Bcl-2 expression in acute myelogenous leukemia: the relation to myeloid antigen expression and response to therapy in Iranian patients*

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Product of Bcl-2 gene prolongs survival of hematopoietic cells by inhibition of programmed cell death. The aim of this study is to examine the expression of the bcl-2 protein in a group of patients with AML and its relation to clinical features and response to therapy.

Slides from the bone marrow or peripheral blood of 70 patients with AML were assessed for the expression of bcl-2 by immunocytochemistry. The expression of myeloid and non-lineage associated markers was detected by indirect immunofluorescence method. Correlation between bcl-2 and markers expression and patients characteristics was determined.

More than 20 % positivity for bcl-2 was found in 22 (31.4 %) patients. Bcl-2 expression showed an association with M4 and M5 subtypes ($p<0.01$) and was correlated with clinical parameters including WBC and platelet count, extramedullary disease and Hb level. Bcl-2 expressing cells were significantly higher in $CD15^+$ and $CD13^+$ patients and lower in $CD11b^+$ and $CD33^+$ cases ($p<0.001$). Complete remission (CR) rate was significantly lower in cases with 20 % or more bcl-2 positivity than others (24.4 % v 75.6 %). A shorter CR duration was observed in bcl-2^+ patients when compared with bcl-2^- ones (571±50 versus 850±17 days)($p=0.0001$).The expression of bcl-2 was also associated with shorter survival ($p=0.0001$). Survival time for bcl-2^+ patients was 831±44 days versus 1119±17 days for bcl-2^- ones. CD11b and CD33 positivity was associated with longer survival whereas CD13 and CD15 positivity was correlated with lower survival ($p<0.007$). In multivariate analysis bcl-2 positivity was associated with poor survival.

Bcl-2 expression showed a prognostic value in our patients indicating that even despite of some differences in treatment regimen, immunocytochemical analysis of this marker is still a simple and inexpensive method for evaluation of prognosis in AML patients. Bcl-2 expression may be related to the expression of differentiation associated markers.

Key words: acute myelogenous leukemia, bcl-2

Bcl-2 oncogene, is located on chromosome 18 and can be activated by 14:18 translocations or expressed at high levels in the absence of gene rearrangements [29]. The protein encoded by bcl-2 gene contributes to neoplastic cell expansion by prolonging cell survival through its ability to block programmed cell death [16, 19]. Many chemotherapeutic agents are thought to exert their genotoxic effects through induction of programmed cell death [12]. Overexpression of bcl-2 therefore may be implicated in resistance to chemotherapy [24]. Bcl-2 is well known in a high proportion of B-cell chronic leukemias and in majority of low-grade non-Hodgkin's lymphoma [15]. Bcl-2 protein has been studied in many types of cancer including breast cancer, prostate cancer and acute lymphoblastic leukemia [18, 22]. Recent reports showed mostly high bcl-2 expression in acute lymphogenous leukemia even by using different methods [30, 27]. However there are few reports concerning the effects of bcl-2 overexpression in the myeloid leukemia with respect to chemotherapy and also differentiation associated markers [8, 10, 31]. In the present work we tried to study bcl-2 expression in relation to treatment outcome and also to the expression of several myeloid and non-lineage associated surface antigens in a group of AML patients.

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Material and methods

Patients. Seventy patients with both *de novo* AML and secondary to myelodysplasia or chronic myelogenous leukemia, aged 10 to 66 years admitted in oncology wards of Shiraz University affiliated hospitals from year 2000 to 2003 were enrolled in this study. Diagnosis was carried out by cytologic criteria according to French-American-British (FAB) and histochemical staining. Patients were treated with standard chemotherapy using the following protocol: cytarabine administered as a continous intravenous infusion at 70 mg/m^2 per day for 7 days, and daunorubicin 40 mg/m^2 or adriamycin 35 mg/m^2 administered intravenously on days 1, 2 and 3. A second course of induction therapy was administered if necessary. The maintenance treatment was 2.5 monthly cycles of 70 mg/m^2 of cytosine arabinoside subcutaneously every 12 hours and 110 mg/m^2 per day of 6-thioguanine orally, each for 7 days.

Sample preparation. Leukemic cells were isolated from peripheral blood or bone marrow aspirates of patients having more than 30 % blasts using ficoll-hypaque gradient centrifugation. Cytocentrifuge samples were prepared and stored at –20 °C. The percentage of blasts was always higher than 90 % after separation.

Immonocytochemistry for bcl-2 expression. Cells were incubated with PBS containing 3 % H_2O_2 to block endogenous peroxidase and then incubated with a 1:50 dilution of monoclonal mouse antibody to human bcl-2 oncoprotein (Dako, Glostrup, Denmark) for 2 hours followed by a 1:100 dilution of goat biotinylated anti-mouse immunoglobulin (Dako) for 1 hour and a 1:200 dilution of peroxidase conjugated streptavidin (Dako) for 45 minutes. During incubations slides were washed thoroughly with PBS. Peroxidase activity was detected using diaminobenzidine (Dako) in 3 % H_2O_2. Cells were counterstained by hematoxylin. Slides were then examined for the percentage of bcl-2 positive cells. K562 cells and cells isolated from chronic leukemia were used as positive control. Negative control was performed for each case using PBS instead of the first monoclonal antibody.

Immunofluorescene for antigens expression. Cells were incubated with predetermined amounts of CD13, CD15, CD11b, and CD33 monoclonal antibodies (Dako, Glostrup, Denmark) for 1 hour and then incubated with goat anti-mouse immunoglobulin conjugated with FITC (Dako). Analysis of the stained cells was carried out using a Zeiss fluorescence microscope. Cases with more than 30 % reactivity were considered positive.

Statistical analysis. Data were analyzed using SPSS software. The relationship between the bcl-2 expression and parameters including surface markers, sex, FAB subtype, CR rate and extramedullary involvement (EXT) (in central nervous system, testis, spleen, liver and lymph nodes) were analyzed by the students t-test. The relashionships of expression of bcl-2 to quantitative parameters of age and blood cell counts were studied by multiple regression analysis. A cutpoint of 20 % bcl-2 positivity was chosen to discriminate bcl-2$^+$ and bcl-2$^-$ cases. Duration of survival was measured from the date of entry into the study. CR duration was calculated from the date CR was achieved. Survival and remission duration curves were plotled according to the method of KAPLAN and MEIER [17] and in different groups were compared by the log-rank test. The respective influence of different parameters on survival or CR duration was calculated according to the Cox-proportional hazard regression method. A predictive value of less than 0.05 was chosen as the level of significance.

Results

Table 1 summarize clinical and laboratory features of patients. The mean survival and remission duration of all patients were 967±156 and 682±197 days, respectively. Of the patients, 20 % had M_0 subtype, 28.6 % had M_1 subtype, 21.4 % M_2, 14.3 % M_3, 10 % M_4, 4.3 % M_5 and 1.4 % M_6. 64 of 70 cases showed bcl-2 expression (range, 2–98 %). The overall frequency of expression of bcl-2 was 22.8±23 %. More than 20 % positivity for bcl-2 was found in 22 (31.4 %) of patients.

Table 1. Clinical and biological features of patients with AML

Age, y	38.7 (10–66)
No. of patients with extramedullary involvement	28 (40)
central nervous system involvement	4 (5.7)
hepatomegaly	20 (28.6)
splenumegaly	19 (27)
White blood cell x 10^9/L	28.9 (10–80)
Platelet x 10^9/L	37.5 (1.4–100)
Hemoglobin level (g/dL)	6.1±3.3
Complete remission duration (days)	682±197
Survival (days)	967±156

The staining intensity of positive cells was variable between individual cases and was correlated with the percentage of positive cells (data not shown).

In Table 2, results of the relationship between different clinical parameters of patients with percentage of bcl-2 expressing cells is presented. Bcl-2 expression showed significant correlation with parameters including WBC count, platelet count, extramedullary involvement and Hb level ($p<0.009$) but not with sex and age. WBC count was highest (>60 x 10^9/l) and platelet count lowest (<13.6 x 10^9) in cases with bcl-2 expression more than 60 %. Hb level was decreased with increased number of bcl-2 positive cells. 50.9 % of cases with more than 20 % bcl-2 expression (bcl-2$^+$) showed EXD compared to 12.5 % of bcl-2$^-$ cases.

The expression of bcl-2 in relation to FAB subtypes of leukemia is presented in Table 3. The mean percentage of bcl-2 positive cells was significantly higher in M_5 followed by M_4 subtype ($p<0.01$).

Table 2. Correlation of bcl-2 expression with hematological characteristics and complete remission rate of patients

	Percentage of bcl-2 expressing cells						
	0–100 (Total)	0–9	10–19	20–39	40–59	60–79	80–100
No. of patients	70	25	22	10	5	5	3
Mean WBC (10^9/L)	28.9	23.8	15.2	39.7	35.2	67.6	60.2
Mean platelet (10^9/L)	37.5	47.5	39.2	41.8	12.8	13.6	9.0
No. of patients in remission	49 (70 %)	21 (84 %)	16 (73 %)	6 (60 %)	3 (60 %)	2 (40 %)	1 (33 %)

Table 3. FAB subtypes and bcl-2 expression

FAB subtype	mean % of bcl-2$^+$ cells	No. of cases with	
		>20 % bcl-2$^+$ cells	<20 % bcl-2$^+$ cells
M_o	20.9	4	10
M_1	17.4	6	14
M_2	15.2	4	11
M_3	9.6	0	10
M_4	56.1	5	2
M_5	79	3	0
M_6	2	0	1

Frequency of expression of myeloid and non-lineage associated antigens was as follows: CD13, 73 %; CD15, 12.9 %; CD33, 71.4 %; CD11b, 47.1 %.

Bcl-2 expression showed significant association with the expression of these markers. The mean percentage of bcl-2 positive cells was significantly higher in CD15$^+$ than CD15$^-$ cases (62.8 v 16.9, $p<0.01$) and CD13$^+$ than CD13$^-$ cases (27.8 v 9.8, $p<0.01$). The mean percentage of bcl-2 expressing cells was significantly lower in CD11b$^+$ than CD11b$^-$ cases (8.9 v 35.2, $p<0.001$) and CD33$^+$ than CD33$^-$ cases (12.4 v 48.9, $p<0.001$).

A comparison of *de novo* and secondary cases of AML revealed that percentage of bcl-2 positive cells in blasts from secondary cases was higher than that in cases of *de novo* leukemia (59.8 v 14.4, $p<0.001$).

Relationship between expression of bcl-2 and response to therapy was studied by determining the CR rate and duration and survival of patients.

The overall CR rate was 70 %. 12 of 22 bcl-2$^+$ cases went into remission compared to 37 of 48 bcl-2$^-$ cases ($p<0.001$). As shown in Table 2, the CR rate was decreased with increase in the number of bcl-2 expressing cells.

The relation of bcl-2 expression to CR duration and survival is shown in Table 4. A shorter CR duration was observed for bcl-2$^+$ patients. CR duration was 571±50 days for bcl-2$^+$ cases compared to 850±17 days for bcl-2$^-$ ones ($p=0.0001$). The expression of bcl-2 was also associated with shorter survival ($p=0.0001$). Survival time for bcl-2$^+$ patients was 831±44 days versus 1119±17 days for bcl-2$^-$ patients. The survival and remission duration curves plotted by Kaplan-Meier method showing the difference between bcl-2$^+$ and bcl-2$^-$ cases are shown in Figure 1.

In multivariate analysis bcl-2 influence remained significant for both CR duration and survival ($p=0.04$).

Other factors including age, EXT, platelet count and Hb level showed a significant correlation with survival. Survival in patients with more than 60 years, platelet count less than $20x10^9$ and Hb level less than 9 g/dl was shorter than others.

As shown in Table 4, CD11b and CD33 positivity was associated with longer survival whereas CD13 and CD15 positivity correlated with shorter survival ($p<0.002$). CR duration was significantly associated with CD33 expression ($p=0.04$) and platelet count ($p=0.021$). The influence of these factors on CR duration and survival was not signifcant in multivariate analysis.

Table 4. Relationship between expression of markers and response to therapy

Antigen	No. of patients	Complete Remission (days)	P	Survival (days)	P
CD_{13}^+	51	739 ± 26		970 ± 26	
CD_{13}^-	19	772 ± 28	0.9	1148 ± 26	0.002
CD_{11b}^+	33	787 ± 19		1143 ± 19	
CD_{11b}^-	37	707 ± 33	0.1	925 ± 33	< 0.001
CD_{15}^+	9	788 ± 23		697 ± 76	
CD_{15}^-	61	745 ± 21	0.8	1078 ± 86	< 0.001
CD_{33}^+	50	785 ± 15		1103 ± 21	
CD_{33}^-	20	599 ± 71	0.04	860 ± 50	< 0.001
Bcl-2$^+$	22	571 ± 50		831 ± 44	
Bcl-2$^-$	48	850 ± 17	0.0001	1119 ± 17	0.0001

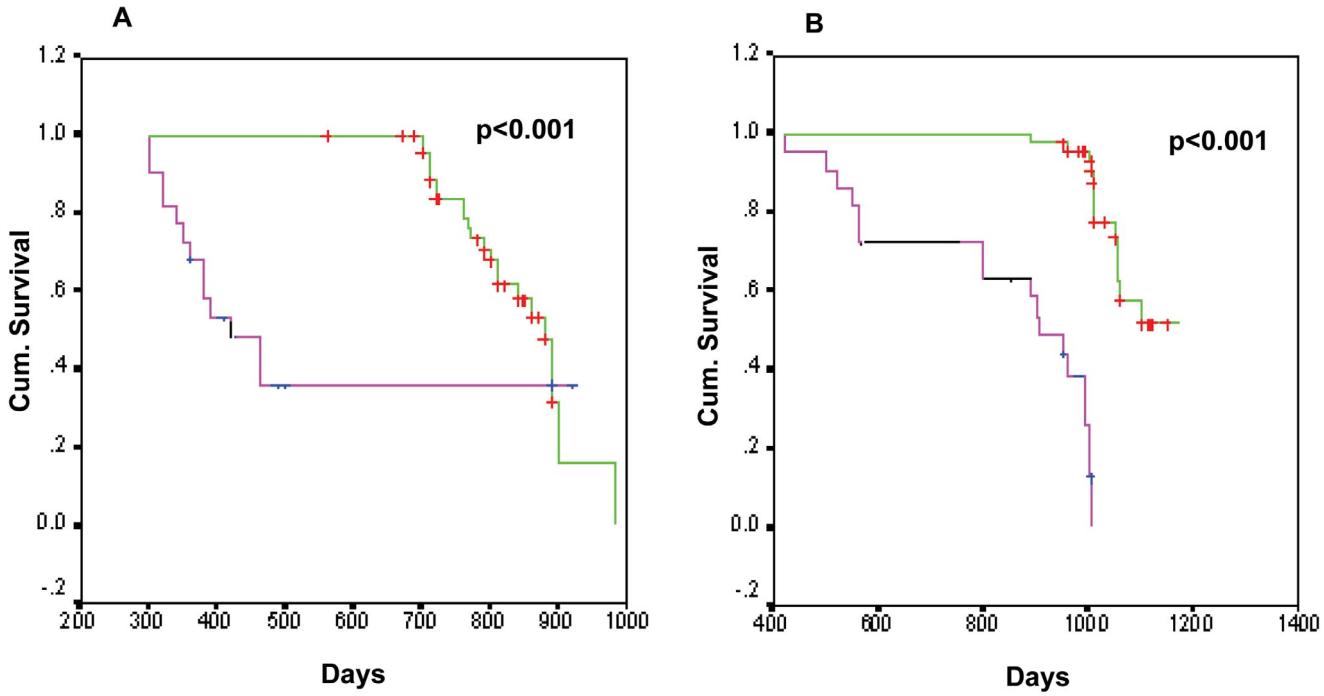

Figure 1. Complete remission duration (A) and survival (B) curves of bcl-2$^+$ (——) and bcl2$^-$ (+++) patients plotted using Kaplan-Meier method. Statistically significant differences are observed.

Discussion

In the present study we observed heterogeneous expression of bcl-2 ranging from 2 to 98 percent. Significant correlation was found between bcl-2 expression and parameters including WBC and platele count, Hb level and myelo-monoblastic FAB subtype. In a previous study using immunocytochemistry higher number of bcl-2 expressing cells in M_4 and M_5 subtypes of AML and in cases with high WBC count were reported which is consistent with the fact that AML of these subtypes is usually characterized with higher number of WBC [7]. However, significant higher value of bcl-2 expression in M_0 and M_1 than in other subtypes using flow cytometry [21, 25] were observed by LAURIA et al and PROWIT et al. No correlation between bcl-2 expression by immunocytochemistry and western blotting with FAB subtypes, sex, age and WBC count was found in two other studies [2, 20]. The difference between these results may be in part due to different methods of study. In our study we considered AML cases with 20 % or more stained cells as positive when it was necessary for statistical analysis to design two categories. But it is likely that some cases with less than 20 % positive cells would be considered as positive by other techniques such as western blot. However, the use of immunocytochemistry allowed us to quantify easily the expression of bcl-2 and to show an association between the number of bcl-2$^+$ cells and other parameters such as WBC, platelet, Hb and treatment outcome.

Previous studies demonstrated mostly expression of bcl-2 as an important poor prognostic indicator of response in AML [3, 31, 34, 35]. In our study the percentage of bcl-2 positive cells was significantly higher in patients who did not achieve remission. Bcl-2 positivity was either by univariate and multivariate analysis correlated with shorter survival and complete remission duration. These data raise the possibility that high values of bcl-2 on myeloid blasts may confer a higher resistance to standard chemotherapy. However, identification of patients with high expression of bcl-2 may be important for a different therapeutic approach [21].

A comparison of *de novo* and secondary cases of AML revealed that percentage of bcl-2 expression in blasts from secondary cases was higher than that in cases of de nove leukemia, which is in agreement with reports that, myelodysplastic related AML has clinically been considered to have a worse prognosis that *de novo* AML [11].

The relationship between bcl-2 expression and immunophenotype of AML has not been fully studied before. In our study we found an inverse association between bcl-2 and CD11b and CD33 expression. Bcl-2 staining of normal bone marrow myeloblasts, promyelocytes, and myelocytes, but neither monocytes nor most polymorphonuclear cells, has been shown by two-color flow cytometric analysis and a large percentage of normal bone marrow bcl-2$^+$ cells shown to express CD33, a marker for myeloid cells as well as CD34, a marker for myeloid progenitor cells [11]. Increased expression of the leukocyte integrin CD11b as well as down-regula-

tion of the gene bcl-2 has been reported to be associated with late stage differentiation of the myeloid lineage [13]. In few studies bcl-2 expression in conjunction with CD34 expression has been investigated in AML [5, 26, 32, 33]. Bcl-2 was correlated with CD34 positivity and both of these markers were introduced as independent prognostic factors for achieving CR in AML.

There were no reports of bcl-2 expression either with CD15 or CD13 association. Our results including increased expression of CD15 and CD13 expression in bcl-2 positive cases indicates that bcl-2 expression on myeloid cells might be regulated in a differentiation-linked manner. Moreover, the relationship found between each of these markers and response to therapy showed that the impact of each marker might be related to the expression of bcl-2. As, CD11b and CD33 markers and their expression was inversely correlated with bcl-2 expression and were related to longer survival in AML cases whereas CD13 and CD15 that were directly correlated with bcl-2 expression were related to shorter survival similar to the bcl-2.

Our results regarding the longer survival of patients with CD11b positivity is consistent with our previous reports showing a favorable relationship between CD11b expression and patient outcome [1]. There was also a trend for higher CR rate in $CD11b^+$ patients. In a recent study by PAIETTA et al, $CD11b^+$ AML was introduced as a new leukemic syndrome with poor prognosis (23). GRIFFIN et al and BRADSTOK et al have reported that $CD11b^+$ cases had shorter periods of remission and shorter survival [4, 14]. The difference between these results may be partly due to different drug regimens used in these studies or different genetic background of patients.

The presence of CD15 has been associated with a better outcome in several studies [9]. In a study performed by COMPOS et al, absence of CD15 was associated with a poor survival [6]. In our study CD15 and CD13 positivity were significantly associated with shorter survival. In two studies performed by GRIFFIN et al and SCHWARZINGER et al, CD 13 positivity has been associated with poor outcome [14, 28].

In conclusion, the association of bcl-2 with myeloid antigens expression showed that bcl-2 expression is related to differentiaton of leukemic cells. We showed that bcl-2 expression on AML cells correlates with biologic findings such as hyperleukocytosis and myelo-monoblastic subtype. Moreover, this expression was associated with poor prognosis and may implicate modified therapeutic strategies in bcl-2 patients.

The prognostic impact of bcl-2 expression may be influenced by other potential prognostically important factors, such as cytogenetics, which were not included in our study. Another parameter that has a potential impact on the prognostic role of bcl-2 expression is the administered therapy. In most studies, cytosine arabinoside and anthracycline based regimens have been used for treatment of AML, but differences in therapy among studies could confound the results.

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