

The Significance of Estimation of Anti- Apoptotic Protein in Acute Leukemia Cases

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Abstract:

Background: Apoptosis is a terminal cell fate that eliminates physiological unneeded or dangerous cells. The Bcl-2 oncoprotein encodes a mitochondrial protein that blocks apoptosis. High levels of Bcl-2 protein are found in different malignancies including acute leukemias and it is thought to be directly involved in the emergence of drug resistance by disrupting &/or delaying apoptotic program and promoting tumor survival.

Objectives: To analyze the significance of any variation in the level of Bcl-2 expression according to disease progress (from time of diagnosis to time of remission or resistance) in cases with acute leukemia, and whether to consider this variation as a prognostic marker.

Methods: Through a prospective study, 25 samples of bone marrow aspirates were taken from different patients who attended the hematology unit of Baghdad teaching hospital in the medical city from December 2005 to July 2006, who proved to have acute leukemia in its different types or subtypes (ALL and AML) & at a variable phases of the disease course. Monoclonal antibody targeted against Bcl-2 oncoprotein via immunocytochemistry staining techniques of these aspirates was performed in order to define the level of Bcl-2 protein carrying cells & looking for any differences in this expression in relation to disease progress or treatment hoping to translate these findings in terms of prognosis.

Results: Despite the heterogenous expression of Bcl-2 protein carrying cells in each case (0-500 cells out of 500 cells) of AML or ALL, it was found to be in positive correlation with the density of blast cell percentage in the marrow ($r=0.515, p=0.008$) unlike the relation with total leucocyte count ($r=0.364, p=0.074$).

Conclusion: A good clinical clue to intractable resistant course of the disease at diagnosis is verified in this study when very high level of Bcl-2 protein carrying cells was found. This finding may have a useful clinical application through the recommendation to change therapeutic strategy, at time of diagnosis, to more aggressive protocols, which is agreed by others. There was no chance to study the application of high level of Bcl-2 protein in terms of long-term survival due to short follow up.

Key words: Apoptosis, proteins, acute leukemias, Bcl-2 oncoprotein, monoclonal antibody, blast cells, resistance, prognosis, antisense oligonucleotides

Introduction:

Apoptosis (Greek: *apo* - from, *ptosis* - falling) was distinguished from traumatic cell death in 1972 by Andrew H. Wyllie while studying tissues with electron microscopes⁽¹⁾. It is a terminal cell fate, a highly regulated "suicide" process that eliminates physiologically unneeded or dangerous cells & may prevent mutation that cause cancer⁽²⁾.

Apoptosis is performed by proteases named caspases & by nucleases, activated by a family that includes positively acting *Bax* & negatively acting *Bcl-2* proteins⁽²⁾.

One of apoptosis mechanisms is through intrinsic or mitochondrial pathway that triggered by internal signals to activate Bcl-2 protein which stimulate a related protein, Bax, to cause leaking out of

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cytochrome C & stimulating caspase family cascade leading to degradation of chromosomal DNA & later phagocytosis of cells⁽³⁾.

Some viruses associated with cancers use tricks to prevent apoptosis of the cells like Epstein-Barr Virus (EBV) in some lymphomas types through the production of a protein similar to Bcl-2 to make them more resistant to apoptosis that enable a cancer cell to continue proliferation, therefore; some B cell leukemia & lymphoma may express high levels of Bcl-2 to block apoptotic signals^(4, 5, 6, 7).

Bcl-2 (B-cell lymphoma-2) family of intracellular protein is the central regulator of caspase activation & its opposing function of anti- & pro-apoptotic members arbitrate the life or death switch.^(2, 8, 9)

It is an integral membrane protein even in healthy cells^(10, 11). (Figure 1). **Bcl-2** is a human proto-oncogene located on chromosome 18 & its product called *Bcl-2*⁽⁴⁾. In the cancerous B cells, the portion of chromosome 18 containing the **Bcl-2** locus has undergone a reciprocal translocation with the portion of chromosome 14 containing the antibody heavy chain locus. This t (14; 18) translocation places the **Bcl-2** gene close to the heavy chain gene enhancer⁽⁴⁾. (Figure 2).

The Bcl-2 gene has been implicated in a number of cancers as well as leukemia & it is also thought to be involved in resistance to conventional cancer treatment. This will support a role for decreasing apoptosis in

pathogenesis, as in addition to its prognostic role in AML^(12, 13). It had been represented in around 87% at diagnosis & about 100% at time of a relapse⁽¹⁴⁾.

Most of chemotherapeutic agent used in treatment will exert their cytotoxic effect by induction of apoptosis. Consequently, the regulation of apoptotic or anti apoptotic pathways is of high clinical importance regarding remission induction & overcoming drug resistance^(15, 16).

AML patients with high expression of **Bcl-2** protein in blast cells exhibit a poor response to chemotherapy. Thus, in vitro, chemo sensitivity of AML cells to cytosine arabinosides, the most active agents in AML therapy, can be increased by **Bcl-2** down regulation using anti sense oligonucleotides, all-trans-retinoic acid (ATRA), interleukin 6 or granulocyte colony stimulating factors (G-CSF)⁽¹³⁾.

In acute lymphoblastic leukemia (ALL) expression of **Bcl-2** protein was found to be associated with prolonged life span of lymphoblast, although initial studies did not show a consistent correlation between **Bcl-2** expression levels in primary leukemic cells & treatment outcome⁽¹⁷⁾.

This study had been designed to analyze the significance of any variation in the level of Bcl-2 expression according to disease progress (from time of diagnosis to time of remission or resistance) in cases with acute leukemia, and whether to consider this variation as a prognostic marker.

Patients and Methods:

Over 8 month duration, from December 2005 to July 2006, 31 samples of bone marrow aspirates were collected from different patients seeking hematological advises in hematology unit of Baghdad teaching hospital in medical city. Some of them were just received with their hematological disorders. Others were known to have acute leukemia although some of them were followed from their time of diagnosis all through time of treatment including induction & consolidation cycles of chemotherapy.

Twenty five samples (80.6%) belong to patients with acute leukemia at different time of their disease courses, while 6 samples (19.4%) were considered as control samples for our study as they were taken from patients complained from hematological problems other than acute leukemia (like megaloblastic anemias, pyrexia of unknown origin & bleeding tendency).

Those with acute leukemias included cases of acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), & acute bilineage leukemia (ABL).

Patients' samples taken at different phases of the disease:

- Nine aspirates were taken at time of diagnosis.
- Three aspirate samples were taken to demonstrate the effect of induction chemotherapy phase only (post induction).
- The rest 13 aspirates samples were taken to manifest the effect of whole course of chemotherapy including induction & consolidation cycles of treatment.

Types of chemotherapy were standard protocols according to the type of leukemia.⁽¹⁸⁾

At each time, 2 samples of bone marrow aspirate were taken in addition to blood sample. The first sent for conventional cytomorphology reporting while the 2nd dropped over a charged slide immediately for immunocytochemistry. The two reports were compared in terms of interpretation concerning the diagnosis, total leucocyte count & differential, number of leukemic cells or blast in both peripheral blood and marrow aspirate in addition to disease activity.

Immunocytochemistry for the detection of Bcl-2 Protein in bone marrow aspirate

The Principle:

A primary antibody reacts with the antigen. A biotinylated secondary antibody then reacts with the primary antibody. This is followed by the attachment of an enzyme-conjugated streptavidin to the biotins on the secondary antibody. The enzyme converts a substrate to a colored reaction product. High levels of signal amplification are achieved due to the binding of multiple units of secondary antibody to each primary antibody, the binding of multiple enzyme-conjugated streptavidin molecules to each secondary antibody, and the enzymatic conversion of the substrate⁽¹⁹⁾.

Materials

1. DAB staining kit (DAKOCYTOMATION)
 - a. Secondary Anti-mouse Antibody conjugated with peroxidase enzyme.
 - b. The DAB solution (3,3 Diamino-benzidine tetrahydrochloride).
 - c. Tris buffer pH 7.6 diluent for DAB.
 - d. H₂O₂ substrate.
 - e. Haematoxylin as counter stain.

2. Monoclonal Antibody:- Anti bcl-2 protein (DAKOCYTOMATION).
3. DPX mounting medium, which is a mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate), and xylene (BDH, England).
4. Positively charged microscope slides, Fisherbrand Superfrost/Plus (Fisher Scientific, USA).
5. Coverslips.
6. Binocular light microscopy (Olympus, Japan).

Hematopathologic interpretation

Slides were examined by light microscope at X400 magnification. Immunostaining was scored according to positively stained cells that impart the brown color

Results:

Thirty one bone marrow aspirate samples were gathered from 2 groups of candidates , 25 of them(80.6%) were patients with acute leukemia of different subtypes & at different phases of their disease while the remaining 6 (19.4%) considered as control group who were presented with diagnoses other than acute leukemia like megaloblastic anemia, pyrexia of unknown origin or bleeding tendency.

The group of patients with acute leukemia included 3 types of leukemia as the following :AML patients were 15 samples(60%) of different subtypes, ALL patients were 8 samples(32%) & acute bilineage leukemia (ABL) from the cytomorphologic report were 2 samples (8%).(Figure 5)

The age of patients group varied from 13-65 years with a mean of 31.0 ± 14.27 y, while control group from 22-63 years & the mean was 33.67 ± 15.6 y.

Thirteen patients were males & 12 were females while only one control was male & other 5 controls were females.

No significant differences in the sample age ($p < 0.689$) & sex ($p < 0.118$) distribution as both patients & control groups are comparable.

Both patient & control samples criteria are comparable in statistical point of view concerning their main features as well as the Bcl-2 immunocytochemistry staining results (as in table 1), which is proved to be of significance considering the number of blast cell % only.

The 3 modes of patient presentation at time of bone marrow aspiration are compared in view of Bcl-2 expression level & the other parameters, as well as analyzed individually between each 2 phases looking for any significant difference in down regulation of the original values in relation to progress of disease management(Tab.2). Undoubtedly , the blast number

in contrast to unstained cells which appear as blue cells out of total 500 cells in the field ⁽¹⁹⁾. Examples of these slides are seen in the provided figures.

STATISTICAL ANALYSIS:

Using student t-test, chi square & analysis of variance as well as correlation study to compare the result of bone marrow aspirate between the reports of hematology lab & the obtained reports from immunocytochemistry for the Bcl-2 proteins ⁽²⁰⁾. P value < 0.05 was considered to indicate statistically significant differences between groups.

differs significantly if we compare their initial presentation at time of diagnosis with the progress in the management(whether post induction or post consolidation therapy), unlike the Bcl-2 expression that is reported to differ significantly only post-consolidation treatment which is the point of reporting remission.

Demonstration of the number of blast cell percentage in the bone marrow aspirate is a well-agreed method for reporting disease remission according to the cytomorphology interpretation only, where it is represented to be less than 3% in even distribution ⁽²¹⁾. Here in this study, we tried to look for any relation between the blast percentage and the expression level of Bcl-2 protein carrying cells, in order to define the remission state of the disease.

As shown in table 2 that all parameters manifest significant differences in comparison between the two states of response according to blast percentage concerning most of the compared measures & interestingly demonstrate that Bcl-2 -ve cell is increased significantly in the aspirate sample at time of response state. In other words, the state of remission shows significant differences in most parameters including Bcl-2 proteins carrying cells as well as Bcl-2 -ve cells when compared with aspirate samples taken at the time of diagnosis or at the time of no remission reporting similar differences in blast percentage in bone marrow or neutrophil count or leukemic cells in peripheral blood. (tab.3)

In brief ,it can be verified that there is significant results in estimation of Bcl-2 protein carrying cells in those patient with acute leukemia in comparison with the presumed normal cells in bone marrow as it shown in the following tables(tab 4.A,4.B) (figure 6 , figure 7).

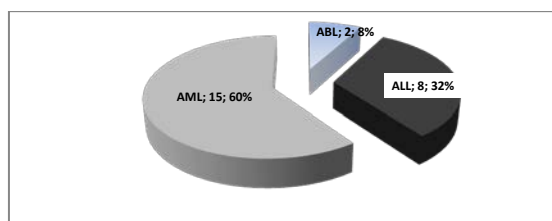


Figure 5 types of leukemic patients in study

Table 1 Comparison between patients & controls concerning Bcl-2 expression

	Control	Leukemic cells	P value
	Mean $\pm$ SD (range)	Mean $\pm$ SD (range)	
Age (years)	33.67 $\pm$ 15.69 (22-63)	31.0 $\pm$ 14.27 (13-65)	0.689
WBC($\times 10^9/l$)	6.466 $\pm$ 6.072 (1.5-14.5)	15.520 $\pm$ 30.714 (0.8-137.0)	0.483
Bcl-2 +ve Brown cells (out of 500 cells)	216.17 $\pm$ 196.61 (0-500)	303.96 $\pm$ 184.84 (0-500)	0.310
Leukemic cells in peripheral blood (%)	0	24.00 $\pm$ 29.34 (0-85)	0.057
Blast in marrow aspirate (%)	1.00 $\pm$ 0.89 (0-2)	35.56 $\pm$ 35.02 (0-98)	0.024*

*Significant difference using t- test for two independent means

Table 2 differences in parameters according to disease phases

	Diagnosis vs Post-induction	Diagnosis vs Post-consolidation	Post-consolidation vs post-induction
Bcl-2 +ve brown cells	0.897	0.041*	0.191
WBC	0.458	0.357	0.614
Neutrophil %	0.0001*	0.0001*	0.082
Leukemic Cells %	0.031*	0.006*	0.272
Blast %	0.019*	0.013*	0.169

* Significant difference using t-test for two independent means. (p $\leq$ 0.05)

Table 3. Comparison of leukemic patients according to bone marrow remission state

	REMISSION STATE IN BONE MARROW		P VALUE
	Yes (BLAST 1-3%) (n=8)	No (BLAST>3%) (n=17)	
Bcl-2 +ve brown cells mean	206.75 $\pm$ 230.54	349.71 $\pm$ 145.08	0.070
Bcl-2 -ve blue cells mean	293.25 $\pm$ 230.54	131.47 $\pm$ 128.31	0.033*
WBC($\times 10^9/l$)	6.112 $\pm$ 3.063	19.947 $\pm$ 36.686	0.303
Neutrophil (% out of WBC)	60.25 $\pm$ 10.73	23.24 $\pm$ 16.66	0.0001*
Leukemic cells in peripheral blood (%)	0.38 $\pm$ 0.74	35.12 $\pm$ 29.67	0.003*
BLAST in marrow aspirate (%)	2.25 $\pm$ 2.25	51.24 $\pm$ 31.97	0.0001*

*Significant difference using t-test for two independent means. (p $\leq$ 0.05)

Table 4 correlation study for leukemic patient

	WBC	Neutrophil %	Leukemic cells % (PB)	Blast % (BM)
Positive	r	0.364	-0.283	0.592**
Brown	P	0.074	0.170	0.002
Negative	R	-0.339	0.350	-0.606**
Blue	P	0.097	0.087	0.001
WBC	r	-0.303	0.602**	0.471*
	P	0.141	0.001	0.018
Neutrophil %	r		-0.704**	-0.623**
	P		0.0001	0.001
Leukemic Cells %	r			0.694**
	P			0.0001

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Discussion:

Although the methods of measuring and categorizing patients have varied, in general, many studies have found that patients with higher levels of Bcl-2 have a lower remission rate, an inferior survival, or both. Most

of the patients involved in these studies were participants in cooperative group trials; consequently, they contained a high percentage of younger patients with favorable types of leukemias. Paradoxically, the

report by Lauria *et al*⁽²²⁾ noted that only little patients with high *Bcl-2* had unfavorable prognosis. Karakas *et al*⁽¹⁴⁾ found that patients with AML arising after MDS were more likely to have undetectable *Bcl-2* mRNA, compared to patients with *de novo* AML. Additionally, they found that patients with no expression of *Bcl-2* had lower remission rates and higher relapse rates than those with intermediate levels of *Bcl-2*. If *Bcl-2* was functioning in the same pathogenic role in leukemias with both favorable and unfavorable cytogenetics, then it would be logical to expect increased, not decreased, frequencies of high *Bcl-2* expression in patients with unfavorable cytogenetics or prior MDS because they traditionally have lower remission rates and inferior survival outcomes. The fact that *Bcl-2* levels may be lower among patients with unfavorable leukemias suggests that *Bcl-2* may be acting differently in different types of leukemia.

Therefore, the role of *Bcl-2* in the leukemogenesis and prognosis of favorable and unfavorable leukemias might be different. Through immunocytochemical staining technique of bone marrow, aspirate samples from 25 patients with acute leukemia at different phases of presentation & treatment schedule. This study tried to find a prognostic implication through estimation of level of expression *Bcl-2* protein carrying cells & its application in term of prognosis & remission, unlike other similar purpose studies that depend on quantitative measurement of *Bcl-2* mRNA using RT-PCR⁽¹⁴⁾ or western blot test⁽²³⁾ or flow cytometry⁽²⁴⁾ but there were no chance to determine the relation of *Bcl-2* level on long term survival in our study, unlike the compared studies, due to short time of follow up of our patients.

The complete remission rate was lower among patients with high *Bcl-2* level, relative to those in the lower level of expression, which is statistically significant in our study unlike Steven *et al*⁽²³⁾ who found that the difference was not statistically significant among similar groups of patients. AML patient with complete remission after consolidation; as seen in this study, have significantly lower expression of *Bcl-2* compared with initial presentation (diagnosis or relapse state) similar to finding in Karakas *et al*⁽¹⁴⁾ & Campos *et al*⁽²⁴⁾.

Conclusions:

It is shown that high *Bcl-2* is expressed on AML cells, and correlates with biologic finding such as blast percent and associated with poor prognosis & it recommends modifying treatment programs & strategies. The level of *Bcl-2* cells may serve as a

The agreement in these results may indicate significant lower probability of achievement of complete remission in AML patient with persistent high levels of *Bcl-2* expression. Most of detected *Bcl-2* carrying cells at presentation may arise from malignant blast population & contains prognostic value for the clinical outcome of AML patients⁽¹⁴⁾ so variable levels of *Bcl-2* estimated to be associated with poor prognosis & treatment outcome^(25,26).

Immunocytochemistry in this study shows the expression of *Bcl-2* to be heterogeneous (0-500) using monoclonal antibody to leukemic cells carrying *Bcl-2* which make it difficult to define 2 groups with a cut off value as positive sample or negative sample while other studies chosen arbitrarily AML with 20% stained cells or more by flow cytometry using anti *Bcl-2* monoclonal antibody binding to leukemic cells as a positive cells for statistical analysis^(19,24) although many cases with less than 20% positive cells would be considered +ve by other techniques such as western blot^(17,23) that use a cut off value equals to 10%.

High levels of *Bcl-2* gene products have also been observed in other lymphoid cell malignancies⁽²⁴⁾ although it appears that *Bcl-2* will prolong lymphoid cell survival by blocking programmed cell death & prolong life span of lymphoblast but not the prognosis in cases of acute lymphoblastic leukemia^(14,17) However; we could not confirm similar reports as the general observation in our study is worse prognosis association with high level of *bcl-2* expression in both AML & ALL. *Bcl-2* protein expression is higher in all relapse ALL than *de novo* ALL⁽¹⁷⁾ in agreement with the concept of the role of *Bcl-2* in chemotherapy induced apoptosis.

It is worth to mention that as there are no similar studies found to use similar technique in estimation of *Bcl-2* protein level via the immunocytochemistry staining of bone marrow aspirate we could not make assessment of the validity of this method since other investigators tried to use more sophisticated procedures, & therefore; the methodological differences, the uniformity of patient selection at presentation stages & treatment protocols variability, with the lack of longer follow up in our study may contribute to the different outcome of these studies

significant prognostic factor for predicting remission independent of sex, WBC count and age.

Therefore, analysis of *Bcl-2* expression accompanying routine bone marrow analysis may be helpful defining high-risk patients group for modifying therapeutic strategies.

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