doi:10.4149/neo_2013_085

Overexpression of Bcl2 protein predicts chemoresistance in acute myeloid leukemia: Its correlation with FLT3

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Received February 6, 2013 / Accepted March 28, 2013

Potential prognostic biomarkers in acute myeloid leukemia (AML) can be identified by understanding the cellular pathway and molecular changes underlying leukemogenesis. Deregulation of apoptosis is one of the important features of AML and to understand the molecular mechanism underlying apoptosis and its contribution to tumor progression, this study aimed to evaluate anti-apoptotic Bcl2 protein expression in AML and correlate with FLT3 parameters for their role in prognosis of disease.

Bcl2 and FLT3 protein expression was quantified by flow cytometry on leukemic blasts in total 174 de novo AML, myelodysplastic syndrome (MDS) and aplastic anemia patients. FLT3 internal tandem duplication (ITD), Tyrosine kinase domain (TKD) point mutations and quantification of mRNA level was carried out using PCR and RT-PCR methods.

The incidence of Bcl2 positivity was 71% in AML patients. Bcl2 positivity was significantly associated with CD34+ and CD117+ AML. Bcl2 positivity tended to be associated with reduced DFS while Bcl2 positivity with FLT3 protein positivity was significantly associated with reduced DFS. In multivariate analysis, Bcl2+ and combined Bcl2+/FLT3 protein+ along with high WBC count emerged as poor prognostic factors for reduced DFS and high blast count for predicting reduced OS. In MDS patients, the incidence of Bcl2 expression was high while in aplastic anemia patients, incidence of Bcl2 expression was low.

Patients with Bcl2 and FLT3 protein positivity showed significantly reduced DFS suggesting parallel role of these proteins in imparting chemoresistance to the leukemic cells.

Key words: Bcl2, FLT3, AML, MDS, aplastic anemia, flow cytometry, PCR, RTPCR

Acute myeloid leukemia (AML) is a highly heterogenous disease [1, 2] and despite recent progress in molecular genetics, the understanding of mechanisms underlying the leukemic growth and response to treatment is limited [2]. This has lead to lack of efficient biomarkers in clinical diagnosis in AML. Biomarkers are required to enable identification of patients who are most likely to benefit from specific treatments and to help improve clinical outcome and treatment design [3]. Potential therapeutic targets and prognostic biomarkers of AML patients can be identified by understanding the cellular pathways and molecular changes involved in malignant transformation.

Apoptosis plays key role in the control of tissue homeostasis, such as rapidly renewing hematopoietic tissue [4] and deregulation of apoptosis may give rise to neoplastic transformation. Several lines of evidence indicate that leukemic cells undergo apoptosis in response to chemotherapy, suggesting an association between therapy-induced apoptosis and therapeutic efficacy in AML [5]. Abnormalities in the apoptotic pathway give survival advantage to the leukemic cells and thereby play major role in development of drug resistance [4].

Major regulators of apoptotic pathway are Bcl2 (B cell lymphoma 2) family of proteins. Some of these proteins (Bcl2, Bcl-XL) are anti-apoptotic while others (Bad, Bax, Bid) are pro-apoptotic. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic Bcl2 proteins [6]. In AML, Bcl2 overexpression is linked with resistance to chemotherapy and short survival [7, 8]. Therefore, detection of Bcl2 protein expression in AML is appealing as a potential prognostic biomarker.

The present study evaluated Bcl2 protein expression in de novo AML, myelodysplastic syndrome (MDS) and aplastic

anemia patients. Further, the protein expression was correlated with clinical and haematologic parameters, disease status and treatment offered along with Fms-like tyrosine kinase-3 (FLT3) mutation status, mRNA and FLT3 protein expression in AML patients.

Patients and methods

Patients. Total 174 patients with de novo AML [N=144], MDS [N=9] and aplastic anemia [N=21], referred to Gujarat Cancer and Research Institute for diagnosis and treatment from 2009 to 2011, were enrolled in the study. Bone marrow or peripheral blood samples were collected in ethylenediamine tetraacetic acid (EDTA) vacuette at the time of diagnosis.

The diagnosis and classification of the acute leukemia samples were based on morphology and cytochemistry according to the French-American-British (FAB) classification and by immunophenotyping as per the criteria of the European Group for the Immunological Characterization of Leukemias [9]. The patients' detailed clinical history was noted from the case files maintained at the Medical Record Department of the Institute. This study was approved by the Institutional Scientific Review Board and Ethics Committee. Patients provided informed consent to use their sample for the study.

Therapy. Out of 144 AML patients enrolled in the study, 76 patients underwent treatment while 68 patients were either lost to follow up or died within a month. Disease remission was achieved in 30% (23/76) while disease relapse was noted in 70% (53/76) of AML patients. The treatment protocols were decided by clinicians of the Institute. Adult AML non-M3 patients were treated with 7+3 protocol, pediatric AML non-M3 patients were treated with Berlin Frankfurt Munster (BFM) 93 and AML M3 patients were treated with All Trans Retinoic Acid (ATRA) and / or Arsenic trioxide in combination with anthracycline. Oral chemotherapy was given to 17 patients. Detailed treatment protocol has been described earlier [10].

Flow cytometry analysis. Surface and cytoplasmic protein expression were studied using bone marrow or peripheral blood samples on intact leukemic blasts by flow cytometric analysis along with immunophenotypic study. The unstained or isotype controls for surface and cytoplasmic antigens were simultaneously stained and CD45 antibody was added in each tube for leukemic blast gating. For the study of cytoplasmic Bcl2 protein expression, 2 ml Lysing solution (1:10 dilution, BD Biosciences) was added to adjusted cell count of 1 x 10^6 mononuclear cells per 100 µl sample and incubated for 15 minutes. Then samples were centrifuged at 400 g for 5 minutes. The supernatant was discarded and 1 ml Perm/Wash buffer (1:10 dilution, BD Biosciences) was added and incubated for 20 minutes. Then samples were centrifuged at 400 g for 5 minutes and the supernatant was discarded. The monoclonal antibodies fluorescein isothiocyanate (FITC) conjugated anti-Bcl2 (Bcl2/100) along with peridinin-chlorophyll protein (PerCp) conjugated anti-CD45 (2D1) (20 µl each) were added to the pellet and incubated for 15 minutes. Then 2 ml phosphate buffered saline (PBS) was added and the samples were centrifuged at 400 g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 500 µl PBS [11].

To study the surface FLT3 protein expression, monoclonal antibody phycoerythrin (PE) conjugated anti-FLT3 (4G8) was added along with FITC conjugated anti-CD34 (8G12) and PerCp conjugated CD45 (20 µl each) to 100 µl sample (1 x 10^6 mononuclear cells) and incubated for 15 minutes. After addition of 2 ml Lysing solution (1:10 dilution, BD Biosciences), the samples were incubated for another 15 minutes. Then samples were centrifuged at 400 g for 5 minutes. The supernatant was discarded and the remaining pellet was washed twice with PBS and then resuspended in 500 µl PBS [11].

For lineage assignment the following combinations of monoclonal antibodies were used as the primary panel: CD22 (S-HCL-1; FITC) / CD34 (8G12; PE) / CD5 (L17F12; Phycoerythrin cyanine 7 (PE-CY7)) / CD10 (HI10a; Allophycocyanin (APC)) / CD19 (SJ25C1; Allophycocyanin cyanine 7 (APC-Cy7)) / CD45, CD7 (4H9; FITC) / CD13 (L138; PE) / CD33 (P67.6; PE-Cy7) / CD117 (104D2; APC) / HLA-DR (L243; APC-Cy7) / CD45, cytoMPO (5B8; FITC) / cytoCD79a (2ST8.5H7; PE) / cytoCD3 (SK7; PE-Cy7) / nTdt (E17-1519; APC) / CD45. All the antibodies and reagents were procured from BD Biosciences (San Jose, USA) and followed the manufacturer's protocol.

Statistical analysis. Statistical analysis was carried out using SPSS statistical software version 17 (SPSS Inc, USA). Pearson's Chi-square test with Pearson's correlation coefficient (r) was used to assess correlation and significance between the two parameters. Univariate survival analysis was carried out by Kaplan and Meier method and Log Rank statistics was used to assess the prognostic significance of disease free survival (DFS) and overall survival (OS). Multivariate survival analysis was performed using Cox regression model with forward stepwise (likelihood ratio) method. The Wald statistics and relative risk [Exp(B)] with 95% confidence interval (CI) for Exp(B) were used to evaluate the prognostic significance. P values ≤ 0.05 were considered significant.

Results

Anti-apoptotic protein Bcl2 expression in acute myeloid leukemia. Bcl2 positivity was noted in 71% (102/144) of AML patients (Figure 1).

Correlation of Bcl2 with clinical and hematological parameters. With clinical parameters, a trend of high incidence of Bcl2 positivity was noted in patients of pediatric (<15 years; 79%, 22/28) and younger (15 to 59 years; 72%, 69/96) age groups as compared to older (≥60 years; 55%, 11/20) age group while with gender, similar incidence of Bcl2 positivity was noted (Table 1).

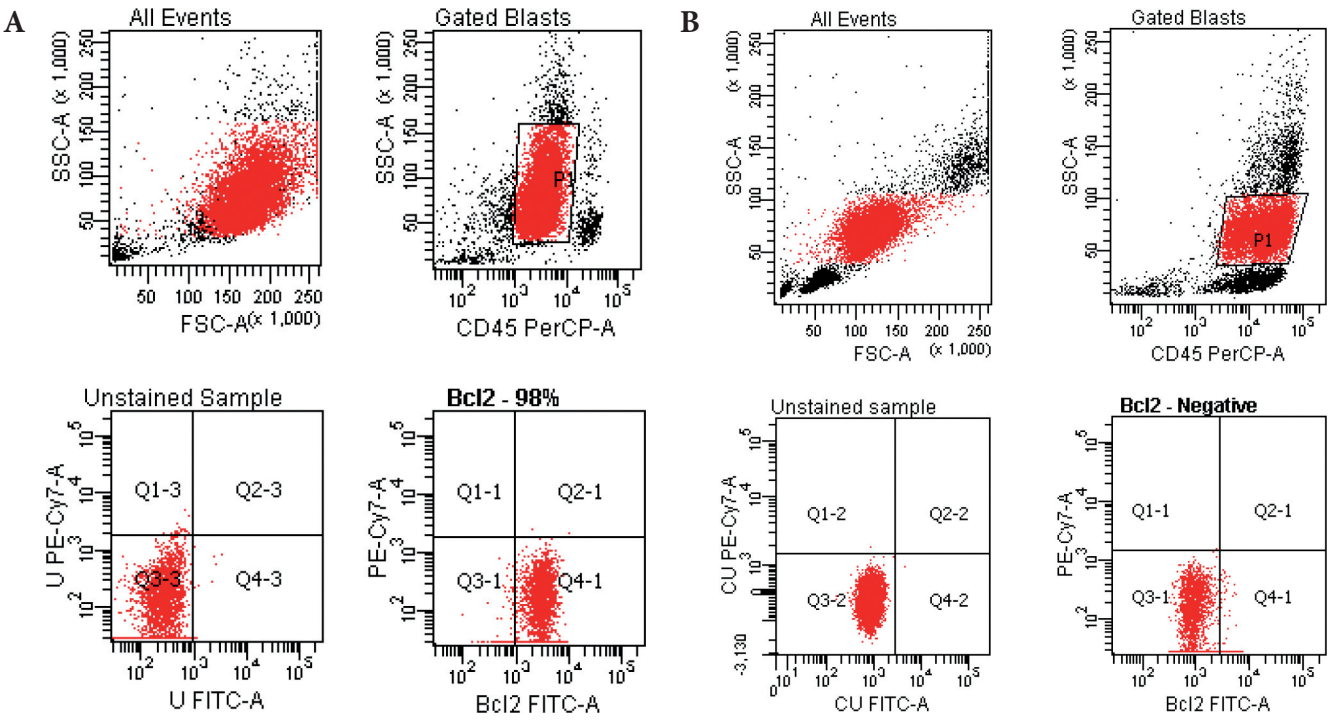

Figure 1. A. Representative flow cytometric dot plots of Bcl2 expression in bone marrow of AML M1 patient (1) FSC versus SSC dot plot; (2) Myeloblasts gated on CD45 PerCp versus SSC dot plot; (3) Unstained sample as negative control; (4) Bcl2 positivity (98%) on myeloblasts. FITC, fluoroscein isothiocyanate; PerCp, peridinin-chlorophyll protein. B. Representative flow cytometric dot plots of Bcl2 negativity in peripheral blood of AML M5a patient (1) FSC versus SSC dot plot; (2) Myeloblasts gated on CD45 PerCp versus SSC dot plot; (3) Unstained sample as negative control; (4) Bcl2 negativity on myeloblasts.

In relation to AML subtypes, significantly high incidence of Bcl2 positivity was noted in patients with AML M4 (94%, 17/18) as compared to M1 (85%, 23/27), M5a (65%, 11/17), M2 (58%, 21/36), M5b (57%, 8/14) and M3 (50%, 6/12) subtypes (X^2=18.71, r=+0.02, P=0.01). Both the patients with AML M6 subtype showed Bcl2 positivity (100%, 2/2) while one patient with AML M7 subtype did not show Bcl2 positivity.

With hematological parameters, a trend of high incidence of Bcl2 positivity was noted in patients with high N:C (nuclear: cytoplasmic) ratio (74%, 64/87) as compared to moderate N: C ratio (50%, 5/10) while incidence of Bcl2 positivity showed no significant difference in subgroups of blast count, WBC count, platelet count, RBC count, hemoglobin level, M:E (myeloid:erythroid) ratio and sudan black B stain (cytochemical stain).

Correlation of Bcl2 with CD34 expression and leukemia associated immunophenotype (LAP). Bcl2 protein when correlated with CD34 (Table 2), significantly high incidence of Bcl2 positivity was noted in CD34+ AML (77%, 67/87; P=0.04) as compared to CD34- AML (61%, 35/57).

Further, Bcl2 when correlated with the myeloid lineage markers (CD13, CD33, CD117 and MPO), significantly high incidence of Bcl2 positivity was noted in CD117+ AML (80%, 86/108; P=0.0001) as compared to CD117- AML (44%, 16/36) while incidence of Bcl2 positivity showed no significant difference in subgroups of CD13, CD33 and MPO.

With non-lineage markers, incidence of Bcl2 positivity showed no significant difference in the subgroups of Tdt and HLA-DR. With respect to aberrant B cell markers, CD19 was expressed in 19% (27/144) of patients whereas CD79a and CD22 were aberrantly expressed in two patients. Incidence of Bcl2 positivity showed no significant difference in the subgroups of CD19. With respect to aberrant T cell markers, CD3 was expressed in 18% (26/144) and CD7 in 14% (19/140) of patients whereas CD2 and CD5 were aberrantly expressed in two and one patient respectively. A trend of high incidence of Bcl2 positivity was noted in CD7+ AML (84%, 16/19) as compared to CD7- AML (69%, 84/121) while showing no significant difference in the subgroups of CD3.

Correlation of Bcl2 protein expression with FLT3 mutation status, FLT3 mRNA transcript and FLT3 protein expression. FLT3 ITD mutations were detected through PCR amplification using mutation specific primers [12]. FLT3 TKD point mutations were detected through RT-PCR followed by restriction enzyme EcoRV digestion. FLT3 ITD mutation to wild type ratio and mRNA transcript levels were calculated based on the quantification of DNA and mRNA. The procedure for detection of these mutations as well as quantification of DNA and mRNA was carried out as described earlier [10].

Table 1. Correlation of Bcl2 expression with clinical and hematological parameters

	N (%)	Bcl2 Negative N (%)	Bcl2 Positive N (%)	X^2	r	P value
All patients	**144**	**42 (29)**	**102 (71)**			
Age	**144**	**42**	**102**			
<15 years	28 (19)	06 (21)	22 (79)	3.28	-0.14	0.19
15 to 59 years	96 (67)	27 (28)	69 (72)			
≥ 60 years	20 (14)	09 (45)	11 (55)			
Gender	**144**	**42**	**102**			
Male	86 (60)	25 (29)	61 (71)	0.001	-0.003	0.97
Female	58 (40)	17 (29)	41 (71)			
Subtypes	**144**	**42**	**102**			
M1	27 (19)	04 (15)	23 (85)	18.71	+0.02	0.01*
M2, M2E0, M2B	36 (25)	15 (42)	21 (58)			
M3	12 (08)	06 (50)	06 (50)			
M4, M4E0	18 (12)	01 (06)	17 (94)			
M5a	17 (12)	06 (35)	11 (65)			
M5b	14 (10)	06 (43)	08 (57)			
M6	02 (01)	00 (00)	02 (100)			
M7	01 (01)	01 (100)	00 (00)			
Unknown	17 (12)	03 (18)	14 (82)			
Blast count (Median= 61%)	**144**	**42**	**102**			
< 61	71 (49)	21 (30)	50 (70)	0.01	+0.009	0.91
≥ 61	73 (51)	21 (29)	52 (71)			
WBC count (x 10^3/µl)	**144**	**42**	**102**			
< 4	07 (05)	01 (14)	06 (86)	1.05	-0.03	0.58
4 to 11	17 (12)	06 (35)	11 (65)			
>11	120 (83)	35 (29)	85 (71)			
Platelet count (x 10^5/µl)	**144**	**42**	**102**			
< 1.5	128 (89)	38 (30)	90 (70)	0.47	+0.04	0.78
1.5 to 4.5	15 (10)	04 (27)	11 (73)			
> 4.5	01 (01)	00 (00)	01 (100)			
RBC count (x 10^6/µl)	**144**	**42**	**102**			
< 3.8	134 (93)	39 (29)	95 (71)	2.63	-0.04	0.26
3.8 to 4.8	09 (06)	02 (22)	07 (78)			
> 4.8	01 (01)	01 (100)	00 (00)			
Hemoglobin level (gm/dL)	**144**	**42**	**102**			
< 9.0	110 (76)	31 (28)	79 (72)	0.21	-0.03	0.64
≥ 9.0	34 (24)	11 (32)	23 (68)			
M:E Ratio	**115**	**34**	**81**			
Normal (2:1 – 4:1)	02 (02)	00 (00)	02 (100)	3.52	+0.11	0.31
Altered	94 (81)	31 (33)	63 (67)			
Increased	16 (14)	03 (19)	13 (81)			
Decreased	03 (03)	00 (00)	03 (100)			
N:C ratio	**97**	**28**	**69**			
Moderate	10 (10)	05 (50)	05 (50)	2.42	+0.15	0.11
High	87 (90)	23 (26)	64 (74)			
Cytochemical stain	**102**	**25**	**77**			
SBB Negative	09 (09)	01 (11)	08 (89)	3.17	-0.16	0.20
SBB weak positive	17 (17)	02 (12)	15 (88)			
SBB Positive	76 (74)	22 (29)	54 (71)			

*P value ≤ 0.05 is significant

M:E ratio, Myeloid:Erythroid ratio; N:C ratio, Nuclear:Cytoplasmic ratio; SBB, Sudan Black B

Table 2. Correlation of Bcl2 with CD34 expression and leukemia associated immunophenotype (LAP)

	N	Bcl2 Negative N (%)	Bcl2 Positive N (%)	X^2	r	P value
Progenitor cell marker						
CD34	**144**	**42**	**102**			
Negative	57	22 (39)	35 (61)	4.06	+0.16	0.04*
Positive	87	20 (23)	67 (77)			
Myeloid lineage markers						
CD13	**144**	**42**	**102**			
Negative	06	01 (17)	05 (83)	0.05 **a**	-0.05	0.81
Positive	138	41 (30)	97 (70)			
CD33	**144**	**42**	**102**			
Negative	08	01 (13)	07 (87)	0.44 **a**	-0.08	0.50
Positive	136	41 (30)	95 (70)			
CD117	**144**	**42**	**102**			
Negative	36	20 (56)	16 (44)	16.17	+0.33	0.0001*
Positive	108	22 (20)	86 (80)			
MPO	**144**	**42**	**102**			
Negative	65	20 (31)	45 (69)	0.14	+0.03	0.70
Positive	79	22 (28)	57 (72)			
Non-lineage markers						
Tdt	**89**	**22**	**67**			
Negative	80	21 (26)	59 (74)	0.34 **a**	+0.10	0.55
Positive	09	01 (11)	08 (89)			
HLA-DR	**144**	**42**	**102**			
Negative	30	10 (33)	20 (67)	0.31	+0.04	0.57
Positive	114	32 (28)	82 (72)			
CD10	**144**	**42**	**102**			
Negative	143	41 (29)	102 (71)	0.21 **a**	-0.13	0.64
Positive	01	01 (100)	00 (00)			
Aberrant B cell markers						
CD79a	**144**	**42**	**102**			
Negative	142	41 (29)	101 (71)	0.0001 **a**	-0.05	1.00
Positive	02	01 (50)	01 (50)			
CD19	**144**	**42**	**102**			
Negative	117	33 (28)	84 (72)	0.27	-0.04	0.59
Positive	27	09 (33)	18 (67)			
CD22	**79**	**19**	**60**			
Negative	77	19 (25)	58 (75)	0.0001 **a**	+0.09	1.00
Positive	02	00 (00)	02 (100)			
Aberrant T cell markers						
CD3	**144**	**42**	**102**			
Negative	118	32 (27)	86 (73)	1.32	-0.09	0.24
Positive	26	10 (39)	16 (61)			
CD2	**59**	**22**	**37**			
Negative	57	22 (39)	35 (61)	0.13 **a**	+0.14	0.71
Positive	02	00 (00)	02 (100)			
CD5	**87**	**21**	**66**			
Negative	86	20 (23)	66 (77)	0.37 **a**	-0.19	0.54
Positive	01	01 (100)	00 (00)			
CD7	**140**	**40**	**100**			
Negative	121	37 (31)	84 (69)	1.11 **a**	+0.11	0.29
Positive	19	03 (16)	16 (84)			

* P value ≤ 0.05 is significant; **a** Yates' Continuity Correction for cell value less than 5

MPO, Myeloperoxidase; Tdt, Terminal deoxynucleotidyl transferase; HLA-DR, Human leukocyte antigen - DR

Patients with Bcl2 positivity showed no significant correlation with FLT3 ITD, FLT3 ITD / WT ratio, FLT3 TKD mutation, mRNA transcript level and FLT3 protein expression (Data not shown). However, Bcl2 positivity showed a trend of high incidence in patients with FLT3 protein positivity (76%, 68/90) as compared to patients with FLT3 protein negativity (63%, 34/54).

Impact of Bcl2 protein on disease status. According to Kaplan and Meier univariate survival analysis (Table 3), with respect to DFS, a trend of high incidence of disease relapse was noted in Bcl2 positive patients (75%, 42/56) as compared to Bcl2 negative patients (55%, 11/20), while with respect to OS, no such trend was observed.

Impact of Bcl2 protein on disease status with respect to FLT3 parameters. Bcl2 negative and positive patients were divided according to FLT3 parameters (Table 3).

With respect to DFS, Bcl2 negative and positive patients showed no significant correlation with subgroups of FLT3 ITD and FLT3 TKD mutation. With FLT3 mRNA transcript levels, Bcl2 negative patients showed no significant correlation while a trend of high incidence of relapse was noted in Bcl2 positive patients with low FLT3 mRNA transcript level (86%, 24/28) as compared to high FLT3 mRNA transcript level (64%, 16/25). With FLT3 protein subgroups, Bcl2 negative patients showed no significant correlation while significantly high incidence of disease relapse was noted in Bcl2 positive patients with FLT3

Table 3. Impact of Bcl2 protein on disease status

		N	Remission	Relapse	N	Alive	Dead
Bcl2							
Negative		20	09 (45)	11 (55)	25	15 (60)	10 (40)
Positive		56	14 (25)	42 (75)	67	44 (66)	23 (34)
			Log Rank=2.44, df=1, P=0.11			Log Rank=0.09, df=1, P=0.75	
Bcl2	**FLT3 ITD**						
Negative	Negative 20		09 (45)	11 (55)	22	15 (68)	07 (32)
	Positive		-	-	03	00 (00)	03 (100)
						Log Rank=13.09, df=1, P=0.0001*	
Bcl2	**FLT3 ITD**						
Positive	Negative 44		10 (23)	34 (77)	53	35 (66)	18 (34)
	Positive 12		04 (33)	08 (67)	14	09 (64)	05 (36)
			Log Rank=0.01, df=1, P=0.91			Log Rank=0.04, df=1, P=0.84	
Bcl2	**FLT3 TKD**						
Negative	Negative 15		08 (53)	07 (47)	19	12 (63)	07 (37)
	Positive 01		00 (00)	01 (100)	01	01 (100)	00 (00)
			Log Rank=2.20, df=1, P=0.13			Log Rank=0.31, df=1, P=0.57	
Bcl2	**FLT3 TKD**						
Positive	Negative 48		12 (25)	36 (75)	58	37 (64)	21 (36)
	Positive 05		01 (20)	04 (80)	05	05 (100)	00 (00)
			Log Rank=0.18, df=1, P=0.66			Log Rank=2.22, df=1, P=0.13	
Bcl2	**mRNA Transcript**						
Negative	$<16 \times 10^5$	09	05 (56)	04 (44)	09	08 (89)	01 (11)
	$\geq 16 \times 10^5$	07	03 (43)	04 (57)	11	05 (45)	06 (55)
			Log Rank=0.64, df=1, P=0.42			Log Rank=4.30, df=1, P=0.03*	
Bcl2	**mRNA Transcript**						
Positive	$<16 \times 10^5$	28	04 (14)	24 (86)	35	21 (60)	14 (40)
	$\geq 16 \times 10^5$	25	09 (36)	16 (64)	28	21 (75)	07 (25)
			Log Rank=3.57, df=1, P=0.06			Log Rank=1.33, df=1, P=0.24	
Bcl2	**FLT3 protein**						
Negative	Negative 09		03 (33)	06 (67)	12	07 (58)	05 (42)
	Positive 11		06 (54)	05 (46)	13	08 (62)	05 (38)
			Log Rank=0.17, df=1, P=0.67			Log Rank=0.0001, df=1, P=0.98	
Bcl2	**FLT3 protein**						
Positive	Negative 21		10 (48)	11 (52)	25	15 (60)	10 (40)
	Positive 35		04 (11)	31 (89)	42	29 (69)	13 (31)
			Log Rank=5.01, df=1, P=0.02*			Log Rank=0.19, df=1, P=0.66	

*P value ≤ 0.05 is significant

DFS, Disease free survival; OS, Overall survival

protein positivity (89%, 31/35; P=0.02) as compared to FLT3 protein negativity (52%, 11/2).

With respect to OS, significantly higher incidence of death was noted in Bcl2 negative patients with FLT3 ITD positivity (100%, 3/3; P=0.0001) as compared to FLT3 ITD negativity (32%, 7/22) while no such trend was seen in Bcl2 positive patients. With FLT3 mRNA transcript level, significantly higher incidence of death was noted in Bcl2 negative patients with high mRNA transcript level (55%, 6/11; P=0.03) as compared to low mRNA transcript level (11%, 1/9) while no such trend was noted in Bcl2 positive patients. Bcl2 negativity and positivity showed no significant correlation with subgroups of FLT3 TKD mutation and FLT3 protein.

Impact of Bcl2 positivity on disease status in relation to treatment offered. Bcl2 expression was correlated with treatment offered in 76 patients. In AML non-M3 adult patients treated with 7 + 3 protocol, with respect to DFS, Bcl2 positive patients showed significantly high relapse rate (79%, 26/33; P=0.04) as compared to Bcl2 negative patients (38%, 03/08). While with respect to OS, no significant difference was noted (Table 4A). In AML non-M3 pediatric patients treated with BFM93 protocol, with respect to DFS, Bcl2 positive patients showed a trend of high relapse rate (57%, 4/7) as compared to Bcl2 negative patients (40%, 2/5). While respect to OS, no significant difference was noted (Table 4B). In case of AML M3 treated with ATRA / Arsenic trioxide protocol, with respect to DFS, inverse trend was observed wherein Bcl2 positive patients showed a trend of high remission rate (100%, 3/3) as compared to Bcl2 negative patients (33%, 1/3). Similarly, with respect to OS, Bcl2 positive patients showed increased incidence of overall survival (100%, 3/3) as compared to Bcl2 negative patients (67%, 2/3) (Table 4C).

Multivariate survival analysis including all parameters. Multivariate analysis including all clinical and hematological parameters, Bcl2 protein and FLT3 parameters was performed. In case of DFS, high WBC count (>11000/μl) entered at step 1 as significant prognostic factor (Wald statistic=4.390, df=1, Exp(B)=2.02, P=0.03) and Bcl2 positivity (≥20%) (Wald statistic=4.426, df=1, Exp(B)= 2.38, P=0.03) at step 2 for predicting disease relapse, while in case of OS, high blast count (≥61%) was found as significant prognostic factor (Wald statistic=11.478, df=1, Exp(B)=4.80, P=0.001) entered at step 1 for predicting poor OS.

Since, Bcl2 positivity with FLT3 protein positivity showed significantly higher incidence of disease relapse, Bcl2 and FLT3 protein expression were combined to form a new parameter with subgroups of Bcl2-/FLT3 protein-, Bcl2+/FLT3 protein-, Bcl2-/FLT3 protein+ and Bcl2+/FLT3 protein+. It was included with above mentioned parameters in multivariate analysis. In case of DFS, combined Bcl2+/FLT3 protein+ entered at step 1 as significant prognostic factor (Wald statistic=4.792, df=1, Exp(B)=1.67, P=0.02) and high WBC count (>11000/μl) (Wald statistic=4.399, df=1, Exp(B)=2.03, P=0.03) at step 2 for predicting disease relapse while in case of OS, high blast count (≥61%) was found as significant prognostic factor (Wald statistic=11.478, df=1, Exp(B)=4.80, P=0.001) entered at step 1 for predicting poor OS.

Comparison of incidence of Bcl2 protein expression in aplastic anemia, myelodysplastic syndrome and acute myeloid leukemia patients. Incidence of Bcl2 protein in aplastic anemia, MDS and AML patients was evaluated. Bcl2 positivity was found to be similar in MDS (80%, 4/5) and AML (71%, 102/144) patients, however it was significantly high (P=0.0001) as compared to aplastic anemia patients (5%, 1/19).

Table 4. Impact of Bcl2 on disease status in relation to treatment offered

A: Adult AML non-M3 patients treated with 7 + 3 protocol

		DFS		OS	
	N	Remission	Relapse	Alive	Dead
Bcl2					
Negative	08	05 (62)	03 (38)	06 (75)	02 (25)
Positive	33	07 (21)	26 (79)	27 (82)	06 (18)
		Log Rank=4.02, df=1, P=0.04*		Log Rank=0.004, df=1, P=0.94	

B: Pediatric AML non-M3 patients treated with BFM93 protocol

		DFS		OS	
	N	Remission	Relapse	Alive	Dead
Bcl2					
Negative	05	03 (60)	02 (40)	04 (80)	01 (20)
Positive	07	03 (43)	04 (57)	06 (86)	01 (14)
		Log Rank=1.91, df=1, P=0.16		Log Rank=0.06, df=1, P=0.80	

C: AML M3 patients treated with ATRA / Arsenic trioxide

		DFS		OS	
	N	Remission	Relapse	Alive	Dead
Bcl2					
Negative	03	01 (33)	02 (67)	02 (67)	01 (33)
Positive	03	03 (100)	00 (00)	03 (100)	00 (00)
		Log Rank=2.50, df=1, P=0.11		Log Rank=1.00, df=1, P=0.31	

*P value ≤ 0.05 is significant; DFS, Disease free survival; OS, Overall survival

Discussion

This study evaluated Bcl2 protein by flow cytometry method in AML patients. High incidence of Bcl2 positivity (71%) was noted which was further correlated with conventional hematologic parameters and evaluated for its clinical relevance as well as correlated with the FLT3 mutations, FLT3 mRNA transcript level and FLT3 protein expression.

Incidence of Bcl2 protein in the present study was 71%. The Bcl2 expression in AML has been studied by flow cytometry [5, 14-19], western blotting [20] and immnunocytochemistry [13, 21, 22] by various groups reporting heterogenous expression in the range of 34 to 87%. With clinical and hematological pa-

rameters, no significant correlation could be obtained. Present study showed a trend of high incidence of Bcl2 positivity in pediatric and younger age group as compared to older (≥60 years) age group which is contradictory to the known fact that increasing age is associated with accumulation of mutations as well as aberrant protein expression.

Bcl2 positivity was found to be significantly high in AML subtype M4 and M1 followed by M5a, M2, M5b and M3. Both patients with AML M6 expressed Bcl2 positivity. High Bcl2 expression was reported by Venditti et al [18] in AML M0, M1 and M6, and by Campos et al [23] in AML M4 and M5 subtypes. Also, Tzifi et al [24] noted Bcl2 positivity in immature AML subtypes and similarly present study findings show significant positive correlation of Bcl2 with CD34 and CD117. Similarly, Poeta et al [5] observed low bax/bcl2 ratio associated with high CD34 and CD117 levels while, association of Bcl2 with CD34 over expression is supported by other study groups [16-18]. Further, Bcl2 positivity tended to be high in high N:C ratio, suggesting association of Bcl2 with immature cell type.

In univariate analysis, a trend of association of Bcl2 over expression with reduced DFS and increased incidence of disease relapse was observed whereas no correlation was noted with OS. Similarly, some study groups [16, 21, 23, 24] demonstrated that increased mean fluorescence intensity and high levels of Bcl2 expression significantly lowered the complete remission rates. With respect to treatment options, in case of AML non-M3 patient group, Bcl2 positivity showed high incidence of disease relapse in adult patients treated with 7+3 protocol and pediatric patients treated with BFM93 protocol. This result is in accordance with study by Tamm et al [25] where they noted that childhood AML patients treated with BFM93 protocol and with Bax:Bcl2 ratio above average, had a shorter overall survival. Present study demonstrated high incidence of disease remission in Bcl2 positive patients with ATRA / Arsenic trioxide treatment protocol. This result is supported by some study groups [26-28] who observed downregulation of Bcl-2 expression after treatment with ATRA in AML patient samples and HL-60 cells.

Bcl2 protein was correlated with FLT3 parameters, where a trend of high incidence of Bcl2 positivity was found with FLT3 protein positivity. Also, when Bcl2 expression in relation to the FLT3 parameters was evaluated for its impact on clinical outcome, FLT3 protein positivity in Bcl2 positive patients showed significantly poor DFS with a high incidence of disease relapse. Similarly, the results of in vitro study by Lisovsky et al [29] demonstrate that stimulation with FLT3-ligand induces proliferation and up-regulates Bcl2. These results suggest that FLT3 protein over expression, irrespective of its mutation status, plays role in sustaining leukemic cell growth by upregulation of Bcl2. Contradictory to the results obtained with DFS, Bcl2 when correlated with OS, Bcl2 negative patients having FLT3 ITD or having high mRNA transcript level showed significantly high incidence of death which suggests that mutation in FLT3 or aberrant expression of FLT3 mRNA downregulated Bcl2 while increasing the aggressiveness of the disease.

In multivariate survival analysis high WBC count and Bcl2 protein expression emerged as significant predictors of high relapse rate and reduced DFS while high blast count was significant predictor of high incidence of death and reduced overall survival. Similarly, Bcl2 has been reported as independent predictive factor of survival by Campos et al [23], independent prognostic factor for achieving complete remission by Lauria et al [16] and independent prognostic factor for reduced OS and EFS by Kornblau et al [20]. Our findings are consistent with studies by Chang et al [30] and Meshinchi et al [31] demonstrating WBC count as significant prognostic factor while blast count was demonstrated to be of prognostic significance for reduced OS by Amin et al [32]. Also, in multivariate analysis, the subgroup of Bcl2+/FLT3 protein+ emerged as significant prognostic factor followed by high WBC count for predicting poor DFS. As discussed earlier, these results further confirm the parallel role of Bcl2 and FLT3 protein over expression in inhibiting apoptosis and inducing chemoresistance in leukemic blasts.

In aplastic anemia patients, incidence of Bcl2 was 5%. Other studies [33, 34] have shown expression of Bcl2 on CD34+ cells in aplastic anemia but apoptosis in such cases mainly being Fas – dependent, the anti-apoptotic activity of Bcl2 becomes ineffective. In MDS patients, Bcl2 protein over expression was found to be 80%. The protein has been studied by other groups through flow cytometry or immunohistochemistry and found to have heterogenous expression in MDS patients. A study by Kurotaki et al [35] demonstrated high Bcl2 over expression in RAEB-t subtypes and suggested that apoptosis was suppressed in late events of MDS.

In summary, incidence of anti apoptotic protein Bcl2 was high in AML suggesting suppression of apoptotic pathway. Its positivity was significantly associated with immature myeloblasts. Further, patients with Bcl2 positivity and FLT3 protein positivity showed significantly reduced DFS suggesting the parallel role of these proteins in imparting chemoresistance to the leukemic cells.

Acknowledgements: We are grateful to Hematopathology Department for providing us with the hematological findings.

References

[1] HEEG S, WALLER CF. Cytogenetic and molecular aberrations as predictive biomarkers in acute myeloid leukemia. Biomarkers in Oncology 2013; 119–130. http: //dx.doi.org/10.1007/978-1-4419-9755-5_6

[2] YANG J, SCHIFFER CA. Genetic biomarkers in acute myeloid leukemia: will the promise of improving treatment outcomes be realized? Expert Review of Hematology 2012; 5: 395–407. http: //dx.doi.org/10.1586/ehm.12.32

[3] GUINN BA, MOHAMEDALI A, MILLS KI, CZEPULKOWSKI B, SCHMITT M et al. Leukemia associated antigens: Their

dual role as biomarkers and immunotherapeutic targets for acute myeloid leukemia. Biomarker Insights 2007; 2: 69–79.

[4] TESTA U, RICCIONI R. Deregulation of apoptosis in acute myeloid leukemia. Haematologica 2007; 92: 81–94. http: //dx.doi.org/10.3324/haematol.10279

[5] POETA GD, VENDITTI A, PRINCIPE M, MAURILLO L, BUCCISANO F et al. Amount of spontaneous apoptosis detected by Bax/Bcl-2 ratio predicts outcome in acute myeloid leukemia (AML). Blood 2003; 101: 2125–2131. http: //dx.doi.org/10.1182/blood-2002-06-1714

[6] DASH P. Apoptosis – Basic Medical Sciences. Available from www.sgul.ac.uk/dept/immunology/~dash.

[7] PARKER JE, MUFTI GJ, RASOOL F, MIJOVIC A, DEVEREUX S et al. The role of apoptosis, proliferation and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. Blood 2000; 96: 3932–3938.

[8] LICHT J, STERNBERG D. The molecular pathology of acute myeloid leukemia. Hematology 2005; 137–142. http: //dx.doi.org/10.1182/asheducation-2005.1.137

[9] BENE MC, CASTOLDI G, KNAPP W, LUDWIG W, MATUTES E et al. Proposals for the immunological classification of acute leukemias. European group for the immunological characterization of Leukemias (EGIL). Leukemia 1995; 9: 1783–1786.

[10] MEHTA SV, SHUKLA SN, VORA HH. Comprehensive FLT3 analysis in Indian Acute Myeloid Leukemia. J Blood Lymph 2012; 2. http: //dx.doi.org/10.4172/2165-7831.1000102

[11] VORA HH, SHUKLA SN, BRAHMBHATT BV, MEHTA SV, PATEL NA et al. Clinical relevance of FLT3 receptor protein expression in Indian patients with acute leukemia. Asian Pacific Journal of Clinical Oncology 2010; 6: 306–319. http: //dx.doi.org/10.1111/j.1743-7563.2010.01322.x

[12] ARMSTRONG SA, MABON ME, SILVERMAN LB, LI A, GRIBBEN JG et al. FLT3 mutations in childhood acute lymphoblastic leukemia. Blood 2004; 103: 3544–3546. http: //dx.doi.org/10.1182/blood-2003-07-2441

[13] SAHU G, JENA RK. Clinical significance of p53 and Bcl-2 in acute myeloid leukemia patients of eastern India. Hematology Reports 2011; 3: e28. http: //dx.doi.org/10.4081/hr.2011.e28

[14] ZHAO Z, ZUBER J, DIAZ-FLORES E, LINTAULT L, KOGAN SC et al. p53 loss promotes acute myeloid leukemia by enabling aberrant self-removal. Genes & Development 2010; 24: 1389–1402. http: //dx.doi.org/10.1101/gad.1940710

[15] CAMPOS L, SABIDO O, RONAULT J, GUYOTAT D. Effects of BCL-2 antisense oligodeoxynucleotides on in vitro proliferation and survival of normal marrow progenitors and leukemic cells. Blood 1994; 84: 595–600.

[16] LAURIA F, RASPADORI D, RONDELLI D, VENTURA MA, FLACCHINI M et al. High bcl-2 expression in acute myeloid leukemia cells correlates with CD34 positivity and complete remission rate. Leukemia 1997; 11: 2075–2078. http: //dx.doi.org/10.1038/sj.leu.2400854

[17] BRADBURY DA, RUSSELL NH. Comparative quantitative expression of bcl-2 by normal and leukemic myeloid cells. Br J Haematol 1995; 91: 374–379. http: //dx.doi.org/10.1111/j.1365-2141.1995.tb05306.x

[18] VENDITTI A, POETA GD, MAURILLO L, BUCCISANO F, PRINCIPE MI et al. Combined analysis of bcl-2 and MDR1 proteins in 256 cases of acute myeloid leukemia. Haematologica 2004; 89: 934–939.

[19] IRISH JM, ANENSEN N, HOVLAND R, SKAVLAND J, BORRESEN-DALE AL et al. FLT3 Y591 duplication and Bcl-2 overexpression are detected in acute myeloid leukemia cells with high levels of phosphorylated wild-type p53. Blood 2007; 109: 2589–2596. http: //dx.doi.org/10.1182/blood-2006-02-004234

[20] KORNBLAU SM, THALL PF, ESTROV Z, WALTERSCHEID M, PATEL S et al. The prognostic impact of BCL2 protein expression in acute myelogenous leukemia varies with cytogenetics. Clinical Cancer Research 1999; 5: 1758–1766.

[21] MAUNG ZT, MACLEAN FR, REID MM, PEARSON AD, PROCTOR SJ et al. The relationship between bcl-2 expression and response to chemotherapy in acute leukemia. Br J Haematol 1994; 88: 105–109. http: //dx.doi.org/10.1111/j.1365-2141.1994.tb04984.x

[22] BENSI L, LONGO R, VECCHI A, MESSORA C, GARAGNANI L et al. BCL-2 oncoprotein expression in acute myeloid leukemia. Haematologica 1995; 80: 98–102.

[23] CAMPOS L, ROUAULT JP, SABIDO O, ORIOL P, ROUBI N et al. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood 1993; 81: 3091–3096.

[24] TZIFI F, ECONOMOPOOULOU C, GOURGIOTIS D, ARDAVANIS A, PAPAGEORGIOU S et al. The role of Bcl2 family of apoptosis regulator proteins in acute and chronic leukemias. Advances in Hematology 2012. http: //dx.doi.org/10.1155/2012/524308

[25] TAMM I, RICHTER S, OLTERSDORF D et al. High expression levels of X-linked inhibitor of apoptosis protein and Survivin correlate with poor overall survival in childhood de novo acute myeloid leukemia. Clinical cancer research 2004; 10: 3737–3744. http: //dx.doi.org/10.1158/1078-0432.CCR-03-0642

[26] ANDREEFF M, JIANG S, ZHANG X, KONOPLEVA M, ESTROV Z et al. Expression of Bcl-2-related genes in normal and AML progenitors: changes induced by chemotherapy and retinoic acid. Leukemia 1999; 13: 1881–92. http: //dx.doi.org/10.1038/sj.leu.2401573

[27] BRADBURY DA, ALDINGTON S, ZHU YM, RUSSELL NH. Down-regulation of bcl-2 in AML blasts by all-trans retinoic acid and its relationship to CD34 antigen expression. Br J Haematol 1996; 94: 671–675. http: //dx.doi.org/10.1046/j.1365-2141.1996.d01-1838.x

[28] PISANI F, DEL POETA G, ARONICA G, VENDITTI A, CARAVITA T et al. In vitro down-regulation of bcl-2 expression by all-trans retinoic acid in AML blasts. Ann Hematol 1997; 75: 145–147. http: //dx.doi.org/10.1007/s002770050332

[29] LISOVSKY M, ESTROV Z, ZHANG X, CONSOLI U, SANCHEZ-WILLIAMS G et al. FLT3 ligand stimulates proliferation and inhibits apoptosis of acute myeloid leukemia cells: regulation of Bcl-2 and Bax. Blood 1996; 88: 3987–3997.

[30] CHANG H, SALMA F, YI QL, PATTERSON B, BRIEN B et al. Prognostic relevance of immunophenotyping in 379 patients

with acute myeloid leukemia. Leukemia Research 2004; 28: 43–48. http: //dx.doi.org/10.1016/S0145-2126(03)00180-2

[31] MESHINCHI S, ARCECI RJ. Prognostic factors and risk based therapy in pediatric acute myeloid leukemia. The Oncologist 2007; 12: 341–355. http: //dx.doi.org/10.1634/theoncologist.12-3-341

[32] AMIN HM, YANG Y, SHEN Y, ESTEY EH, GILES FJ et al. Having a higher blast percentage in circulation than bone marrow: clinical implications in myelodysplastic syndrome and acute lymphoid and myeloid leukemias. Leukemia 2005; 19: 1567–1572. http: //dx.doi.org/10.1038/sj.leu.2403876

[33] CALLERA F, GARCIA AB, FALCAO RP. Fas-mediated apoptosis with normal expression of bcl-2 and p53 in lymphocytes from aplastic anemia. Br J Haematol 1998; 100: 698–703. http: //dx.doi.org/10.1046/j.1365-2141.1998.00625.x

[34] ISMAIL M, GIBSON FM, GORDON-SMITH EC, RUTHERFORD TR. Bcl-2 and Bcl-x expression in the CD34+ cells of aplastic anaemia patients: relationship with increased apoptosis and upregulation of Fas antigen. British Journal of Hematology 2001; 113: 706–712. http: //dx.doi.org/10.1046/j.1365-2141.2001.02810.x

[35] KUROTAKI H, TSUSHIMA Y, NAGAI K, YAGIHASHI S. Apoptosis, bcl-2 expression and p53 accummulation in myelodysplastic syndrome, myelodysplastic-syndrome-derived acute myelogenous leukemia and de novo acute myelogenous leukemia. Acta haematol 2000; 102: 115–123. http: //dx.doi.org/10.1159/000040984