Original Article

The Influence of Bcl-2 and Myeloid Antigen Expression on Response to Therapy in Childhood Acute Lymphoblastic Leukemia

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Abstract

Background: The possible prognostic significance of the expression of a variety of markers has been investigated in acute lymphoblastic leukemia (ALL).

Methods: In the present study we investigated the prognostic significance of CD13 and CD33 myeloid antigens (MY) aberrantly expressed on the blasts of ALL patients and Bcl-2 anti- apoptotic molecule expression in childhood ALL.

Results: Aberrant expression of MY occurred in 8.8% of cases. Variant levels of Bcl-2 were expressed in patients (44.2±25.5%), with more than 20% positivity for Bcl-2 in 64.7% of patients. Bcl-2$^+$ patients survived 959±242 days compared to 1059+230 days for Bcl-2$^-$ patients (P=0.2). Corresponding data for complete remission duration was 682±170 and 716±173 days (P=0.3), respectively, indicating no significant association between survival and complete remission duration of patients with expression of the Bcl-2 molecule. Analysis of clinical response according to MY expression, however, showed significant association with survival and complete remission duration. MY$^+$ patients had shorter complete remission duration (383±58 days) and survival (473±68 days) than MY$^-$ patients (complete remission duration, 724±144 days; survival, 1045±186 days; P<0.001). Expression of Bcl-2 along with MY was not associated with a significant decrease in survival or complete remission duration.

Conclusion: Results of this study indicated that expression of MY was a poor prognostic factor in childhood ALL. Bcl-2 expression in MY$^+$ patients could not influence the response to therapy.

Keywords: acute lymphoblastic leukemia, Bcl-2, myeloid antigens, outcome

Introduction

Acute lymphoblastic leukemia (ALL) is a common malignancy with relatively high rate of mortality in children.[1] The etiology of this disease is not clearly known. However, failure of lymphocyte differentiation and a defective apoptosis lead to overproduction of immature lymphocytes. More than 80% of patients respond to therapy but some patients experience refractory or recurrent disease. Resistance of ALL blasts to chemotherapy or defects in apoptosis may account for refractory disease in these patients.[2] Apoptosis, or programmed cell death, is a biological process essential for tissue homeostasis and normal development of multicellular organisms.[3] Consequently, perturbation of the regulation of apoptosis may lead to the development of diseases such as cancer. Apoptosis is regulated by a complex network of interacting molecules. Bcl-2 anti-apoptotic protein is a member of the Bcl-2 protein family that plays pivotal roles in the decision and execution phases of apoptosis. This molecule can prevent the release of cytochrome *c* from mitochondria thereby blocking caspase activation and apoptosis.[3,4] Bcl-2 has been shown to protect cancer cells from the effects of chemotherapy.[5] This protein has been studied in different types of cancer and hematological malignancies including follicular lymphoma, acute myelogenous leukemia (AML) and chronic lymphocytic leukemia.[6–8] Association of higher Bcl-2 levels with poorer responses to chemotherapy in some of these neoplasms has been demonstrated. Recent reports have shown high Bcl-2 expression in ALL; however, the prognostic value of Bcl-2 is still controversial.[9–13]

Aberrant expression of myeloid antigens (MY) CD13 and CD33 on lymphoblasts has been investigated in previous studies.[14–17] Although the regulating mechanisms are unclear, however, MY expression in ALL can be assumed to be a feature of early differentiation stage and/or lineage indecisiveness.[14] The clinical significance of MY expression on leukemic lymphoblasts has yielded contrasting results. Data from several reports indicated that the presence of MY on lymphoblasts of ALL patients is predictive of a poor response to therapy.[15–17] In contrast, are reports that show no prognostic value for the expression of myeloid markers in these patients.[18,19]

Considering the possible prognostic values of Bcl-2 expression in ALL, in the present study we investigated the impact of the expression of Bcl-2 in combination with CD13 and CD33 MY on the clinical and biological features and response to therapy in a group of Iranian patients with ALL.

Materials and Methods

Study patients

In this study, 34 patients with ALL diagnosed by morphologic studies of bone marrow aspiration and biopsy according to the French-American-British (FAB) criteria and cytochemical staining were enrolled. They were 17 girls and 17 boys (ages 2 – 11) with a mean age of 6.28±2.8 years. Informed consent was obtained from patients and/or their guardians. The protocol

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Table 1. Clinical and biological feature of patients with ALL in relation to the expression of bcl-2 and myeloid antigens.

Variables	Total	MY^+ ALL	MY^- ALL	*P*-value
No. of patients	34	3 (8.8%)	31(91.2%)	—
Age (year)	6.28±2.8	6.6±4	6.24±2.7	0.8
Male/Female	17/17	0/3	17/14	0.23
WBC x 10^9/L	25.6±19.9	29.7±13.3	25.2±20.5	0.7
Platelet x 10^9/L	38.3±36.8	56.7±55	36.5±35.3	0.4
CRD (days)	694±169	383±58	724±144	<0.001
Survival (days)	994±242	473±68	1045±186	<0.001
CR rate	31(91%)	3 (9.7%)	28 (91.3%)	1
No. patients with EXD	20 (58.8%)	2 (10)	18 (90%)	1
Bcl-2 positivity	44.2±25.5	50±33	43.6±25.2	0.7
Bcl-2 expression > 20%	22 (64.7%)	2 (9%)	20 (91%)	1

Values are mean (range), mean (%) or mean±SD. MY=myeloid antigen positive, CR=complete remission, CRD=complete remission duration, EXD=extra medullary disease.

was approved by the Ethical Committee of Shiraz University of Medical Sciences. Clinical and laboratory features of patients are summarized in Table 1. Remission induction included vincristine, prednisone, L-asparaginase, and doxorubicin. For intrathecal chemotherapy, methotrexate was administered once weekly for six times. Leukemic cells were isolated from peripheral blood or bone marrow aspirates of patients before chemotherapy by using ficoll-hypaque gradient centrifugation. In order to have an adequate number of blasts for immunofluorescence, we chose patients with more than 30% blasts. After twice washing, cell concentration was adjusted to 1×10^6/mL and then cytocentrifuged samples were prepared and stored at -20°C. The percentage of blast cells was always higher than 90% after separation.

Analysis of expression of markers

Biotin-Avidin immunocytochemistry was used to study Bcl-2 expression. Slides were incubated with phosphate buffered saline (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 for two hours followed by a 1:100 dilution of goat biotinylated anti-mouse immunoglobulin for one hour and a 1:200 dilution of peroxidase conjugated streptavidin for 45 minutes. All monoclonal antibodies and reagents of the experiments were purchased from Dako, Glostrup, Denmark. During incubation periods, slides were washed thoroughly with PBS. Peroxidase activity was detected using diaminobenzidine in 3% H_2O_2. Cells were counterstained by hematoxylin after which slides were examined for the percentage of Bcl-2 positive cells. Staining intensity was scored as weak (1+), moderate (2+), and strong (3+). The staining index was calculated by multiplying the percentage of positive cells by staining intensity. The positive control consisted of cells isolated from chronic lymphocytic leukemia and the negative control for each case was an unrelated monoclonal antibody. Due to the variation in Bcl-2 expression levels, we analyzed the patients using a cut-off criterion of 20% or more blast cells that expressed Bcl-2 molecule. For analyzing the expression of the lymphoid and MY, an indirect immunofluorescence analysis on isolated blood or marrow mononuclear cells using specific monoclonal antibodies was performed. CD7, CD10, CD13, CD19, and CD33 monoclonal antibodies were used in this study. Cells (1×10^6/mL) were treated with predetermined amounts of monoclonal antibodies. After a two hour incubation, cells were washed and then a 1:100 dilution of fluorescein conjugated goat anti-mouse immunoglobulin was added. After an hour of incubation at dark the cells were washed and analyzed by a Ziess fluorescence microscope. Samples were considered positive when more than 20% of the cells stained with particular antibody.

Statistical analysis

Data were analyzed using Statview Statistical Software Package (SPSS, Abaus Concepts, Berkeley, CA, USA). The relationship between the expression of markers and parameters including sex, FAB subtype, and extramedullary disease (EXD) in the central nervous system, testis, spleen, liver, and lymph nodes were analyzed by Fisher's exact test. The relationship of expression of markers to quantitative parameters of age, Hb, platelets, and white blood cell counts (WBC) were studied by independent samples *t*-test. Duration of survival was measured from the date of entry into the study and complete remission duration (CRD) calculated from the date complete remission was achieved. Survival and remission duration curves were plotted according to the method of Kaplan and Meier[20] and in different groups were compared by the log-rank test. The respective influence of different parameters on survival or CRD 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

ALL subtypes

According to the criteria of FAB classification, 3% of patients had L1 subtype, 91% L2, and 6% had L3 subtype. Using specific monoclonal antibodies it was determined that 17.6% of cases expressed more than 70% CD7 (T-ALL) and 82.4% expressed more than 70% CD19 antigen (B-ALL). CD10 molecule was expressed in 21 of 28 $CD19^+$ patients (75%).

Bcl-2 expression

Results of Bcl-2 expression are shown in Table 1. Variant levels of Bcl-2 were expressed in patients (range 8 – 80%). The overall frequency of expression of Bcl-2 was 44.2±25.5% and the overall staining index was 85.6±73.7. A cut off point of 20% Bcl-2 positivity was chosen to discriminate $Bcl\text{-}2^+$ and $Bcl\text{-}2^-$ cases. $Bcl\text{-}2^+$ patients were 22 out of 34 (64.7%). Expression of Bcl-2 showed no significant correlation with different FAB subtypes of ALL. Bcl-2 was expressed in 6/6 of $CD7^+$ patients and 16/28 (57.1%) of $CD19^+$ cases. Statistical analysis of the Bcl-2 expression showed no significant association with the expression of lymphoid and MY.

CD13 and CD33 myeloid antigen expression

A low number of patients (3, 8.8%) were positive for at least one of the MY expression (MY^+ALL) (Table 1). Frequency of

Table 2. Relationship between expression of markers and response to therapy.

Antigen	No. of patients	CRD (days)	*P*-value	Survival (days)	*P*-value
$CD13^+$	1	450±115		550±227	
$CD13^-$	33	701±166	—	1008±230	—
$CD33^+$	3	383±58		473±68	
$CD33^-$	31	724±144	<0.001	1045±186	<0.001
$Bcl\text{-}2^+$	22	682±170		959±245	
$Bcl\text{-}2^-$	12	716±173	0.3	1059±230	0.2

Values are mean±SD. *P* values are based on log-rank tests comparing complete remission duration (CRD) and survival of patients with and without expression of marker.

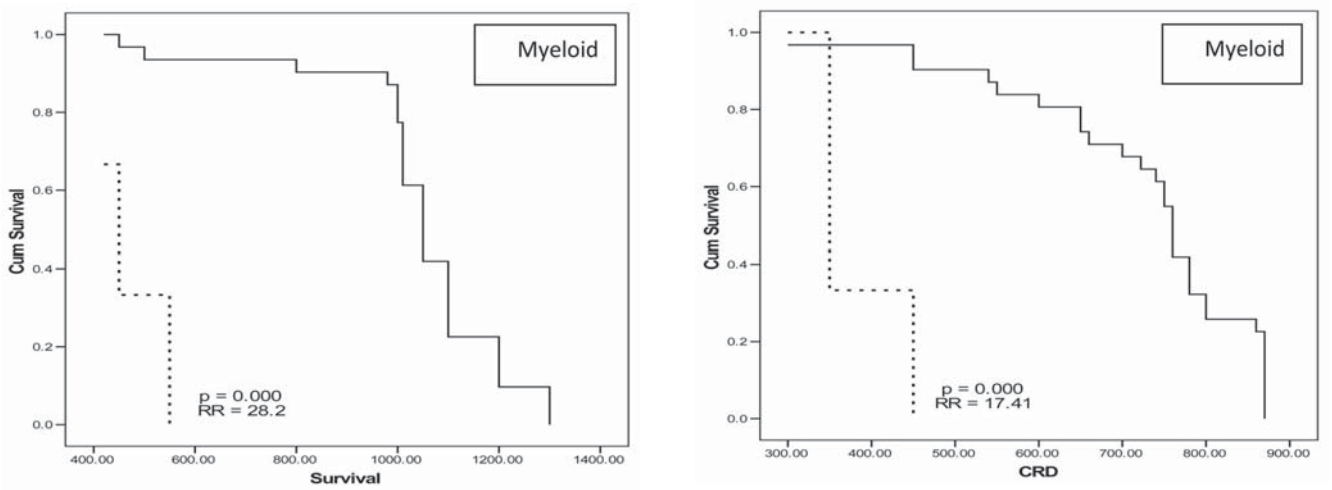

Figure 1 . Survival and complete remission duration (CRD) curves for myeloid antigens (CD13 and/or CD33) plotted using Kaplan-Meier method. Significant differences are observed. Expression of marker is shown by (- -) and lack of expression shown by (−). RR=relative risk.

expression of CD13 and CD33 MY was 3% and 8.8%, respectively. All MY^+ patients were $CD33^+$. No significant correlation between expression of myeloid markers and expression of lymphoid markers was found.

Clinical and biological characteristics of patients

The clinical and biological features of patients including WBC and platelet count, gender, and age did not significantly differ between $Bcl\text{-}2^+$ and $Bcl\text{-}2^-$ patients. Expression of Bcl-2 in high-risk group patients, including children who have WBC count of 50,000/μL or more (four patients) compared to those with less than 50,000/μL showed no significant difference. Among other factors that affect the high risk group, Bcl-2 showed a significant correlation with CD7 positivity. All T-ALL patients were $Bcl\text{-}2^+$ (*P*=0.001). Study of clinical response according to Bcl-2 expression in patients with ALL revealed that although the overall survival for $Bcl\text{-}2^+$ cases (959±245 days) was less than that of $Bcl\text{-}2^-$ (1059±230 days), the difference was not significant (*P*=0.2). CRD did not also significantly differ between $Bcl\text{-}2^+$ (682±170 days) and $Bcl\text{-}2^-$ (716±173 days) patients (*P*=0.3). Of patients, 11 out of 22 $Bcl\text{-}2^+$ achieved CR (*P*=0.1). Bcl-2 index did not correlate with survival or CRD (data not shown).

Results of the study of the relationship between MY expression and disease outcome are shown in Table 2. CD33 antigen showed significant association with survival and CRD. Survival for $CD33^+$ patients (473±68 days) was shorter than $CD33^-$ patients (1045±186, *P*<0.0001) indicating an unfavorable outcome in MY^+ ALL patients when compared with MY^- ones. A similar result was obtained for survival in a $CD13^+$ case (550±227 days) versus $CD13^-$ patients (1008±230 days). In multivariate analysis the influence of CD33 MY remained significant (*P*<0.001; Figure 1).

In Figure 2 the effects of MY and Bcl-2 expression on survival and CRD of patients is demonstrated. As shown, both survival and CRD in $MY^+Bcl\text{-}2^+$ patients are shorter than $MY^-Bcl\text{-}2^-$ cases (*P*<0.001). The influence of different combinations of MY and Bcl-2 on survival and CRD of patients are noted (Figure 2). Survival or CRD of $MY^+Bcl\text{-}2^+$ patients were similar to a $MY^+Bcl\text{-}2^-$ patient; however, statistical analysis due to the low number of cases was not possible.

In this study, overall survival and CRD were not associated with WBC count, platelet count, and other parameters (data not shown).

Discussion

The clinical importance of aberrant expression of two MY including CD13 and CD33 was examined in children with ALL. We found that 8.8 % of all cases expressed at least one of these myeloid markers. The frequency of MY expression in ALL cases has been reported to vary with age and a range of 7% to 25% in childhood ALL and up to 40% in adult ALL has been suggested.[21,22] According to MY expression when we divided our patients into MY^+ and MY^- cases a significant difference between MY^+ and MY^- patients in regard to CRD and survival was observed, which indicated the impact of the myeloid molecules on response

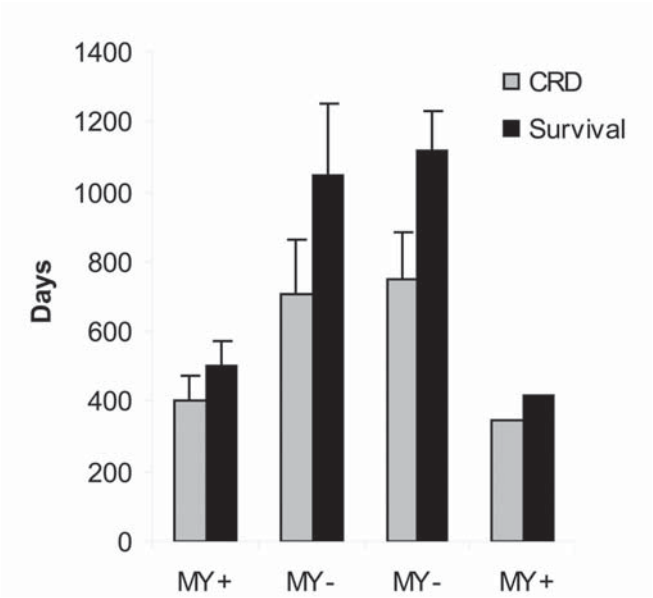

Figure 2. Comparison between survival and complete remission duration (CRD) of various groups of patients according to Bcl-2 and myeloid CD13 and/or CD33 (MY) antigen expression. $MY^+Bcl\text{-}2^+$ cases compared with $MY^-Bcl\text{-}2^-$ and $MY^-Bcl\text{-}2^+$ is significant (P<0.001).

to therapy. Aberrant expression of MY have been previously observed both in adult and childhood ALL. In adult ALL a few early studies have shown an inferior outcome for MY^+ compared to MY^- ALL patients, the other published series demonstrated no prognostic correlation using high-dose chemotherapy regimens between these two groups.[23] In children, similarly, prognostic significance of expression of MY has been studied with conflicting results.[24] Wiersma et al. have shown the clinical importance of MY expression in childhood ALL.[25] Similarly, Cantú-Rajnoldi et al. showed the overall incidence of childhood ALL expressing MY were low, but they reported evidence that this co-expression may be related to an unfavorable clinical course.[26] In contrast, myeloid-associated antigen expression lacks prognostic value in childhood ALL treated with intensive multiagent chemotherapy in two other studies.[24,27] In a recent study on Malaysian patients MY co-expression was fairly common and constituted 23% of childhood ALL within the Malaysian population, but it was not an adverse risk factor in childhood ALL.[28] These results indicated that the clinical and biologic significance of myeloid positive ALL is different among studies. Differences in definitions of MY expression, immunophenotyping methods, study populations and treatment regimens may explain the discordant results.

Bcl-2 anit-apoptotic protein expression has been associated with tumor cell survival and resistance to chemotherapy in hematological malignancies.[29] In ALL, despite several investigations, the biological role and prognostic value of Bcl-2 antigen expression on leukemic blasts still remains poorly defined. In our study, we analyzed a series of 34 patients with ALL using a cut-off criterion of 20% or more blast cells that expressed Bcl-2 molecule. Of cases, 64.7% were positive for Bcl-2. In contrast to a study reporting the lower frequency of expression of Bcl-2 in T rather than B-lineage ALL,[30] all cases of CD7 positive ALL (T-ALL) in our study expressed Bcl-2. We found that neither age, sex, EXD, Hb, WBC, platelet nor immunological phenotype was associated with high levels of Bcl-2 protein expression. In agreement with these results, none of the high-risk features predictive of poor treatment outcome such as high WBC count, organomegaly and T-lineage immunophenotype was associated with high levels of Bcl-2 expression in another study performed by Uckun et al.[31]

As results of our study showed, Bcl-2 expression in ALL blasts was not associated with poor clinical outcome. In previous studies, Bcl-2 expression in clinical samples has yielded contrasting results. In a study performed by Wutcher et al. expression levels of Bcl-2 did not predict the response to induction chemotherapy and relapse rate in childhood ALL.[32] Also, in a 28 untreated cases of ALL, high expression of Bcl-2 was not an unfavorable factor for disease outcome.[33] In contrast, lower Bcl-2 expression has been shown to be associated with poorer outcome by Coustan-Smith et al.[34] The inconsistency observed in these results could be probably due to technical differences, patient selection and other important factors, such as the number of cases screened, differences in therapy among the studies and cytogenetics, which was not included in our study.

As results of our study showed, MY expression has stronger impact on the prognosis of ALL than Bcl-2. To study the impact of MY expression in combination with Bcl-2 expression on the prognosis of the patients, survival and CRD of $MY^+Bcl\text{-}2^+$ patients were compared to that of MY^+ $Bcl\text{-}2^-$ cases. As results of the study showed survival and CRD in the two groups were almost similar, suggesting that Bcl-2 expression could not be an additive prognostic factor to MY expression for predicting the response to therapy in childhood ALL. A limitation of our study was the relatively small sample size. Indeed, study on a larger sample of patients is necessary to confirm these results. It is worthy to note that in our previous study on adult Iranian patients with ALL, we found that Bcl-2 expression in ALL blasts was associated with poor clinical outcome. Besides, MY expression was a more powerful poor prognostic factor compared to Bcl-2 protein and the Bcl-2 molecule was also shown to influence survival of MY^+ patients.[35] These differences between the role of Bcl-2 on disease outcome in children and adults may come from the fact that pediatric and adult ALL are distinct diseases based on their molecular characteristics.

In conclusion, MY expression was a stronger predictor of treatment outcome rather than Bcl-2 in childhood ALL. Further studies with larger numbers of patients may provide additional information regarding the clinical impact of Bcl-2 and MY expression in pediatrics and adult ALL.

Disclosures: None

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