463	
CD34 index							
median	6076	1649	340	1	1	1	0.00001
range	1-95100	1-38200	1-26496	1-23661	1-9462	1-8480	
CD71 index							
median	80.5	220.7	178.4	470.5	638.1	404.6	0.00001
range	6-1923	1-5975	11-1699	87-3142	16-4512	155-7630	

the selected ones were those which correlated significantly with clinical outcome. Based on these observations we grouped our AML cases into 4 classes as follows: 1) [bcl-2 index <800, MDR1 index <300] (double negative); 2) [bcl-2 index >800, MDR1 index <300] (single positive$^{bcl-2/+\ MDR1-}$); 3) [bcl-2 index <800, MDR1 index >300] (single positive$^{bcl-2+/MDR1+}$); 4) [bcl-2 index > 800, MDR1 index >300] (double positive). These categories were analyzed for prognostic purposes.

Cytogenetic analysis

Karyotyping was carried out on GTG-banded chromosomes and reported using the ISCN-1995 nomenclature.[23] Karyotypic findings were classified according to the SWOG/ECOG criteria.[24] The fluorescence *in situ* hybridization (FISH) technique was used to investigate the presence of bcr/abl rearrangement, as previously described.[25]

Statistical analysis

The relationships of bcl-2, MDR1, CD71 and CD34 indices with FAB were assessed by the Kruskal Wallis test. Spearman's rank test was used to explore the correlation of bcl-2 and MDR1 indices with CD34 and CD71 indices. Correlation between the 4 different classes of AML (double negative, single positive$^{bcl-2+/MDR1-}$, single positive$^{bcl-2+/MDR1+}$, double positive) and CR rate, white blood cell count (WBC), FAB classification and cytogenetics were based on Pearson's χ^2 or two-tailed Fisher's exact test. Overall survival (OS) was calculated from the date of diagnosis to the date of death or last follow-up. Disease-free survival (DFS) was measured from the date of CR to the date of relapse or death. The patients who received an autologous or allogeneic stem cell transplant were censored at the time of stem cell infusion. The log-rank test was applied for comparison of OS or DFS involving multiple groups. A logistic regression model was used to assess the independent effect of dichotomous co-variables on CR. A Cox proportional hazards regression model was also applied to evaluate the independent effect of covariables on OS and DFS. Only the variables proving significant in univariate analysis were included in the regression model.

Results

Immunophenotyping

Table 2 shows the distribution of bcl-2 and MDR1 indices within the different FAB classes. The bcl-2 index was significantly higher in M0 (median 1732, range 241-10025), M6 (median 1606, range 290-3616) and M1 (median 1437, range 146-7604) subtypes ($p = 0.00001$), whereas the MDR1 index was higher in M5 (median 851, range 6-4975) and M4 (median 397, range 3-3920), than in the other FAB subgroups ($p = 0.00001$). The value of the MDR1 index was lowest in AML-M0 (median 23, range 1-1035). To investigate these aspects further, we also calculated the CD34 and CD71 indices. We assumed that since high levels of bcl-2 index were found in immature AML such as M0 and M1, the CD34 index would also be very high in these categories; in addition, CD71 antigen expression was used as an indirect tool to explore the cell-cycle status.[26] Indeed, a significantly higher value of CD34 index was actually observed in AML-M0 (median 6076, range 1-95100) and M1 (median 1649, range 1-38200) than in the other FAB groups ($p = 0.00001$). In keeping with this, we also observed a direct correlation between CD34 and bcl-2 index ($r = 0.4$, $p = 0.00026$). By contrast, the value of CD71 index was low in AML-M0 (median 80.5, range 6-1923) and M1 (median 221, range 1-5975), whereas it was high in AML M5 (median 638, range 16-4512) and M4 (median 470, range 87-3142) ($p = 0.00001$); as expected there was a

direct correlation between CD71 and MDR1 index (r = 0.42, p = 0.00001). Thus, this first step of analysis demonstrated that the association of bcl-2, CD34 and lack of proliferative activity may help in defining *genuine myeloid* subsets of AML such as M0 and M1. Conversely, the expression of MDR1 and CD71, and negativity for CD34 tended to constellate in monocytoid AML such as M4 and M5. However, in 54 (21%) of 256 cases, a bcl-2 index > 800 and an MDRI index > 300 coexisted, suggesting that the two mechanisms are not mutually exclusive. Following these observations, we grouped our AML cases into 4 categories based on the combination of bcl-2 and MDR1 index values: (i) double negative; (ii) single positive$^{bcl-2+/MDR1-}$; (iii) single positive$^{bcl-2-/MDR1+}$; (iv) double positive. These four classes were differently distributed within the FAB classification (Table 3); the higher incidence of double negative cases was observed in the M2 class whereas double pos cases occurred more frequently in the M4, M5 and M6 subgroups. Seventy-eight percent and 71% of M0 and M1, respectively, showed single positive$^{bcl-2+/MDR1-}$ expression whereas 30% and 36% of M4 and M5, respectively, carried a single positive$^{bcl-2-/MDR1+}$ pattern; all the differences were statistically significant (p=0.00001).

Table 3. Distribution of double negative, single positive$^{bcl-2+MDR1-}$, single positive$^{bcl-2-MDR1+}$ and double positive cases within FAB categories; all the differences were statistically significant (p=0.00001).

FAB	Double negative	Single positive bcl-2+MDR1-	Single positive bcl-2-MDR1+	Double positive
M0%	7	78	0	15
(n./total)	(2/28)	(22/28)	(0/28)	(4/28)
M1%	9	71	9	11
(n./total)	(5/55)	(39/55)	(5/55)	(6/55)
M2%	47	27	15	12
(n./total)	(28/60)	(16/60)	(9/60)	(7/60)
M4%	15	30	30	25
(n./total)	(7/44)	(13/44)	(13/44)	(11/44)
M5%	9	17	36	38
(n./total)	(5/58)	(10/58)	(21/58)	(22/58)
M6%	36	27	0	36
(n./total)	(4/11)	(3/11)	(0/11)	(4/11)

Cytogenetics

Karyotype evaluation was successful in 187 of the 256 patients, for an overall success rate of 73% (Table 1). A good, intermediate and poor prognosis karyotype was found in 8%, 55% and 37% of the patients, respectively. In the double positive group, 57% (20/35) of the cases had a poor prognosis karyotype, 37% (13/35) an intermediate one, and only 6% (2/35) of the patients had a good prognosis karyotype.

Conversely, in the double negative group, a poor prognosis karyotype was observed in 26% (9/35) of the patients, whereas intermediate and good prognosis karyotypes were found in 57% (20/35) and 17% (6/35) of the cases, respectively. In the single positive$^{bcl+/M021-}$ and single positive$^{bcl\ 2/MDR1+}$ group, intermediate karyotype was the prevalent finding, occurring in 56% (45/80) and 65% (24/37) of the cases, respectively. All the differences were statistically significant (p = 0.04) (Table 4).

Table 4. Distribution of double negative, single positive$^{bcl-2+MDR1-}$, single positive$^{bcl-2-MDR1+}$ and double pos cases according to karyotypic prognostic group; all the differences were statistically significant (p=0.04).

Karyotype	Double neg	Single pos $^{bcl-2+MDR1-}$	Single pos $^{bcl-2-MDR1+}$	Double pos
good%	17	6	5	6
(n./total)	(6/35)	(5/80)	(2/37)	(2/35)
intermediate%	57	56	65	37
(n./total)	(20/35)	(45/80)	(24/37)	(13/35)
poor%	26	38	30	57
(n./total)	(9/35)	(30/80)	(11/37)	(20/35)

Prognostic parameters

Overall, 123 of 240 (51%) patients receiving chemotherapy achieved CR. When the patient population was split by age (≤ or > 60 years), a CR rate of 63% (74/117) and 40% (49/123) was observed in younger and older patients, respectively (p = 0.0003). In univariate analysis and regardless of age, cytogenetics, FAB classification, bcl-2 index 800 and MDR1 index ≥ 300 were found to be significantly associated with CR rate (p = 0.000002, 0.0034, 0.0084 and 0.0032, respectively). Unfavorable cytogenetics and an MDR1 index≥ 300 were also associated with a shorter duration of OS (p = 0.0015 and 0.024, respectively). When the analysis was broken down according to the status of the combination of bcl-2 and MDR1 indices, we observed that 28% (15/54) of patients belonging to the double positive category achieved CR, whereas for double negative, single positive$^{bcl-2+/MDR1-}$ and single positive$^{bcl-2-/MDR1+}$ categories, the CR rate was 69% (31/45), 52% (23/44) and 56% (54/97), respectively (p = 0.00038). The unfavorable outcome of double positive patients held true even when analysis was performed by age. In fact, among younger patients, 37% (10/27) of those who were double positive achieved CR as compared to 80% (16/20), 74% (34/46) and 58% (14/24) respectively, of those who were double negative, single positive$^{bcl-2+/MDR1-}$ and single posi-

Table 5. Response to therapy according to bcl-2 and MDR1 index status in younger and elderly patients.

	Double negative	*Single positive bcl-2⁺/MDR1-*	*Single positive bcl-2-/MDR1⁺*	*Double positive*	*P*
Age ≤60 yrs					
CR %	80	74	58	37	0.005
n.CR/total	16/20	34/46	14/24	10/27	
Age > 60 yrs					
CR %	60	39	45	18	0.02
n.CR/total	15/25	20/51	9/20	5/27	
Total					
CR %	69	56	52	28	0.00038
n.RC/total	31/45	54/97	23/44	15/54	

tive$^{bcl-2-/MDR1+}$ (p = 0.005). Similarly, in the elderly group, 18% (5/27) of patients who tested double positive had a CR, whereas 60% (15/25), 39% (20/51) and 45% (9/20), respectively, of those double negativity, single positive$^{bcl-2+/MDR1-}$ and single positive$^{bcl-2-/MDR1+}$ did so (p = 0.02) (Table 5). Interestingly, no difference was observed between the younger and elderly group as regard to the frequency of occurrence of double positive cases (23% vs 22%). Finally, all relevant prognostic variables such as age, FAB classification, bcl-2 index, MDR1 index, double negativity, single positive$^{bcl-2+/MDR1-}$, single positive$^{bcl-2-/MDR1+}$, double positivity and karyotype were pooled into a multivariate model to determine their role as independent prognosticators. This analysis showed that double positive status, karyotype and age independently affected the frequency of CR (p = 0.008, 0.00074 and 0.004, respectively); karyotype was also shown to influence OS and DFS (Table 6).

Table 6. Multivariate analysis of variables with proven prognostic relevance in the univariate model. For each cytogenetic risk group the median durations of overall survival and disease-free survival are also reported.

	Complete Remission	*Duration of overall survival-*	*Duration of relapse free survival*
Karyotype	0.00074	0.009	0.0064
Good		26.7 months (range 1-125)	13.7 months (range 1-124)
Intermediate		5.7 months (range 1-86)	5 months (range 1-85)
Poor		4.6 months (range 1-34)	4 months (range 1-29)
Age	0.004	N.S.	N.S.
Double positive	0.008	N.S.	N.S.
Bcl-2 index > 800	N.S.	N.S.	N.S.
MDR1 index > 300	N.S.	N.S.	N.S.
Double negative	N.S.	N.S.	N.S.
Single positive$^{bcl-2+/MDR1-}$	N.S.	N.S.	N.S.
Single positive$^{bcl-2/MDR1+}$	N.S.	N.S.	N.S.
FAB	N.S.	N.S.	N.S.

Discussion

Proteins related to both apoptosis and multidrug resistance are among the most widely characterized drug resistance mechanisms leading to unsuccessful treatment of AML. In this study we systematically analyzed the expression of bcl-2 and MDR1 in 256 consecutive cases of *de novo* AML. Using values of ≥ 800 and ≥ 300 to denote bcl-2 and MDR1 expression, respectively, we were able to describe 4 different classes of AML with peculiar biological and clinical characteristics. Among double negative AML, M2 subtypes and favorable karyotypes predominates; this translates into a higher CR rate. Single positive$^{bcl-2+/MDR1-}$ cases were significantly associated with M0, M1 AML and CD34 antigen expression. This observation is in keeping with previous reports[9-11,27-29] and points to abrogation of apoptosis as a potential active process in CD34⁺ AML. In addition, the low value of CD71 index signifies minimal rates of proliferation. In line with these findings, our group has recently demonstrated that immature AML, such as AML-M0 and M1, express low levels of the pro-apoptotic protein bax, this pattern being significantly associated with CD34 and CD117 positivity.[10] On a speculative ground, this phenotypic profile supports the hypothesis of a relation between CD34⁺ AML and disorders such as myelodysplastic syndromes (MDS); this holds true especially in elderly patients.[30] Increased expression of bcl-2, a shift from excess to inhibition of apoptosis and a fall in the number of S-phase cells have been associated with leukemic transformation in refractory anemia with excess blasts.[31] This indicates that in most cases of MDS the evolution toward AML arises from genomic lesions that inhibit the apoptotic control machinery rather than promoting cell proliferation. In contrast to the single positive$^{bcl-2+/MDR1-}$ cases, there are single positive$^{bcl-2-/MDR1+}$ AML which are characterized by an MDR1 index ≥ 300; in these cases the CD71 index is also very high and CD34 is not expressed. It may well be that monocytoid AML have a high proliferative rate and use MDR1 protein as a preferred mechanism of chemoresistance; in fact, 66% of these cases qualified as M4/M5 subtypes.[21] Finally, roughly 20% of our cases showed simultaneous expression of bcl-2 and MDR1 (index ≥ 800 and 300, respectively). There was no correlation between these double positive cases and age or CD34 expression. This subset represents a discrete entity characterized by a convergence of M4/M5 subtypes, unfavorable karyotype and a significantly lower CR rate.

In conclusion, the combined analysis of bcl-2 and MDR1 allowed different classes of AML to be identified, with distinct clinico-biological features. Bcl-2 is overexpressed in $CD34^+$ AML and may represent a key protein involved in the process of chemoresistance. This might have therapeutic implications since the use of apoptosis inducers may be indicated for improving treatment outcome. Conversely, MDR1 is overexpressed in $CD34^-$ AML suggesting that the use of specific modulators may be appropriate in this group of patients. However, the occurrence of the two proteins is not mutually exclusive since their combined expression defines a distinct subset of AML with a very poor prognosis. This indicates that several different mechanisms of chemoresistance may be operating simultaneously in some AML. Indeed, the contribution of other proteins, such as lung-resistance protein and multidrug related resistance protein, needs to be evaluated.

AV, GDP, LM, FB. contribution to conception, design and interpreting data; AT, MID, CM, BN, LO: contribution to conducting the work and analyzing the results; CC, PP: contribution to conducting karyotypic and FISH analysis; SA: supervised the project. All authors contributed to the design of the study and revision of the manuscript. Primary responsibility for the publication: AV. The authors reported no potential conflicts of interest.

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