

Bax, Bcl-2, and Bax/Bcl-2 as prognostic markers in acute myeloid leukemia: are we ready for Bcl-2-directed therapy?

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Purpose: Many anticancer drugs induce apoptosis in malignant cells, and resistance to apoptosis could lead to suboptimal or no therapeutic benefit. Two cytoplasmic proteins, B-cell lymphoma protein 2 (Bcl-2)-associated X (Bax) and Bcl-2, act as a promoter and an inhibitor of apoptosis, respectively. Both Bax and Bcl-2 as well as their ratio have been regarded as prognostic markers in various cancers. However, conflicting results have been reported. A clear understanding of apoptosis has also become crucial due to reports about anti-Bcl-2 chemotherapy. We explored the relationship of Bax and Bcl-2 gene expression and their ratio with the therapeutic response in acute myeloid leukemia (AML) patients.

Patients and methods: Bone marrow and/or blood samples from 90 AML patients treated with cytarabine and daunorubicin were included. Expression of Bax and Bcl-2 was determined through real-time polymerase chain reaction by using $\Delta\Delta C_t$ method of relative expression.

Results: Bax and Bcl-2 expression among marrow and blood samples correlated with each other ($r_s=0.5$, $p<0.01$). Although bone marrow expression of Bax and Bcl-2 tended to remain higher among responders (median 1.01 and 0.29, respectively) as compared to non-responders (median 0.66 and 0.24, respectively), the difference failed to reach statistical significance ($U=784.5$ and 733 ; $p=0.68$ and 0.28 , respectively). Conversely, Bax/Bcl-2 ratio was higher among poor responders (median 3.07 vs 1.78), though again failed to reach statistical significance ($U=698.5$, $p=0.07$).

Conclusion: Expression of Bax and Bcl-2 does not differ significantly among AML patients treated with cytarabine and daunorubicin in terms of remission, relapse, resistance, overall survival, and disease-free survival, thus questioning the utility of emerging anti-Bcl-2 therapy.

Keywords: anthracyclines, cytarabine, Bcl-2, Bax Bcl-2 ratio, anti Bcl-2 therapy, BH3 mimetic inhibitors

Introduction

Acute myeloid leukemia (AML) is frequently a fatal malignancy in adults.^{1,2} Chemotherapy is the primary treatment of AML, which commonly employs cytarabine and anthracyclines such as daunorubicin.³ It is typically administered in two phases: an induction phase followed by a consolidation phase. After induction chemotherapy is given, the patient's bone marrow and blood are analyzed for complete remission (CR) between days 21 and 28. Unfortunately, a substantial number of patients do not respond to chemotherapy. In addition, AML patients who respond to chemotherapy often relapse later.³⁻⁶ Relapse is defined as $>5\%$ blast cells in bone marrow, or reappearance of blast cells in blood, or development of blasts from any sites other than bone marrow after CR is achieved. Relapse occurs usually within first 3 years from the end of the chemotherapy,³ especially in young patients.⁵ Different relapse rates have been reported, ranging between 21% and 39%, from various parts of the world.^{7,8} Thus, resistance to

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chemotherapy is a common observation and major obstacle in treating AML patients. Even though patients may respond to anticancer drugs, their overall survival (OS) remains low.^{5,9}

Anticancer drugs eradicate cancer cells either by disrupting cellular pathways vital for cell survival or by activating programmed cell death (apoptosis). Apoptosis is the final executor of many anticancer drugs. This cell suicide can be achieved by extrinsic or intrinsic pathways. The extrinsic pathway is mainly involved in controlling the cell turnover as well as eliminating mutant cells, while the intrinsic pathway is involved in antineoplastic drug action.^{10,11} DNA strand breaks accumulate after chemotherapy which trigger the intrinsic pathway of apoptosis in cancer cells.¹¹ In apoptosis, B-cell lymphoma protein 2 (Bcl-2)-associated X (Bax) protein activates the cascade of reactions by releasing cytochrome c from the mitochondria that helps in successive activation of caspases and ultimately leads to cell death. Bcl-2 is believed to prevent Bax from releasing cytochrome c, thus restricting downstream activation of apoptotic machinery. This will result in cell survival but at the same time Bcl-2 is also involved in retiring proliferating cells back to G₀ phase of the cell cycle.¹² Both Bax and Bcl-2 are cytoplasmic proteins.¹³ Release of cytochrome c from mitochondrial matrix is considered to work as an inevitable call for cell death.¹⁴ DNA fragmentation activates Bax and inhibits Bcl-2 through p53.^{10,11}

Dysregulation of apoptosis in cancer cells is considered to be one of the mechanisms of multidrug resistance.¹⁰ Many studies have investigated Bax and Bcl-2 in AML as well as other cancers and have yielded conflicting results. It has been proposed that high Bax and/or low Bcl-2 as well as high Bax/Bcl-2 ratio favors apoptosis and hence may lead to a favorable outcome,^{15–19} whereas others have reported contradictory observations.^{18,20,21} Thus, despite ongoing research, our understanding of this process and its impact on therapeutic outcome is still inadequate. This is further complicated by the current focus to develop anti-Bcl-2 drugs and their anticipated role in cancer therapy.^{22,23} Hence, we designed this study to explore the relationship of chemotherapeutic response to Bax and Bcl-2 gene expression in newly diagnosed AML patients using their bone marrow as well as peripheral blood samples and investigate their role as biomarkers of chemotherapy outcome.

Patients and methods

Patients and samples

We recruited 135 AML patients, diagnosed according to WHO criteria and treated at National Institute of Blood

Diseases and Bone Marrow Transplantation (NIBD&BMT), Karachi, during 2011–2017. All prospective AML patients, including acute promyelocytic leukemia (M3), were inducted into the study if they received an induction chemotherapy comprising only standard 3+7 regimen (daunorubicin 45 mg/m² on days 1–3; cytarabine 200 mg/m² on days 1–7). High-dose Ara-C was administered in the consolidation phase after the patient achieved CR. The study was approved by the Ethical Review Board at NIBD&BMT in accordance with the Declaration of Helsinki. All patients provided written informed consent to participate in this research. Any participant under the age of 18 years had parental or legal guardian written informed consent confirmed. Bone marrow and blood samples of the patients were collected separately. Samples from 45 patients were excluded for various reasons, such as hemolysis or no RNA yield. Thus, samples from 90 AML patients were available for analysis. Of those, 82 patients provided bone marrow samples and 77 provided peripheral blood samples, while 70 patients provided both bone marrow and peripheral blood samples. Gene expression was analyzed in bone marrow and peripheral samples separately without pooling.

Cell separation and storage

A total of 2 mL of bone marrow and/or blood samples from patients and only blood sample from control subjects were collected in EDTA tubes. White blood cell pellets were isolated by using Lymphocyte Separation Medium (LSM[®]; Corning-Cellgro, Manassas, VA, USA), which contains Ficoll and sodium diatrizoate (density=1.077 g/mL). The cell pellets were immediately stored in RNALater[®] (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C overnight and then stored at –80°C until RNA extraction.

RNA extraction, quantification, and cDNA synthesis

For RNA extraction, samples were thawed and washed with phosphate-buffered saline (PBS). Total RNA was extracted by using RNeasy Plus Mini Kit[®] from Qiagen (NV, Venlo, the Netherlands) according to the manufacturer's instructions. RNA was quantified by using Qubit[®] RNA HS Assay Kits and Qubit[®] 2.0 fluorometer (Thermo Fisher Scientific) according to the manufacturer's protocol. A total of 1 µg of RNA was utilized to synthesize cDNA by using Revert Aid First Strand cDNA Synthesis kit #K1622 (Thermo Fisher Scientific) according to the manufacturer's protocol. The cDNA was either analyzed through real-time PCR or stored at –40°C until analysis.

Real-time PCR, primers, and probes

We used VeriQuest Probe® qPCR Master Mix (Affymetrix, Santa Clara, CA, USA), and real-time PCR was run by using Eco Illumina® System version 5.0.16.0 (Illumina, San Diego, CA, USA). The expression of the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the internal control. Primers and probes were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). The reporter dye in the probe was 6-carboxyfluorescein (FAM), and the quencher was 6-carboxytetramethylrhodamine (TAMRA) with an intermediate ZEN-BQI. The primers and probes used for Bcl-2 were 5'-TCCGATCAGGAAGGCTAGAGTT-3' (forward), 5'-TCGGTCTCCTAAAAGCAGGC-3' (reverse), 5'-(FAM)/56-FAM/CC CAG AGC A/ZEN/T CAG GCCGCC AC/3IABkFQ/(TAMRA)-3' (Probe), for Bax were 5'-CCGC-CGTGGACACAGAC-3' (forward), 5'-CAGAAAACATGTCAGCTGCCA-3' (reverse), 5'-(FAM)/56-FAM/CC CCC CGA G/ZEN/A GGT CTT TTTCCG AC/3IABkFQ/(TAMRA)-3' (Probe), and for GAPDH were 5'-GAAGGTGAAGGTCCGAGTCA-3' (forward), 5'-GAAGATGGTGATGGGATTC-3' (reverse), 5'-(FAM)/56-JOEN/CC GAC TCT T/ZEN/G CCC TTCGAA C/3IABkFQ/(TAMRA)-3' (Probe).²⁴ The concentration was 6 pmol/mL for primers and 4 pmol/mL for probes. The reaction volume was 20 µL, which consisted of 10 µL Master Mix, 2.4 µL cDNA, 4 µL PCR water, 1.2 µL each of both forward and reverse primers, and 1.2 µL of probe. Thermal profile for real-time PCR was 50°C for 2 minutes, 95°C for 10 minutes followed by 45 cycles of 95°C for 30 seconds and 60°C for 60 seconds. Remaining cDNA was stored at -40°C. Real-time PCR determined the threshold (Cq values) for Bcl-2, Bax, and GAPDH. Relative quantification of the genes was done by $2^{-\Delta\Delta C_t}$ method.²⁵

Statistical analysis

The statistical analyses were done on SPSS version 19.0 (IBM Corporation, Armonk, NY, USA). The data were given as frequencies and percentages, or median and interquartile range, where applicable. Mann–Whitney *U*-tests were used to analyze the differences between the groups where applicable. Spearman's correlations (r_s) were also computed between variables. Kaplan–Meier survival analysis (log-rank test) was carried out to explore the relationship between gene expression and survival. The significance level was set at 5%, and a *p*-value <0.05 was considered significant.

Results

The baseline characteristics (Table 1) show that male to female ratio was 2.7 to 1, and the most common age group

was 15–40 years (68.8%). The most common AML subtype was “AML with maturation” (M2) (48.9%), followed by acute promyelocytic leukemia (APL) (M3) (18.9%). Special investigations for diagnosis or prognosis were carried out in patients as part of their diagnostic workup. Such investigations could only be performed for limited number of patients because of financial constraints. The myeloperoxidase (MPO) test was carried out in 84.4% of patients to determine the myeloid lineage, and the majority of patients were positive. FLT3 mutation and MLL translocation are considered adverse prognostic markers, while NPM1 mutation and PML-RAR translocation are considered favorable prognostic markers. The FLT3 test was performed in 42 patients out of which only seven were found positive, while MLL was performed in 15 patients out of which five were positive. NPM1 was investigated in 13 patients who were all negative. PML-RAR was performed in nine patients where five were positive. Karyotyping was performed in 49 patients, and the findings are given in Table 1. Karyotyping was categorized according to ELN classification. A total of 37.8% of patients showed resistance to chemotherapy, while 62.2% of patients achieved remission, and 34% of patients who achieved remission (21% overall) later presented with relapse. As described above, patients who achieved remission and did not relapse during the study period were labeled as “good responders” (GR) to therapy. All those patients who were either resistant to chemotherapy or relapsed later were grouped together as “poor responders” (PR). Survival of the study subjects was also noted. At the end of the study, 42 patients died while 44 were alive and four patients could not be traced for survival status.

To determine whether gene expression in bone marrow and peripheral blood correlated, we computed Spearman correlation coefficients because of wide variation in expression levels (Table 2). It is evident that Bax and Bcl-2 in bone marrow correlated significantly with their corresponding blood levels, but not consistently with ratios. Difference in gene expression was explored by using analysis of variance with post hoc Dunnett's test, which did not show any significant differences (Tables S1 and S2). Since, the gene expression data are not normally distributed, the data are presented using the median and interquartile range (Table 3) and expressed as boxplots on a logarithmic scale (Figure 1). Furthermore, we categorized the patients into either high or low expressers (HE or LE) of Bax and Bcl-2 based on the normalized expression, as described above²⁵ being greater than or up to 1, respectively, for meaningful analysis. HE and LE were analyzed through

Table 1 Baseline characteristics of study population (N=90)

Parameters	N	%
Age groups		
<15 years	3	3.3
15–40 years	62	68.9
41–60 years	24	26.7
>60 years	1	1.1
Gender		
Male	66	73.3
Female	24	26.7
AML classification (WHO)		
APL (M3) with t(15;17)	17	18.9
Translocation 6;9	2	2.2
AML with minimal differentiation (M0)	2	2.2
AML without maturation (M1)	15	16.7
AML with maturation (M2)	44	48.9
Acute myelomonocytic leukemia (M4)	2	2.2
Acute panmyelosis with fibrosis	1	1.1
Myeloid proliferations related to Down syndrome	1	1.1
Unknown	6	6.7
AML classification (prognostic)		
APL (M3)	17	18.9
Other (poor)	67	74.4
Unknown	6	6.7
Ethnicity		
Urdu	36	40.0
Sindhi	28	31.1
Pashto	9	10.0
Punjabi	3	3.3
Baloch	6	6.7
Gujrati/Katchhi	4	4.4
Unknown	4	4.4
MPO status		
Negative	14	15.6
Positive	62	68.9
Unknown	14	15.6
FLT3 mutation		
Negative	35	38.9
Positive	7	7.8
Unknown	48	53.3
NPM1 mutation		
Negative	13	14.4
Unknown	77	85.6
PML-RAR mutation		
Negative	4	4.4
Positive	5	5.6
Unknown	81	90.0
MLL mutation		
Negative	10	11.1
Positive	5	5.6
Unknown	75	83.3
Karyotyping		
Poor	18	20.0
Good	7	7.8
Normal	24	26.7
Unknown	41	45.6
Total	90	100.0
Karyotyping (prognostic)		
Unfavorable karyotype	18	20.0
Favorable karyotype	31	34.4
Unknown	41	45.6

(Continued)

Table 1 (Continued)

Parameters	N	%
Sample type		
Pre-chemotherapy sample	34	37.8
Post-chemotherapy sample	56	62.2
Therapeutic response		
Resistant	34	37.8
Relapse	19	21.1
Persistent remission	37	41.1
Survival status		
Dead	42	46.7
Alive	44	48.9
Unknown	4	4.4
Final outcome		
Poor	53	58.9
Good	37	41.1

Abbreviations: AML, acute myeloid leukemia; MPO, myeloperoxidase; APL, acute promyelocytic leukemia; PML-RAR, promyelocytic leukemia/retinoic acid receptor alpha; MLL, mixed lineage leukemia.

the Mann–Whitney *U*-test (Table 4). Bax/Bcl-2 ratio in marrow was found significantly higher in all other AML subtypes combined as compared to APL ($p<0.001$) and in MPO positive as compared to MPO negative patients ($p=0.03$). Mean ranks for Bax and Bcl-2 were higher in the GR group as compared to the PR group suggesting an overall better apoptosis response, but failed to reach statistical significance. However, Bax/Bcl-2 ratio was higher in the PR group (mean rank 44.1 vs 37.9; $p=0.07$) apparently due to relatively lower Bcl-2 values. Overall, Bax and Bcl-2 were expressed higher among GR as compared to PR (Figure 1) but did not reach statistical significance. The median and interquartile ranges of Cq values of GAPDH, the house-keeping gene used as control in our experiments, are also considered for comparison. It is noteworthy that although GAPDH Cq values are plotted on a linear scale, Bax and Bcl-2 gene expression data could be plotted on logarithmic scale, suggesting a skewed distribution.

A Kaplan–Meier analysis (Figure 2) was carried out on categorically transformed gene expression to explore its relationship with overall as well as disease-free survival (DFS) up to 1 year (52 weeks). We found no significant difference in DFS or OS according to gene expression.

Discussion

In this study, we observed no significant association of Bax or Bcl-2 expression with remission, DFS, or OS among AML patients. However, a higher Bax/Bcl-2 ratio was associated with poor chemotherapy outcome, despite higher absolute values of Bax and Bcl-2 in “good response” group. However, there are certain trends that need to be discussed, despite not

Table 2 Spearman correlation between gene expression in bone marrow and peripheral blood

Parameters			Bone Marrow			Blood		
			Bax	Bcl-2	Bax/Bcl-2	Bax	Bcl-2	Bax/Bcl-2
Bone marrow	Bax	rs	1	0.906**	-0.215	0.510**	0.432**	0.053
		N	82	82	82	69	69	69
	Bcl-2	rs		1	-0.563**	0.500**	0.503**	-0.108
		N		82	82	69	69	69
Blood	Bax/Bcl-2	rs		1	-0.233	-0.405**	0.414**	
		N		82	69	69	69	
	Bax	rs			1	0.847**	0.097	
		N			77	77	77	
Bcl-2	rs				1	-0.331**		
	N				77	77		
Bax/Bcl-2	rs					1		
	N					77		

Notes: Correlation is significant at the 0.05 level (two-tailed). **Correlation is significant at the 0.01 level (two-tailed).

Abbreviation: rs, Spearman rho.

being statistically significant. It is interesting to note that while some studies, like ours, have shown that these markers have no association with clinical outcome, others have shown a positive or negative association. Table 5 compares various contemporary studies in this regard. A number of factors could underlie this contrast, such as sample size, technique used, and pathology of the patients.

Studies have shown that increased Bax expression is a good predictor being a pro-apoptotic marker, as it is associated with a higher CR rate (n=34) in AML patients,²⁶ while in acute lymphoblastic leukemia (ALL), a low Bax expression was associated (n=14, immunoblotting) with relapse.¹⁵ In contrast, increased Bax expression has been found in association with decreased DFS (n=47, Western blotting)²⁷ and OS (n=232, real-time PCR).²⁸ However, a recent study conducted on ALL patients (n=26; real-time PCR)²⁹ and another study on endometrial cancer (n=89; immunohistochemistry) have shown no significant correlation of Bax expression with chemotherapy outcome, in agreement with our observations.²⁰

Similarly, Bcl-2 has been investigated in many cancers, yielding conflicting results. A recent study reported that decreased expression of Bcl-2 was found to be associated with a better CR rate in ALL but not in AML patients, which supports our observations.³⁰ However, they found that increased Bcl-2 expression was associated with relapse and higher bone marrow blast counts in both AML and ALL patients. It is notable that they had a small sample size (ALL=16 and AML=24) investigated using flow cytometry. Similarly, Kornblau et al³¹ has reported in a larger sample of AML patients (n=198; Western blotting) that high Bcl-2 expression was associated with a shorter median survival

in the favorable and intermediate cytogenetic group, while a longer median survival as well as remission duration in the poor cytogenetic group. We found that there was a tendency of higher Bcl-2 levels in APL patients as compared to other patient subtypes, as well as in those with persistent remission as compared to poor responders, although it failed to reach statistical significance (Table 4). However, others have reported significant differences. For example, it has been observed that Bcl-2-positive patients had better OS as compared to Bcl-2-negative patients in AML,³² colorectal carcinoma,³³ and breast cancer³⁴ patients. In contrast, another study with a smaller number of ALL and AML patients (n=26) showed no significant correlation with CR rate, DFS, or OS.²⁹ Similarly a larger study (n=502) conducted on prostate patients using immunohistochemistry did not find any significant correlation between Bcl-2 expression and clinical outcome.¹⁷ Likewise, other studies on malignancies such as ALL,¹⁵ endometrial cancer,²⁰ head and neck cancers,³⁵ ovarian cancer,^{21,36} hepatocellular carcinoma,³⁷ and colorectal carcinoma¹⁸ did not find significant association of Bcl-2 expression and clinical outcome. It is noteworthy that although being anti-apoptotic, it is easy to understand that a lower expression of Bcl-2 could translate into a better clinical outcome, but the paradoxical association with poor clinical outcome is difficult to explain. A partial explanation could be given by an observation that in mouse liver, Bcl-2 inhibits carcinogenesis and delays the growth of proliferative foci, thereby leading to a favorable outcome.³⁸ This is contrary to the widely accepted notion that Bcl-2 may be involved in the development of cancer and chemoresistance. Interestingly, Kasimir-Bauer et al,³⁹ who investigated the expression of Bcl-2, P-glycoprotein,

Table 3 Median and interquartile ranges of gene expression

Grouping variable	n	Bone marrow				Blood				
		Bax		Bcl-2		Bax		Bcl-2		
		Bax	Bcl-2	Bax/Bcl-2	Bcl-2	Bax	Bcl-2	Bax/Bcl-2		
Age group	82									
<40 years	60	0.66	0.26	2.51	0.21	0.77	0.21	3.20	(1.78-6.25)	
>40 years	22	1.01	0.25	5.42	0.19	0.62	0.19	4.12	(1.83-7.21)	
Gender	82									
Male	58	1.11	0.30	2.76	0.21	0.77	0.21	3.66	(1.83-6.84)	
Female	24	0.44	0.14	2.73	0.16	0.71	0.16	2.65	(1.53-7.95)	
AML classification	82									
APL (M3)	17	1.20	0.71	1.13	0.22	0.59	0.22	2.54	(1.12-5.88)	
AML with maturation (M2)	40	0.92	0.24	2.97	0.19	0.76	0.19	4.29	(1.93-7.27)	
AML without maturation (M1)	14	0.58	0.33	3.99	0.09	0.55	0.09	4.99	(1.67-20.99)	
Other AML subtypes	11	0.32	0.10	3.07	0.44	0.88	0.44	1.85	(0.87-2.68)	
Pre-/post-chemotherapy sample	82									
Pre-chemotherapy	32	1.02	0.33	2.21	0.16	0.71	0.16	4.12	(1.39-5.89)	
Post-chemotherapy	50	0.57	0.25	3.12	0.20	0.78	0.20	2.79	(1.81-7.62)	
Myeloperoxidase status	71									
Negative	13	1.20	0.20	3.82	0.18	0.95	0.18	5.50	(1.26-8.9)	
Positive	58	0.53	0.21	3.05	0.16	0.62	0.16	3.66	(1.92-7.24)	
FLT3 mutation	39									
Negative	33	0.76	0.15	3.02	0.20	0.73	0.20	3.89	(1.45-7.52)	
Positive	6	0.55	0.26	2.47	0.15	0.64	0.15	2.89	(2.27-4.15)	
Karyotyping	46									
Poor	16	0.65	0.26	2.83	0.46	1.95	0.46	3.91	(1.07-4.99)	
Good	7	1.02	0.41	1.33	0.07	0.59	0.07	3.75	(1.32-1797.1)	
Normal	23	0.76	0.16	4.12	0.17	0.84	0.17	3.49	(1.47-8.81)	
Remission status	82									
Resistant	32	0.48	0.22	3.07	0.19	0.77	0.19	2.89	(1.82-8.34)	
Relapse	15	1.02	0.30	3.07	0.10	0.33	0.10	3.49	(1.92-8.81)	
Remission	35	1.01	0.29	1.78	0.25	0.72	0.25	3.17	(1.39-6.46)	
Final outcome	82									
Poor	47	0.66	0.24	3.07	0.18	0.76	0.18	3.35	(1.89-8.46)	
Good	35	1.01	0.29	1.78	0.25	0.72	0.25	3.17	(1.39-6.46)	

Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia.

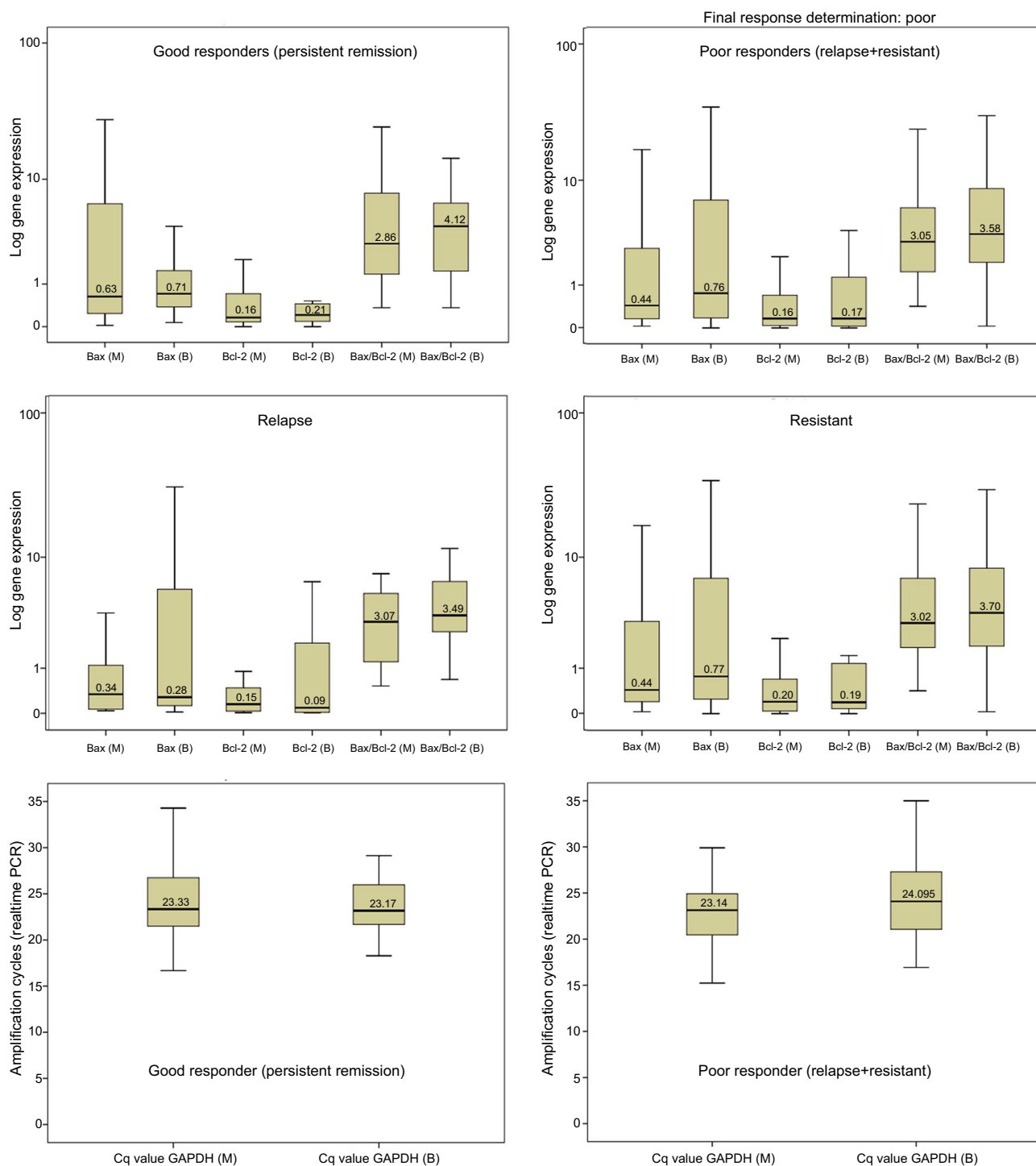


Figure 1 Median and interquartile ranges of gene expression. **Abbreviations:** B, blood; M, bone marrow.

multidrug resistance-related protein, p53, and heat shock protein 27 in AML patients (n=124), reported that although the individual markers did not correlate with OS, a combination of at least two proteins does so.

Likewise, Bax and Bcl-2 as a ratio (Bax/Bcl-2 or otherwise) has been widely studied as a prognostic marker of

chemotherapy success, as discussed below. One of the reasons to study Bax and Bcl-2 as a ratio was lack of clear evidence of them being a reliable marker when used alone, as mentioned earlier. A study on AML patients has shown that a higher Bax/Bcl-2 ratio is associated with a higher CR rate and a lower Bax/Bcl-2 ratio is associated with a poor outcome.⁴⁰

Table 4 Gene expression–Mann–Whitney *U*-test

Parameters	N	Groups	Groups, N	Mean rank	Sum of ranks	p-value
AML class: APL vs others (poor)						
Bone marrow						
Bax	78	APL	17	43.94	747.00	0.29
		Others	61	38.26	2334.00	
Bcl-2	78	APL	17	45.56	774.50	0.10
		Others	61	37.81	2306.50	
Bax/Bcl-2	78	APL	17	28.94	492.00	<0.001
		Others	61	42.44	2589.00	
Peripheral blood						
Bax	72	APL	14	30.71	430.00	0.17
		Others	58	37.90	2198.00	
Bcl-2	72	APL	14	34.14	478.00	0.50
		Others	58	37.07	2150.00	
Bax/Bcl-2	72	APL	14	36.36	509.00	0.96
		Others	58	36.53	2119.00	
MPO status						
Bone marrow						
Bax	71	Negative	13	39.62	515.00	0.42
		Positive	58	35.19	2041.00	
Bcl-2	71	Negative	13	41.65	541.50	0.13
		Positive	58	34.73	2014.50	
Bax/Bcl-2	71	Negative	13	29.58	384.50	0.03
		Positive	58	37.44	2171.50	
Peripheral blood						
Bax	67	Negative	12	38.75	465.00	0.26
		Positive	55	32.96	1813.00	
Bcl-2	67	Negative	12	35.88	430.50	0.59
		Positive	55	33.59	1847.50	
Bax/Bcl-2	67	Negative	12	32.92	395.00	0.72
		Positive	55	34.24	1883.00	
Final response: poor vs good						
Bone marrow						
Bax	82	Poor	47	40.69	1912.50	0.68
		Good	35	42.59	1490.50	
Bcl-2	82	Poor	47	39.60	1861.00	0.28
		Good	35	44.06	1542.00	
Bax/Bcl-2	82	Poor	47	44.14	2074.50	0.07
		Good	35	37.96	1328.50	
Peripheral blood						
Bax	77	Poor	46	40.24	1851.00	0.49
		Good	31	37.16	1152.00	
Bcl-2	77	Poor	46	39.54	1819.00	0.73
		Good	31	38.19	1184.00	
Bax/Bcl-2	77	Poor	46	39.64	1823.50	0.64
		Good	31	38.05	1179.50	

Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; MPO, myeloperoxidase.

The authors also reported that a low Bax/Bcl-2 ratio was characterized by relatively immature cell types (AML-M0 and M1 subtypes; CD34 >20%) and poor risk cytogenetics (complex patterns, hyperploid or polyploidy, numerical or structural deletions of chromosome 5 or 7, trisomy 8, t(9;22), 11q23 rearrangements, etc). In another large study on AML patients, lower Bax/Bcl-2 was associated with lower OS.²⁸

Other hematological malignancies have been studied in relation to the Bax/Bcl-2 ratio. In a study on a limited number of ALL patients (n=26), the high-risk group (n=9) had a higher Bax/Bcl-2 ratio at diagnosis than at remission, whereas no such association was observed in the median risk group.²⁹ Others reported that decreased Bax/Bcl-2 ratio was associated with relapse in ALL patients (n=14).¹⁵ Hogarth and Hall observed an

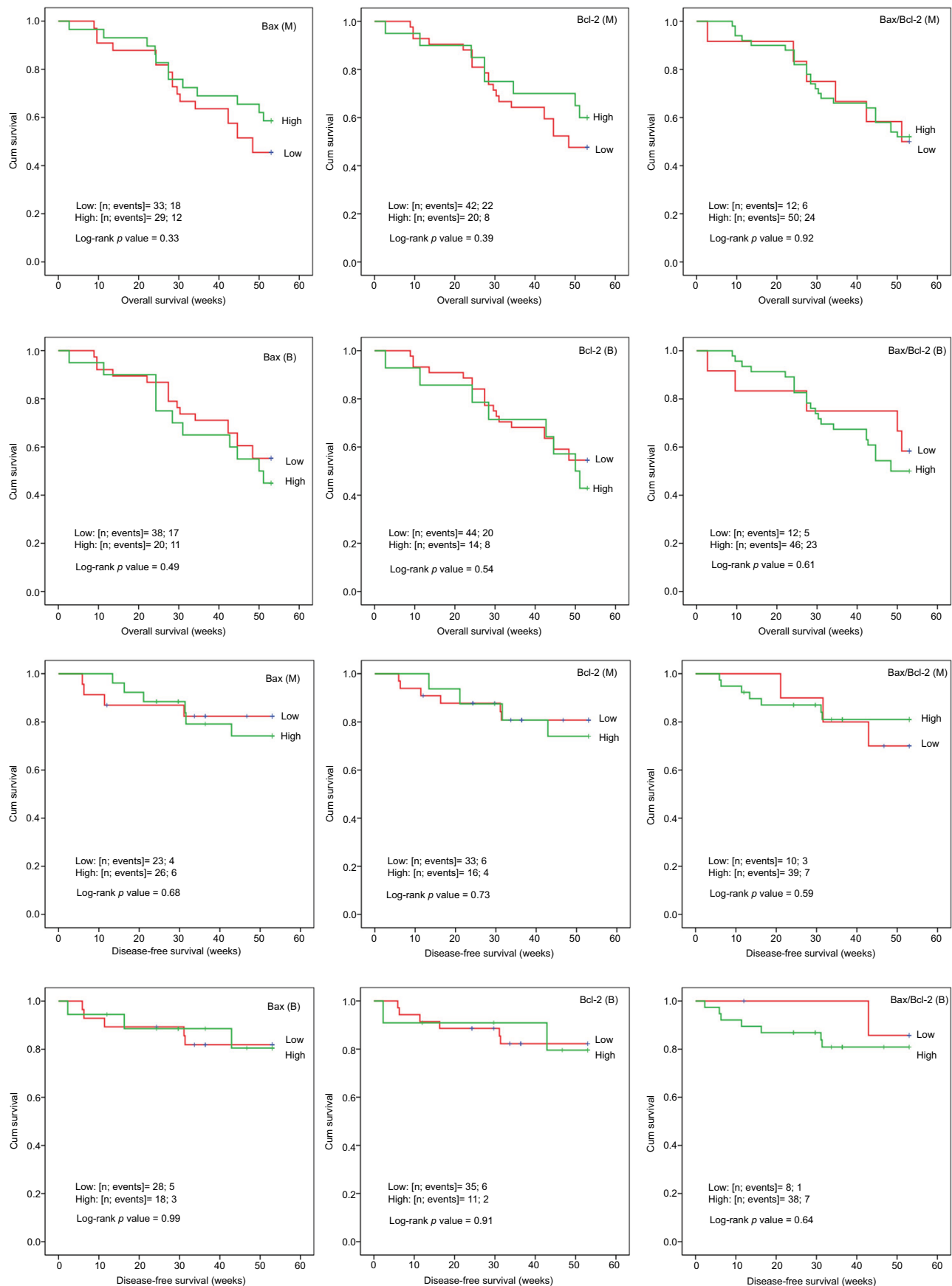


Figure 2 Kaplan–Meier survival curves according to gene expression.
Abbreviations: Cum, cumulative; B, blood; M, bone marrow.

Table 5 Comparison of contemporary literature on Bax, Bcl-2, and Bax/Bcl-2 ratio with our study

Study	Disease	No. of subjects	Technique	Findings
Bax expression				
Bekhet et al ²⁶	AML	34	RT-PCR	↑ Bax associated with ↑ CR
Garcia et al ³⁷	Hepatocellular carcinoma	70	IHC	↑ Bax associated with ↑ OS
Prokop et al ¹⁵	ALL	14	Immunoblotting	↓ Bax associated with relapse
Hogarth and Hall ²⁷	ALL	47	WB	↑ Bax associated with ↓ DFS
Köhler et al ²⁸	AML	232	RT-PCR	↑ Bax associated with ↓ OS
Kaparou et al ²⁹	AML	26	RT-PCR	No correlation with outcome
Peiró et al ⁴⁵	Endometrial cancer	89	IHC	No correlation with outcome
Bcl-2				
Kornblau et al ³¹	AML	198	WB	Expression associated with ↓ median survival if favorable/intermediate cytogenetics & ↑ median survival if poor cytogenetics
Aref et al ³⁰	AML and ALL	16 (ALL); 24 (AML)	FCM	↓ Expression → ↑ CR only in ALL, expression → ↑ relapse and blast % in both AML and ALL No significant correlation with OS in both AML and ALL
Sahu and Jena ³²	AML	110	IHC; blood only	Bcl-2 positive → better OS
Huang et al ³³ (meta-analysis)	Colorectal carcinoma	7658	IHC; 40 articles	↑ Bcl-2 associated with longer OS/DFS/RFS, if received adjuvant therapy before surgery
Hwang et al ³⁴	Breast cancer	7230	Meta-analysis	Positive Bcl-2 → favorable clinicopathologic features, OS & DFS
Kaparou et al ²⁹	AML & ALL	26	RT-PCR	No significant correlation between Bcl-2 expression and clinical outcome
Khor et al ¹⁷	Prostate cancer	502	IHC	
Prokop et al ¹⁵	ALL	14	Immunoblotting	
Peiro et al ⁴⁵	Endometrial cancer	89	IHC	
Casado et al ³⁵	Head and neck cancers	43	IHC	
Sagarra et al ³⁶	Epithelial and serous ovarian cancer	90	IHC	
Palmer et al ²¹		132	IHC	
Garcia et al ³⁷	Hepatocellular carcinoma	70	IHC	
Katkoori et al ¹⁸	Colorectal adenocarcinoma	112	IHC	
Pierce et al ³⁸	Mouse liver			Bcl-2 inhibits carcinogenesis and delays the growth of proliferative foci
Bax/Bcl-2				
Scopa et al ⁴⁶	Rectal carcinoma	49	IHC	↓ Bax/Bcl-2 → radioresistance
Del Poeta et al ⁴⁰	AML	255	FCM	↑ Bax/Bcl-2 ratio is associated with ↑ CR and ↓ Bax/Bcl-2 ratio is associated with AML-M0 & MI and CD34 >20%
Köhler et al ²⁸	AML	232	RT-PCR	↓ Bax/Bcl-2 associated with ↓ OS
Kasimir-Bauer et al ³⁹	AML	124	FCM	A combination of at least two proteins out of Bcl-2, P-gp, MDR, p53, and hsp27 correlates with OS
Kaparou et al ²⁹	ALL	26	RT-PCR	↑ Bax/Bcl-2 at diagnosis than at remission in high-risk group patients, no such association observed in the medium-risk group At diagnosis, Bax/Bcl-2 ratio in children >10 years and with WBC count, DNA index <1.16 (at diagnosis & remission) & del(9p)
Prokop et al ¹⁵	ALL	14	Immunoblotting	↓ Bax/Bcl-2 ratio → relapse
Hogarth and Hall ²⁷	ALL	47	WB	↑ Bcl-2/Bax and Mcl-1/Bax in B lineage as compared to T lineage. Bax → relapse

(Continued)

Table 5 (Continued)

Study	Disease	No. of subjects	Technique	Findings
Katkoori et al ¹⁸	Colorectal adenocarcinoma	112	Immunophenotyping	↓ Bax/Bcl2 ratio → better OS
Khodapasand et al ⁴¹	Colon rectal cancers	22	RT-PCR	The Bax/Bcl2 ratio decreased with increasing age and in colon cancers (vs sigmoid colon and rectosigmoid)
Khor et al ¹⁷	Prostate cancer	502	IHC	“Negative Bcl-2/Normal Bax” → better response rate to short-term androgen therapy (not long-term androgen therapy)
Our study	AML	90	RT-PCR	Bax/Bcl-2 ratio did not differ among GR and PR ↓ Bax/Bcl-2 ratio in APL (vs others) in marrow ↓ Bax/Bcl-2 ratio in MPO negative in marrow samples

Abbreviations: AML, acute myeloid leukemia; RT-PCR, real-time polymerase chain reaction; IHC, immunohistochemistry; ALL, acute lymphoblastic leukemia; WB, Western blotting; GR, good responders; PR, poor responders; MPO, myeloperoxidase; OS, overall survival; DFS, disease-free survival; CR, complete remission.

increased Bcl-2/Bax ratio (reversed ratio, translating into low Bax/Bcl-2) and myeloid cell leukemia factor 1 (Mcl-1)/Bax ratio in B lineage as compared to T lineage cells.²⁷ They also reported that high Bax expression was associated with a higher probability of relapse in ALL patients (n=47). Similarly, the Bax/Bcl-2 ratio has been studied in solid organ tumors, again with conflicting results. In rectal carcinoma (n=49), a lower Bax/Bcl-2 ratio was found associated with radio resistance,¹⁶ while others reported that a lower Bax/Bcl-2 ratio correlated with better OS in colorectal adenocarcinoma (n=112).¹⁸ However, the ratio shows variability with age and site of involvement, as reported by a small study (n=22).⁴¹ Considering the skewed distribution of data in many studies and its possible role in conflicting results, some authors have attempted to rather categorize the expression data. In a large study among prostate cancer patients (n=502), a multivariate analysis found that Bcl-2 overexpression was not related independently to mortality and metastasis. However, a combination of “negative bcl-2 and normal Bax” was associated with slightly better response rate to short-term androgen therapy but not with long-term androgen therapy.¹⁷

In our study, Bax/Bcl-2 ratio was not significantly different among good and poor responders; however, APL, a subtype of AML with better prognosis than all other subtypes combined, showed significantly lower Bax/Bcl-2 ratio as compared to other subtypes (mostly M0, M1, and M2) ($p < 0.001$) in bone marrow samples. When considering Bax and Bcl-2 expression separately rather than as a ratio, it was observed that their expression was higher in APL patients as compared to other patient subtypes, although it failed to reach statistical significance. It is noteworthy that APL patients had a favorable outcome as denoted by significantly lower

rates of relapse ($\chi^2(1) = 6.3, p = 0.012$), poor final therapeutic response ($\chi^2(1) = 15.5, p < 0.001$), and mortality ($\chi^2(1) = 4.3, p = 0.05$) as compared to other AML subtypes combined (data not shown). Likewise, bone marrow Bax/Bcl-2 ratio was lower in MPO-negative as compared to MPO-positive patients ($p = 0.03$), where MPO was significantly positive among AML subtypes with poor prognosis (subtypes other than APL) ($\chi^2(1) = 13.7, p < 0.001$), and those who had relapse ($\chi^2(1) = 4.3, p = 0.033$), or had poor final therapeutic response ($\chi^2(1) = 8, p = 0.007$) (data not shown). Thus, APL subtype and negative MPO were found as good prognostic factors but the Bax/Bcl-2 ratio was lower in those subgroups.

Thus, we can conclude from the above discussion that the expression pattern of Bax, Bcl-2, and their ratio not only differs between various cancers but even within the same cancer. Such conflicting reports might suggest a more complicated role of Bax, Bcl-2, or their ratio and needs further investigation. It is evident that some of the inconsistencies could be explained by the type of cancer, the source and size of the sample, the variance of data, and treatment options or techniques used.

Recently, multiple studies have focused on the development of anti-Bcl-2 drugs.^{22,23} Although in vitro results show some positive findings, the ultimate efficacy needs to be evaluated cautiously due to the conflicting results discussed above. Hence, we recommend that the process of apoptosis should first be explored in depth to understand the role of different members of apoptotic pathway before conducting clinical trials on patients with new potential therapeutic agents.

Our study has some limitations as discussed below. We included patients available from a single hematology center. The survival analysis was available up to only 1 year. A long-term follow-up is required for more conclusive results.

Another limitation is that we focused on only Bax and Bcl-2 among various apoptosis-related proteins. As discussed above, both are frequently studied members of apoptosis-related proteins but still have conflicting evidence in terms of utility in cancer chemotherapy, thus making them a logical choice to study in resource-constrained situations like ours. Recently, Mcl-1, a Bcl-2 family member has gained importance as a regulator of apoptosis.⁴² Mcl-1 has multiple variants due to alternative splicing. One of them is “long isoform” (Isoform 1) that inhibits apoptosis while two other “short isoforms” (Isoforms 2 and 3) promote apoptosis. This is interesting that the same gene codes for both anti- and pro-apoptotic protein isoforms which regulate each other as well.⁴³ However, it has been shown in vitro that Bcl-2 rather than Mcl-1 is a predominant marker in leukemia.⁴⁴ Further studies should be designed to investigate the role of Mcl-1 and other members of apoptosis regulating proteins in relation to chemotherapy response in AML patients.

This study has important advantages. The sample size was larger than many contemporary studies. We used a dual approach to study both the actual expression and their ranking, as discussed above. Furthermore, we analyzed the expression in bone marrow and blood separately, thus avoiding the bias of sample pooling. Such an approach is based on the fact that AML is a disease in bone marrow rather than peripheral blood; therefore, bone marrow but not peripheral blood should logically be the appropriate substrate in such studies. It is also evident that a difference of results between the two types of samples supports our approach. Another advantage was inclusion of concurrent bone marrow and blood samples from the majority of patients. Therefore, we were able to compare the two types of samples confidently. Finally, the patients in our study received similar treatment, thus allowing direct statistical comparisons to be made. We used real-time PCR which is more sensitive and accurate than immunohistochemistry, Western blotting, and flow cytometry that are used in many studies.

Conclusion

The findings of our study suggest that there is no significant association between the expression of Bax and Bcl-2 and their ratio with clinical response, or with 1 year DFS and OS. Although both Bax and Bcl-2 had higher expression in those with persistent remission (good response group), a higher Bax/Bcl-2 ratio was more common among those who were resistant or had relapse (poor response group) (Table 4). The overall picture reflects a complex balance between Bax

and Bcl-2, and hence their ratio could be misleading if interpreted incautiously. Based on our findings, we recommend that development of anti Bcl-2 therapy should be pursued with extreme caution in patients until the role of Bcl-2 is more clearly understood in cancer-specific apoptosis. Our findings suggest that for these types of analyses, pooling of bone marrow and blood samples is likely to generate a bias. Thus, the body of literature that reported findings based on pooled bone marrow and blood samples should be revisited to confirm their conclusions.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1: One-way ANOVA test to analyze Bax and Bcl-2 expression difference between various outcomes

Gene expression	Remission status	N	Mean	SE	95% CI		p-value
					Lower	Upper	
Bax expression marrow	Resistant	31	7.23	13.25	-0.24	14.71	0.49
	Relapse	15	3.75	1.69	0.12	7.39	
	Remission	31	13.35	304.25	-0.57	27.28	
	Total	77	9.02	130.31	2.78	15.26	
Bcl-2 expression marrow	Resistant	31	4.89	5.90	-2.06	11.85	0.53
	Relapse	15	3.48	1.94	-0.69	7.65	
	Remission	31	12.65	362.11	-4.07	29.37	
	Total	77	7.74	155.35	0.60	14.88	
Ratio Bax/Bcl-2 marrow	Resistant	31	3609.67	2792.84	2276.34	9495.67	0.32
	Relapse	15	3.18	0.55	1.99	4.37	
	Remission	31	5.56	1.08	3.15	7.97	
	Total	77	1456.10	1095.80	-867.58	3779.78	
Bax expression blood	Resistant	30	10.59	11.89	-2.08	23.26	0.56
	Relapse	14	4.14	1222.59	-0.70	8.99	
	Remission	28	4.66	1330.16	-0.45	9.78	
	Total	72	7.03	583.28	1.48	12.59	
Bcl-2 expression blood	Resistant	30	3.84	11.44	-1.28	8.95	1.00
	Relapse	14	4.14	239.17	-2.93	11.21	
	Remission	28	3.92	2608.46	-2.92	10.76	
	Total	72	3.93	1051.18	0.42	7.43	
Ratio Bax/Bcl-2 blood	Resistant	30	659.46	10.43	-673.06	1991.98	0.68
	Relapse	14	9.93	54.06	-2.62	22.48	
	Remission	28	259.33	6846.27	-263.95	782.61	
	Total	72	377.56	2759.30	-196.11	951.23	

Abbreviation: SE, standard error.

Table S2 Post hoc analysis (Dunnett's two-tailed): persistent remission was considered as control group

Dependent variable	Remission status (a)	Remission status (b)	Mean difference (a-b)	SE	95% CI		p-value
					Lower	Upper	
Bax expression marrow	Resistant	Remission	-6.12	7.01	-21.99	9.75	0.60
	Relapse	Remission	-9.60	8.68	-29.25	10.05	0.45
Bcl-2 expression marrow	Resistant	Remission	-7.76	8.03	-25.94	10.42	0.54
	Relapse	Remission	-9.17	9.94	-31.68	13.34	0.57
Ratio Bax/Bcl-2 marrow	Resistant	Remission	3604.11	2595.18	-2274.13	9482.35	0.29
	Relapse	Remission	-2.38	3213.55	-7281.28	7276.52	1.00
Bax expression blood	Resistant	Remission	5.93	6.25	-8.24	20.09	0.55
	Relapse	Remission	-0.52	7.78	-18.17	17.13	1.00
Bcl-2 expression Blood	Resistant	Remission	-0.08	3.97	-9.09	8.93	1.00
	Relapse	Remission	0.23	4.95	-11.00	11.45	1.00
Ratio Bax/Bcl-2 Blood	Resistant	Remission	400.13	647.12	-1067.06	1867.32	0.77
	Relapse	Remission	-249.40	806.11	-2077.05	1578.25	0.94

Abbreviation: SE, standard error.

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