ABSTRACT

Objective: The primary cause of treatment failure in acute myeloid leukemia (AML) is the emergence of both resistant disease and early relapse. The bcl-2 gene encodes a 26-kDa protein that promotes cell survival by blocking programmed cell death (apoptosis). In the present study, bcl-2 protein expression was evaluated in newly diagnosed AML patients and correlated with the induction of remission and overall survival (OS), in an attempt to define patients who might benefit from modified therapeutic strategies.

Patients and methods: Pretreatment cellular bcl-2 protein expression was measured in bone marrow samples obtained from 68 patients of newly diagnosed acute myeloid leukemia and 10 healthy controls by western blotting.

Results: The mean bcl-2 protein expression was significantly higher in patients (0.686 ± 0.592) compared to controls (0.313 ± 0.016) (p=0.002). The overall survival for patients with mean bcl-2 expression of less, and more than or equal to 0.315, was 67% and 56%, respectively, with no significant difference between the two groups (p=0.86).

Conclusion: Even though we did not observe a significant difference in overall survival between patients with high and low levels of bcl-2, modulation of this protein might still be considered as an option for enhancing the effectiveness of conventional chemotherapy.

Key Words: Acute myeloid leukemia (AML) – Bcl-2 – prognosis – Western blot.

INTRODUCTION

The primary cause of treatment failure in acute myeloid leukemia is the emergence of both resistant disease and early relapse. Among the most frequent causes of these phenomena are defects in the mitochondrial-mediated apoptotic pathway. This pathway is regulated by the bcl-2 family of anti-apoptotic (bcl-2, bcl-xl, mcl-1) and prop-apoptotic proteins (bax, bad, bak) [1].

The bcl-2 oncogene was initially described because of its involvement in the translocation [t(14,18)] present in most follicular lymphomas, where it is juxtaposed to the JH region of the Ig heavy chain gene [2]. This rearrangement results in the production of high levels of normal bcl-2 transcripts, which seems to be a major factor in malignant transformation [2,3]. Despite the lack of t(14,18), high levels of bcl-2 mRNAs or bcl-2 protein have also been detected in other malignancies [4].

The bcl-2 gene encodes a 26-kDa protein that promotes cell survival by blocking programmed cell death (apoptosis) [5]. It has been shown that bcl-2 gene over-expression in human leukemia cell lines conferred a high resistance to a variety of chemotherapeutic agents by abrogating mitochondrial cytochrome C release and activation of caspase-3 [6].

Moreover, it has been shown that AML blasts with autonomous proliferation in culture express high levels of bcl-2 and have increased resistance to chemotherapy [7]. Additionally, those patients with autonomous blast growth in vitro were found to have a poor clinical response and a reduced survival [8,9].

Given the current poor results of conventional chemotherapy in AML, the search for
Innovative approaches based on mechanisms of actions targeting specific pathways involved in leukemogenesis has rapidly progressed. Bcl-2 antisense oligonucleotides are now being under evaluation in phase III trials in combination with chemotherapy in newly diagnosed AML [10,11].

In the present study, we evaluated bcl-2 protein expression in newly diagnosed AML patients and correlated it with the induction of remission and overall survival, in an attempt to define patients who might benefit from modified therapeutic strategies.

**PATIENTS AND METHODS**

**Patients:**

Between June and August 2006, 90 patients with newly diagnosed untreated AML were seen at the National Cancer Institute (NCI), Cairo University. Bone marrow and/or peripheral blood samples were obtained prior to therapy from 71 consecutive patients. Insufficient sample material was obtained from 3 patients, leaving a sample size of 68. Diagnosis of AML was based on morphologic evaluation, cytochemical staining, and immunophenotyping (IPT) according to the French American British (FAB) and the European Group for the Immunological Characterization of Leukemias (EGIL) systems [12,13]. For control, ten peripheral blood samples were obtained from donors of bone marrow transplantation. Informed consent and institutional review board approval were taken.

**Methods:**

**Protein extraction:** Protein extraction was performed as previously described [14]. Theuffy coat cells were lysed in Triton X-100 lysis buffer containing 300mM NaCl, 50mM TrisHCl, pH 7.6, 0.5% Triton X-100 and 10mM PMSF, incubated for 45-60min on ice with frequent vortexing, and centrifuged at 13,000 rpm for 15min at 4ºC. The supernatant was collected and the protein concentration was measured by the Bradford method [15].

**Western blot analysis:** 50µg protein was fractionated by electrophoresis on 12% SDS polyacrylamide gels under non-reducing conditions and electro-transferred to nitrocellulose membranes. After blocking with 5% nonfat powdered milk in TBS containing 0.1% Tween-20 (TBS-T), the membranes were incubated for one hour at room tempreature (RT) with anti-bcl2 (Ab-1) mAb (Calbiochem, Darmstadt, Germany). After washing with TBS-T, the membranes were incubated for one hour at RT with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences, Illinois, USA). After an extensive rinsing with TBS-T, immunoreactive protein bands were visualized with a chemiluminescence-based procedure using the ECL detection kit according to manufacturer instructions (Amersham Biosciences, Illinois, USA). The integrated optical density of the resulting bands was measured by densitometry and the expression of bcl-2 protein was compared to that of the control group. Representative blots are shown in Figs. (1,2).

**Statistics:**

Data were analyzed using the SPSS statistical package version 12. The Chi-square test (Fisher’s exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between the two groups was done by the Mann-Whitney test. The Kaplan-Meier method was used for survival analysis, with the log-rank test to compare two survival curves. ROC curves were plotted for identification of the cut off values of the tested variables. p-values equal to or less than 0.05 were considered significant [16].
RESULTS

Expression of BCL-2 in patients and controls:

Patients included 52 (76.5%) adults with a median age of 37 years (range 16-76 years) and 16 children with a median age of 13 years (range 1.8-15 years) (Table 1).

The median bcl-2 protein expression was significantly higher in patients 0.408 (0.215-3.040) compared to controls 0.312 (0.292-0.350) ($p=0.002$). In the patients group, the median bcl-2 expression level was significantly higher in males compared to females ($p=0.006$) and was higher in FAB subtypes (M1+M2) and M3 compared to M4+M5 with border line significance ($p=0.06$) (Table 2).

When patients were divided in two groups according to the optimum cutoff point [(0.315) sensitivity of 80%, 95% confidence interval (78.2-80.5%) and specificity of 70%, 95% CI (68.7-71.3%)], there was no association between bcl-2 expression with age, hepatomegaly, splenomegaly, initial white blood cell count (WBC), platelet count (PLT), percentage of bone marrow blasts, and hemoglobin level (Hb) (Table 3).

Relation between bcl-2 expression and response to induction therapy:

The overall CR rate was 19.1%. The mean bcl-2 expression was lower (0.586±0.324) in samples from patients in whom remission was attained versus (0.710±0.639) when remission was not attained, although no significant difference was reached ($p=0.864$).

Relation of bcl-2 expression and overall survival (OS):

The median overall follow up period was 1.23 month, with a minimum of three days and a maximum of 41 months. The overall survival at one year was 43.1% for all patients.

The OS for patients with mean bcl-2 expression of less than 0.315 was 48.0%, and for patients with mean bcl-2 expression more than or equal to 0.315 was 42.2% at a one-year follow-up period, with no significant difference between the two groups ($p=0.86$) (Fig. 3).
The biologic basis of drug resistance and relapse in AML is not well understood and prognosis is still largely based on descriptive parameters. Several lines of evidence indicate that apoptosis plays a role in response to chemotherapy, suggesting an interaction between therapy-induced apoptosis and therapeutic efficacy in AML [17].

Earlier reports showed that CD34+ AML fractions are more resistant to apoptosis than are the corresponding CD34- AML fractions, and this was paralleled by higher bcl-2, bcl-xL, Mcl-1, p-gp, and lower bax expression levels. The apoptosis resistant protein profile and the reduced ability to undergo apoptosis in cases of AML with high CD34 expression indicated that the poor clinical outcome is, at least partly, caused by a higher percentage of cells with increased resistance to apoptosis [18].

In the present study, we have analyzed bcl-2 protein expression in newly diagnosed cases of AML using western blotting, as a determinant of response to therapy and prognosis. The product of the bcl-2 proto-oncogene is suggested to contribute to leukemogenesis by prolonging the life span of progenitor cells [19]. Bcl-2 does not only inhibit apoptosis, but also restrains cell cycle entry, and these two functions can be genetically associated. This anti-proliferative effect of bcl-2 can provide additional cyto-protection for malignant cells because proliferating cells are more vulnerable to apoptotic stimuli. Therefore, agents that can overcome the inhibitory effect of bcl-2 on cell cycle entry could be useful adjuncts to the current chemotherapeutic drugs [20].

In line with previous reports [21-25], the mean bcl-2 protein expression was significantly higher in AML patients as compared to controls.
(\(p=0.002\)). In these cases, the mean level of bcl-2 expression in the (M1+M2) FAB subtypes was higher than that in (M4+M5) subtypes with borderline significance (\(p=0.06\)), denoting that bcl-2 was more frequently expressed in the immature forms of the granulocytic leukemias.

Similar findings were previously reported by Lauria, et al. [25] who found significantly higher mean bcl-2 fluorescence intensity in M0 and M1 FAB subtypes as compared to M2, M3 or to M4 and M5 cases. In 1998, Karakas, et al. [26] reported significantly lower levels of bcl-2 mRNA expression in monocytic AMLs (M4 and M5). In support of these findings is the work of Delia, et al. [23], showing that the bcl-2 protein levels among normal myeloid cells are inversely related to maturation, i.e., a large fraction of myeloblasts and promyelocytes are bcl-2 positive whereas metamyelocytes and polymorphonuclear cells are mostly bcl-2 negative, while monocytes are totally negative.

We attempted to correlate the bcl-2 expression with response to chemotherapy, but the remarkably low CR rate made a reliable analysis difficult. Although not reaching the level of significance, and in line with previous reports [21,26], we found that patients in CR had a lower mean bcl-2 expression as compared to those who did not attain CR. The overall survival for patients with a mean bcl-2 expression of less than 0.315 (Cut off) was higher than those with mean bcl-2 expression of more than or equal to 0.315, although not reaching the level of significance. On the contrary, other studies [18,19,25] reported that bcl-2 expression may serve as a significant prognostic factor for predicting remission and long term outcome.

This discrepancy might be attributed to the notion that over expression of only one protein, possibly involved in resistance, is not sufficient to significantly influence the prognosis or long term survival in AML, but the expression of more than one protein, i.e. bcl-2, p53, heat shock protein 27 (HSP27), and p-gp is predictive of reduced overall survival [28].

Furthermore, the discrepancy might be due to the different levels of sensitivity of the various techniques used. Some reports claim that the conventional techniques, such as immunocytochemistry and Western blot, cannot provide reliable quantitative information but flow cytometry is well suited both for the precise, semiquantitative measurement of bcl-2 oncoprotein expression and for estimation of cellular heterogeneity, frequently found in AML [17].

In conclusion, we confirm presence of high levels of bcl-2 expression in Egyptian AML patients. Even though we did not observe a significant difference in overall survival or complete remission rates between patients with high and low levels of bcl-2, modulation of these proteins might still be considered as an option for enhancing the effectiveness of conventional chemotherapy. High bcl-2 expression observed in most cases could afford a protective effect against drug-induced apoptosis. Decreasing levels through emerging antisense technology may prove to be highly successful.

REFERENCES

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