Mcl-1 expression has in vitro and in vivo significance in chronic lymphocytic leukemia and is associated with other poor prognostic markers

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Bcl-2 family proteins play a critical role in the regulation of apoptosis in chronic lymphocytic leukemia (CLL). However, their association with established prognostic markers is unknown. In this study, we analyzed the expression of Bcl-2, Bax, and Mcl-1 in 185 CLL patients and evaluated their relationship with other prognostic markers, in vitro sensitivity to fludarabine, and clinical outcome. Mcl-1 expression was significantly correlated with stage of disease ($P < .001$), lymphocyte doubling time ($P = .01$), V_H gene mutation status ($P < .001$), CD38 expression ($P < .001$), and ZAP-70 expression ($P = .003$). In addition, Mcl-1 and Mcl-1/Bax ratios showed strong correlations with in vitro resistance to fludarabine ($P = .005$ and $P < .001$, respectively). Furthermore, elevated Mcl-1 expression and Mcl-1/Bax ratios were predictive of time to first treatment in the whole cohort ($P < .001$ and $P < .001$, respectively) and in stage A patients only ($P = .002$ and $P = .001$, respectively). Taken together, our data show that Mcl-1 is a key controller of in vitro drug resistance and is an important regulator of disease progression and outcome in CLL. It therefore represents a promising therapeutic target in this incurable condition. The close correlation between Mcl-1 expression and V_H gene mutation status, CD38 expression, and ZAP-70 expression offers a biologic explanation for their association with adverse prognosis. (Blood. 2008; 112:3807-3817)

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by a very heterogeneous clinical course, which has led to a search for prognostic markers that can reliably predict disease progression.[1-3] Many adverse prognostic factors have been identified, including advanced clinical stage at presentation,[4,5] short lymphocyte doubling time,[6] specific cytogenetic lesions ($11q^-/17p^-$),[7] expression of CD38,[8,9] ZAP-70,[10,11] and unmutated V_H genes.[12,13] However, very little is known about how these factors affect the biology of CLL and lead to an inferior prognosis.

CLL is often described as a disease of failed apoptosis[14-16]; but although it is widely accepted that CLL cells accumulate in vivo, they rapidly undergo apoptosis in vitro suggesting that prosurvival signals emanating from the microenvironment play a major role in preventing apoptosis.[17-20] The molecular mechanisms that regulate apoptosis in CLL are complex, but the importance of the Bcl-2 family of apoptosis-regulating proteins in CLL has been established for more than a decade.[21-23] We and others have found that overexpression of the antiapoptotic protein Bcl-2 is a hallmark of CLL,[24-26] and it has long been thought that this is caused by hypomethylation of the promoter region of the bcl-2 gene.[27] However, since the discovery of micro RNA (miR) species, it has been shown that miR-15 and miR-16 are deleted or down-regulated in the majority of CLL cells; both of these miRs negatively regulate Bcl-2 at the posttranscriptional level.[28]

High levels of Bcl-2 have been associated with shorter overall survival in previously treated patients and increased chemoresistance to treatment with fludarabine.[15,16] Indeed, Bcl-2 expression has been shown to be an independent prognostic factor in CLL.[23] However, the relative expression of Bcl-2 and the proapoptotic protein Bax appears to be a more important determinant of CLL cell apoptosis; Bcl-2/Bax ratios correlate with progressive disease and resistance to chlorambucil and fludarabine in vitro.[24,29,30] Further, we have previously shown that chemoresistance to chlorambucil is mediated, at least in part, by in vitro selection of subclones with high Bcl-2 expression and low Bax expression[31] and that a common polymorphism in the *BAX* gene causes lower Bax protein expression, resulting in increased Bcl-2/Bax ratios.[32] This group of patients had a shorter overall survival once treatment was initiated.

More recently, another Bcl-2 family protein, Mcl-1, has also been implicated in the regulation of apoptosis in CLL.[33-35] Mcl-1 has been strongly associated with a failure to achieve a complete response to chlorambucil, fludarabine, and rituximab in vitro and in vivo.[36,37] In addition, targeted down-regulation of Mcl-1 triggers apoptosis in a number of B-cell tumor models[38,39] and has been shown to enhance rituximab-mediated apoptosis and complement-dependent cytotoxicity.[37]

Furthermore, Bcl-2 family proteins, particularly Mcl-1, appear to be dynamically regulated by a wide array of cellular processes, including ligation of the B-cell receptor and signaling via CD40, vascular endothelial growth factor (VEGF), and the PI-3 kinase/AKT pathway.[35,40-42] Given that the capacity of CLL cells to respond to these signals is strongly linked to a number of important

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prognostic markers, we set out to evaluate their relationship with Bcl-2 family proteins in a cohort of 185 patients. We also evaluated the association between Bcl-2 family proteins and in vitro responses to fludarabine and assessed the independent prognostic value of these proteins regarding clinical outcome.

Methods

Patient samples

Peripheral blood samples from 185 patients with CLL attending the clinic at Birmingham Heartlands Hospital (112 male and 73 female) were obtained with the patients' informed consent in accordance with the ethical approval granted by the South East Wales Research Ethics Committee (06/WSE03/23) in accordance with the Declaration of Helsinki. A total of 54% (100 of 185) of patients had previously received treatment, but none had been treated for at least 3 months before protein analysis. Therapies given were chlorambucil, fludarabine, FC (fludarabine and cyclophosphamide), FCR (fludarabine, cyclophosphamide, rituximab), CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone), and alemtuzumab. Clinical stage at diagnosis was based on the Binet system,[5] and indications for treatment were consistent with the published guidelines.[43] All flow cytometry and Western blot analyses were performed on freshly collected peripheral blood samples, and V_H gene mutation analysis and ATM and p53 mutational analyses were performed on paired frozen samples collected at the same time point.

Flow cytometric analysis of CD38 and ZAP-70 expression

Zap-70 expression was quantified using a modification of the flow cytometry method previously described.[44] Samples were incubated with anti–CD3-PE (Dako United Kingdom, Ely, United Kingdom), CD5-allophycocyanin (APC) (Dako United Kingdom), CD19-PE cy5 (Dako United Kingdom), and ZAP-70-Alexa Fluor 488 (Invitrogen, Carlsbad, CA) antibodies. The cells were then analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and at least 10 000 events were acquired from each sample. The percentage of ZAP-70–positive cells was calculated using the $CD3^+$ T-cell population to establish the lower limit of ZAP-70 expression. $CD5^+/CD19^+$ cells above the $CD3^+$ threshold were considered ZAP-70-positive; the cut-off for ZAP-70 positivity was 20%. CD38 expression was evaluated by triple color immunophenotyping using CD5-FITC (Dako United Kingdom), CD38-PE (Invitrogen), and CD19-APC (Invitrogen) antibodies, and the threshold for positivity was 20%.

Genetic analysis

Peripheral blood samples from patients with advanced stage disease underwent fluorescent in situ hybridization analyses looking for trisomy 12, and deletions in chromosomes 6q, 11q, 13q, and 17p (Abbott Laboratories, Abbott Park, IL). Karyotype analysis was also performed in some cases after stimulation for 3 to 5 days with phorbol esters.

Mutation analysis of ATM and TP53 genes

Genomic DNA was obtained from 5×10^6 mononuclear cells using QIAamp DNA blood minikit (Qiagen, Dorking, United Kingdom). DNA was diluted to give a final DNA concentration of 50 to 100 ng/μL. Polymerase chain reactions were performed for each of 62 *ATM* coding exons and flanking intronic sequence using 60 primer pairs, and *TP53* exons 3 to 10 were amplified using 5 primer pairs according to the previously described methods.[45]

V_H gene mutation analysis

RNA was extracted from the patient samples using Qiagen RNeasy mini kit (Qiagen). Complementary DNA was synthesized using the Reverse-iT kit (ABgene, Epsom, Surrey, United Kingdom). The V_H gene mutational status of the CLL patients was analyzed according to the method previously described.[45] The resulting polymerase chain reaction products were sequenced using BIG dye terminator sequencing kit version 3 (Applied Biosystems, Foster City, CA) and the sequences analyzed using the after public databases: Immunoglobulin BLAST (http://www.ncbi.nlm.nih.gov/igblast/) and IMGT (http://imgt.cines.fr/IMGT_vquest/vquest?livret = 0&Option = humanIg). The sequences with a germ line homology of 98% or higher were regarded as unmutated and less than 98% as mutated.

Detection of Bcl-2 family protein expression by flow cytometry

Samples from all 185 patients with CLL were processed for triple immunofluorescent staining using Bcl-2, Mcl-1, or Bax antibodies in conjunction with CD5 and CD19 antibodies within 4 hours of sample collection. Briefly, 1×10^6 cells were incubated with anti-CD5 phycoerythrin-conjugated and anti-CD19 phycoerythrin cyanine 5-conjugated antibodies or isotype-matched controls (Dako United Kingdom). The cells were fixed and permeabilized using a commercially available kit (Dako United Kingdom) and then labeled with one of the following antibodies: anti-Bcl-2 fluorescein isothiocyanate (FITC)-conjugated antibody (Dako United Kingdom), anti-Bax FITC-conjugated antibody, anti–Mcl-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or an appropriate isotype-matched control (Dako United Kingdom). The Mcl-1 labeled cells were subsequently subjected to secondary labeling with a FITC-conjugated antibody (Dako United Kingdom). At least 10 000 cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences), and nonspecific binding was excluded by gating using isotype control antibodies. Gating of the $CD5^+/CD19^+$ cells was performed in the analyses to quantify the specific protein expression in the leukemic cells. The mean fluorescent intensity was determined for each protein using WinMDI software (J. Trotter, Scripps Research Institute, La Jolla, CA), and these values were then converted to molecules of equivalent soluble fluorochrome using a calibration curve to standardize the data.

Detection of Bcl-2 family proteins by Western blotting

Aliquots of CLL lymphocytes (3×10^7 cells) were treated with whole-cell lysis buffer (10 mM Tris-HCl, 250 mM sodium chloride, 50 mM sodium fluoride, 0.5% Triton X-100, 0.1% sodium dodecyl sulfate, 10% glycerol, 1× protease inhibitor mixture, 1 mM phenylmethysulfonyl fluoride, 100 μM sodium orthovandate, 2 mM iodoacetic acid, and 5 mM $ZnCl_2$). Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Each sample (12 μg protein) was separated on a 4% to 12% Bis-Tris acrylamide gel and transferred to polyvinylidene fluoride membrane (Bio-Rad). Western blots were probed with antibodies against Bcl-2 (clone 124; Dako United Kingdom), Mcl-1 (clone 22; Santa Cruz Biotechnology), and Bax (clone O.N.18; Santa Cruz Biotechnology), in phosphate-buffered saline with 5% nonfat milk. The secondary antibodies were horseradish peroxidase–conjugated sheep anti–mouse IgG and donkey anti–rabbit IgG (GE Healthcare, Little Chalfont, United Kingdom), and detection was performed by an enhanced chemiluminescence method.

Cell culture and in vitro drug sensitivity testing

Freshly isolated primary CLL B cells were cultured in RPMI with 10% fetal calf serum and L-glutamine, penicillin, and streptomycin. Cells were maintained at 37°C in an atmosphere containing 95% air and 5% CO_2 (vol/vol). CLL cells (10^6/mL) were treated with either vehicle or fludarabine (10^{-7}-10^{-5} M) for 48 hours. Subsequently, cells were labeled with CD19-APC (Invitrogen) and then resuspended in 200 μL binding buffer containing 4 μL annexin V–FITC (Bender Medsystems, Vienna, Austria). Apoptosis was quantified in the $CD19^+$ CLL cells using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Statistical analysis

The relationship between Bcl-2, Bax, and Mcl-1 expression and known prognostic factors as well as time to first treatment and overall survival were explored through Wilcoxon rank sum tests for the categorical variables

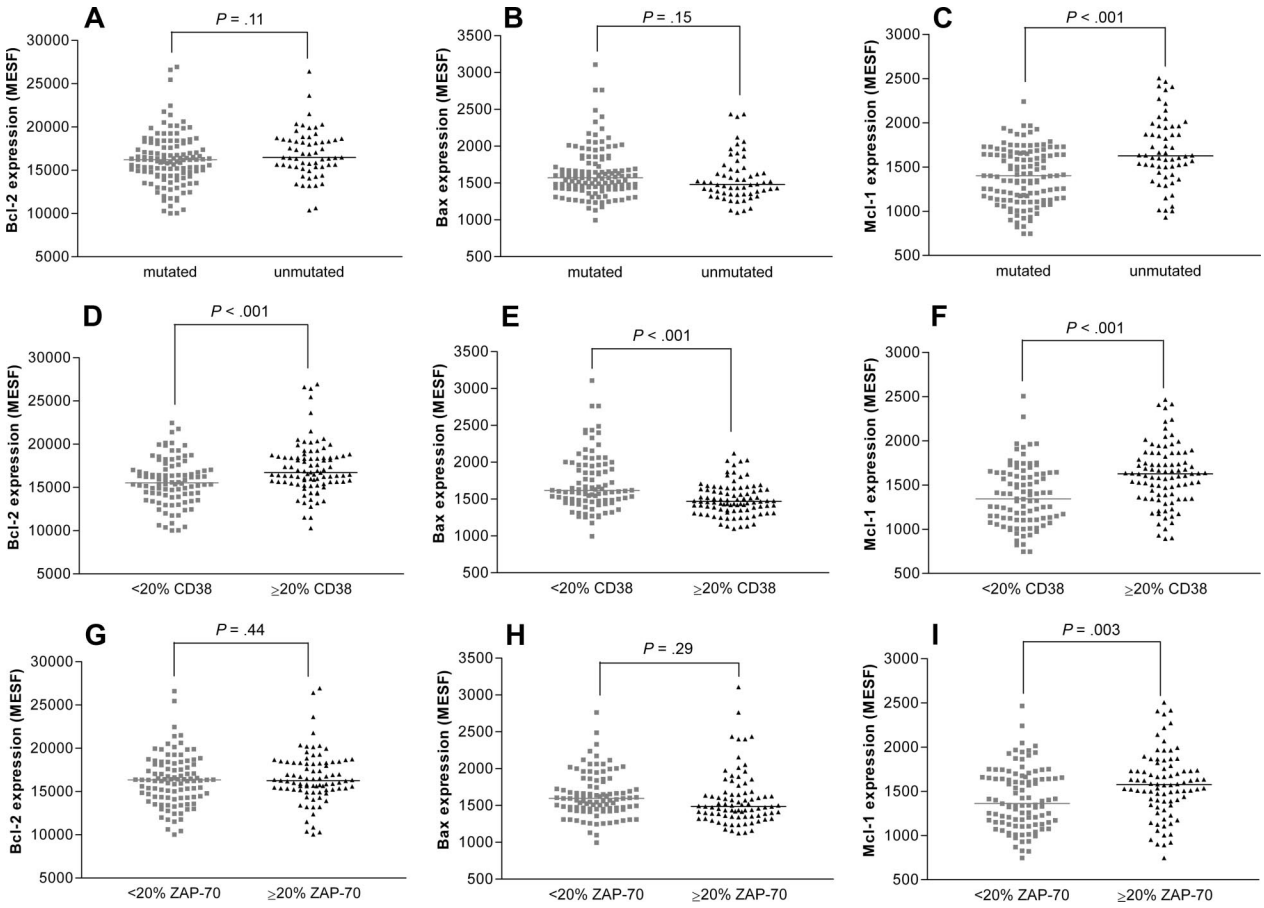

Figure 1. Correlation of Bcl-2 family proteins with V_H gene mutation status, CD38 expression, and ZAP-70 expression. Samples from all 185 patients were analyzed for Bcl-2, Bax, and Mcl-1 protein expression by flow cytometry. The expression of each protein was compared with V_H gene mutation status (A-C), CD38 expression (D-F), and ZAP-70 expression (G-I). Bcl-2 and Bax expression were not significantly associated with V_H gene mutation status or ZAP-70 expression but were associated with CD38 expression. In contrast, Mcl-1 expression was correlated with V_H gene mutation status, CD38 expression, and ZAP-70 expression.

Binet stage, LDT, CD38, ZAP-70, V_H gene mutation status, and cytogenetics and through Spearman rank correlation for the continuous variable of age at diagnosis. Overall survival and progression-free survival times were calculated from date of diagnosis, and curves were constructed using the method of Kaplan and Meier and the log-rank test was used to assess any differences between patient and tumor characteristics. Cox regression

Table 1. Comparison of Bcl-2, Bax, and Mcl-1 expression in prognostic subsets of the CLL patient cohort

	n	Bcl-2 (median IQR)	P*	Mcl-1 (median IQR)	P*	Bax (median IQR)	P*
Stage			.015		< .001		< .001
A	129	15 890 (14 302-18 119)		1414 (1132-1659)		1596 (1463-1850)	
B/C	56	16 903 (15 849-18 664)		1630 (1383-1858)		1413 (1309-1591)	
LDT			.38		.01		.02
Less than 12	17	16 564 (15 547-18 262)		1622 (1448-1829)		1511 (1400-1588)	
More than 12	112	15 940 (14 306-18 119)		1390 (1132-1646)		1601 (1466-1881)	
Not evaluable	56	16 488 (15 611-18 627)		1605 (1352-1789)		1436 (1309-1640)	
CD38			< .001		< .001		< .001
20% or more	91	16 713 (15 646-18 543)		1627 (1375-1843)		1468 (1352-1633)	
Less than 20%	94	15 531 (14 020-17 074)		1343 (1107-1635)		1615 (1477-1963)	
Genetics			.19		.09		.004
$11q^-/17p^-$	26	16 903 (15 313-19 042)		1648 (1231-1954)		1415 (1336-1522)	
N/O	118	16 242 (14 757-18 371)		1503 (1207-1729)		1588 (1448-1866)	
V_H status			.06		< .001		.14
98% or more	59	16 491 (15 429-18 619)		1632 (1503-1963)		1475 (1352-1698)	
Less than 98%	126	16 209 (14 374-17 932)		1401 (1147-1653)		1578 (1436-1767)	
ZAP-70			.44		.003		.29
20% or more	84	16 263 (15 241-18 336)		1576 (1388-1746)		1485 (1358-1660)	
Less than 20%	101	16 338 (14 332-18 147)		1362 (1149-1657)		1596 (1451-1793)	

$11q^-/17p^-$ indicates any FISH or karyotypic abnormality of 11q or 17p and/or a mutational defect of ATM or p53; N, normal cytogenetics; O, other cytogenetic abnormality than $11q^-/17p^-$.

*P values not including "Not evaluable."

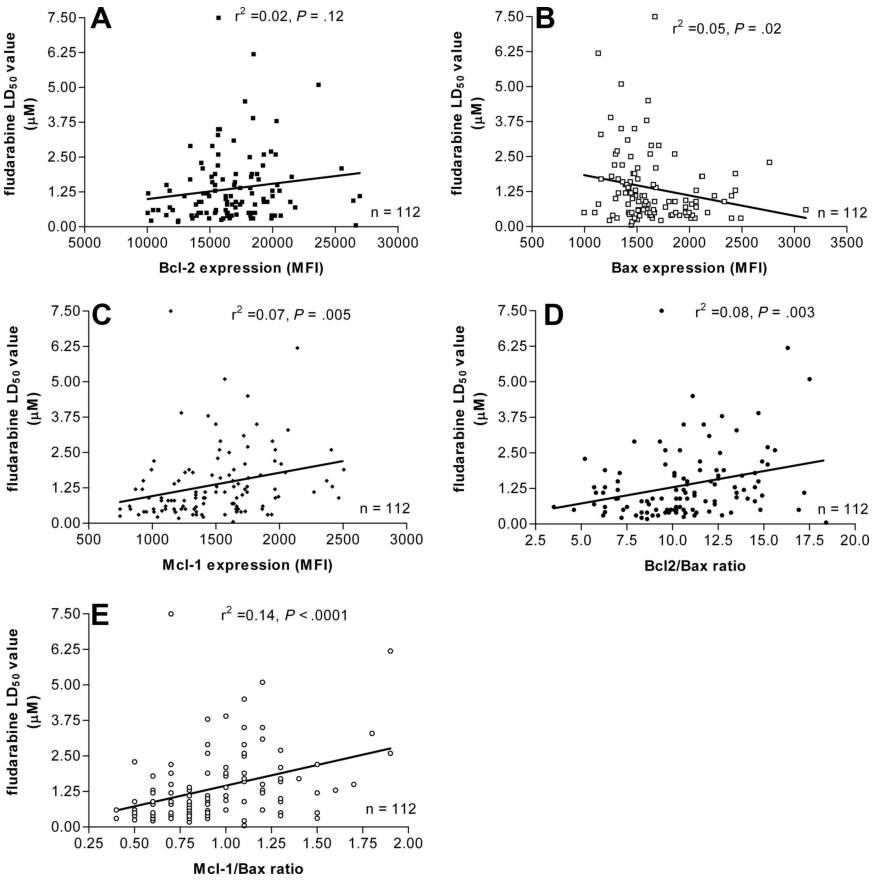

Figure 2. Correlation between Bcl-2 family proteins and in vitro sensitivity to fludarabine. Samples from 112 CLL patients were evaluated for in vitro sensitivity to fludarabine. LD_{50} values, the concentration of fludarabine required to kill 50% of the cells in culture, were then plotted against Bcl-2 family protein expression levels as continuous variables. (A) Bcl-2 expression showed no correlation, (B) Bax was negatively correlated, and (C) Mcl-1 was positively correlated with in vitro sensitivity to fludarabine. (D) Bcl-2/Bax ratios and (E) Mcl-1/Bax ratios showed even stronger correlations with in vitro drug responses.

analysis determined important independent prognostic factors for time to first treatment and overall survival. Statistical analysis was carried out using Prism 3.0 (GraphPad Software, San Diego, CA) and SAS statistical software (SAS Institute, Cary, NC).

Results

Flow cytometric and Western blot analyses of Bcl-2 family proteins

We have previously shown a good correlation between flow cytometry-based assays and Western blot analysis for the quantitation of Bcl-2 and Bax.[32,46] In this study, we evaluated the constitutive expression of Mcl-1 by flow cytometry in all 185 samples and validated the results in a smaller sample (26 patients) by Western blot using the same antibody. Figure S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) shows the gating strategy for defining CLL lymphocytes and the resulting histograms for Mcl-1 expression derived from 8 representative patient samples. The corresponding Western blot analyses together with actin-normalized densitometric ratios for each blot are also shown together with a comparison of the 2 methodologies ($r^2 = 0.66$).

The relationship between Bcl-2 family expression and prognostic markers

In this study, we quantified the constitutive expression of Bcl-2, Bax, and Mcl-1 in a single-center cohort of 185 CLL patients and determined whether these proteins correlated with known prognostic factors. The clinical features of the cohort are summarized in Table S1. The clinical features of the patient group were representative of a standard single center CLL cohort, with the majority of patients having Binet stage A disease at diagnosis (70%) and mutated V_H genes (68%).

When we split the patient cohort into categorical subsets defined by known prognostic markers, a number of statistically significant differences were found between the expression of Bcl-2, Bax, and Mcl-1 (Figure 1; Table 1). In terms of V_H gene mutation status and ZAP-70 expression, only Mcl-1 was differentially expressed in unmutated/mutated and ZAP-70^+/ZAP-70^- subsets ($P < .001$ and $P = .003$; Figure 1C and I, respectively). In contrast, all 3 Bcl-2 family proteins were differentially expressed in our CLL cohort when categorized in terms of CD38 ($< 20\%$/ $\geq 20\%$ expression; Figure 1D-F). Furthermore, Bcl-2 and Mcl-1 were significantly higher and Bax was significantly lower in patients with advanced Binet stage ($P = .015$, $P < .001$, and $P = .001$ respectively). In addition, low Bax and high Mcl-1 expression was associated with shorter LDT (only previously untreated patients included; $P = .02$ and $P = .01$, respectively). Low Bax expression was also associated with adverse cytogenetics ($11q^-/17p^-$, n = 26; $P = .004$) consistent with the notion that Bax expression is transcriptionally regulated by the p53/ATM pathways (Table 1).

Bcl-2 family expression and in vitro drug sensitivity to fludarabine

A number of previous studies have shown a link between Bcl-2 family expression and in vitro drug sensitivity. Here we present the largest single-center analysis of this association in primary CLL cells (n = 112). Bcl-2 expression was not significantly associated with in vitro response

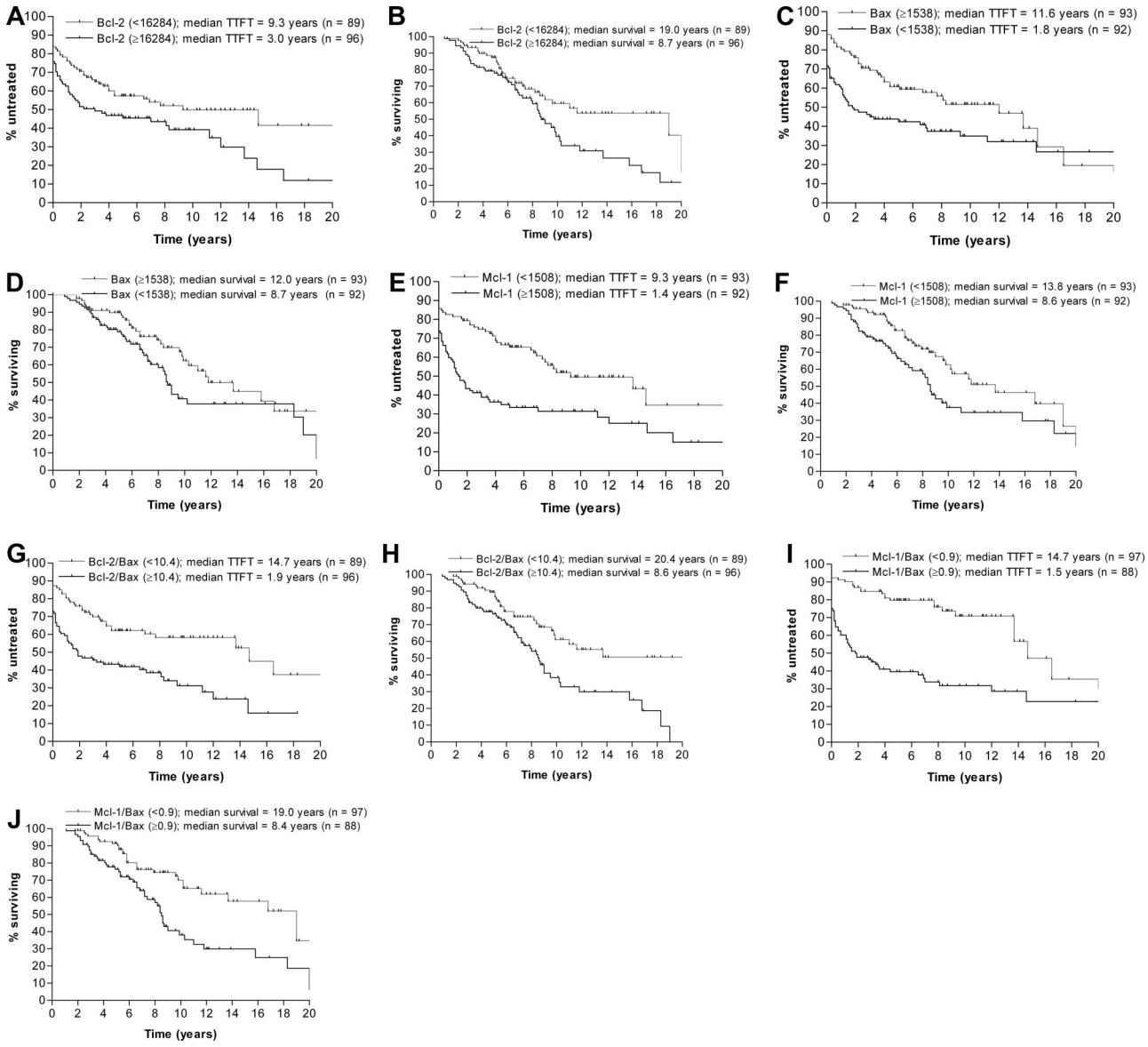

Figure 3. Kaplan-Meier curves for time to first treatment and overall survival for the patient cohort defined by median expression of Bcl-2 family proteins. All of the Bcl-2 family proteins were prognostic for time to first treatment (A,C,E,G,I), and Bcl-2 (B), Bax (D), Mcl-1 (F), Bcl-2/Bax ratios (H), and Mcl-1/Bax ratios (J) were prognostic for overall survival. The hazard ratios, confidence intervals, and *P* values for each parameter are shown in Table 2.

to fludarabine ($r^2 = 0.02$, $P = .12$; Figure 2A). In contrast, Bax expression was inversely correlated and Mcl-1 was positively correlated with in vitro response to this chemotherapeutic agent ($r^2 = 0.05$, $P = .02$ and $r^2 = 0.07$, $P = .005$; Figure 2B and C, respectively). Furthermore, the combined analysis of Bcl-2/Bax ratios and Mcl-1/Bax ratios improved the correlation with fludarabine sensitivity ($r^2 = 0.08$, $P = .003$ and $r^2 = 0.14$, $P < .001$; Figure 2D and E, respectively).

Longitudinal analysis of Bcl-2 family expression

Our previous studies have shown that intrapatient expression of both Bcl-2 and Bax may vary over the clinical course particularly after chemotherapy.[24] These observations were extended here to include a longitudinal analysis of Mcl-1 expression. Figure S2 shows the concurrent expression profiles of Bcl-2, Bax, and Mcl-1 in 11 patients in a time course spanning 48 months. Bcl-2 expression showed an upwards trend that was particularly marked in patients who received chemotherapy during the period of study (5 of 11). Conversely, Bax expression showed a downward trend, especially in the treated patient group. In contrast, Mcl-1 expression remained relatively stable. It is noteworthy that samples were not analyzed for Bcl-2 family protein expression until at least 3 months after treatment, in this way, avoiding transient changes in protein expression induced by chemotherapy.

Bcl-2 family expression and time to first treatment

Figure S3A,C,E shows Kaplan-Meier curves for time to first treatment (TTFT) for V_H gene mutation status, CD38 expression ($< / \geq 20\%$), and ZAP-70 expression ($< / \geq 20\%$). In keeping with previous studies, our cohort showed a reduced TTFT for patients with unmutated V_H genes, more than or equal to 20% CD38 expression, and more than or equal to 20% ZAP-70 expression. To assess the impact of Bcl-2 family protein expression on disease progression in CLL, we categorized the patients into subsets above and below the median expression value for each protein. Kaplan-Meier curves for TTFT were then plotted and are presented in Figure 3. In addition, log-rank analysis yielded hazard

Table 2. Comparison of prognostic factors in terms of time to first treatment and overall survival

	Time to first treatment					Overall survival			
Parameter	**n**	**Median, y**	**HR**	***P***	**95% CI**	**Median, y**	**HR**	***P***	**95% CI**
V_H status			2.9	< .001	3.2-9.6		3.1	< .001	2.8-9.3
Less than 98%	126	11.2				13.7			
98% or more	59	0.6				5.9			
CD38			2.8	< .001	2.1-4.8		2.9	< .001	1.9-4.6
Less than 20%	94	16.5				Undefined			
20% or more	91	1.8				8.0			
ZAP-70			1.6	.01	1.1-2.6		2.1	< .001	1.4-3.5
Less than 20%	101	12.0				15.8			
20% or more	84	3.0				8.0			
Bcl-2			1.6	.02	1.1-2.5		1.7	.01	1.1-2.7
Less than 16 284	89	9.3				19.0			
16 284 or more	96	3.0				8.7			
Bax			1.6	.01	1.2-2.6		1.6	.05	0.9-2.5
1538 or more	93	11.6				12.0			
Less than 1538	92	1.8				8.7			
Mcl-1			2.1	< .001	1.5-3.4		1.8	.008	1.2-2.9
Less than 1508	93	9.3				13.8			
1508 or more	92	1.4				8.6			
Bcl-2/Bax			2.0	< .001	1.4-3.3		2.1	< .001	1.4-3.4
Less than 10.4	96	14.7				20.4			
10.4 or more	89	1.9				8.6			
Mcl-1/Bax			3.2	< .001	2.1-4.7		2.4	< .001	1.5-3.6
Less than 0.9	97	14.7				19.0			
0.9 or more	88	1.5				8.4			

HR indicates hazard ratio; and CI, confidence interval.

ratios and *P* values for each dataset, and these are summarized in Table 2. All 3 Bcl-2 family proteins analyzed showed significantly different TTFT in subsets defined by their median expression. Of the 3 proteins, Mcl-1 expression was the most prognostic for TTFT with the above median expression subset having a median TTFT of 1.4 years compared with 9.3 years for the below median expression subset (hazard ratio = 2.1; $P < .001$). Furthermore, Mcl-1/Bax ratios were a superior discriminator of TTFT as evidenced by an increased hazard ratio (HR = 3.2; $P < .001$). Indeed, comparison with V_H gene mutation status, CD38 expression, and ZAP-70 expression revealed that Mcl-1/Bax ratios were more prognostic for TTFT than all 3 of these parameters. However, in multivariate analysis, none of the Bcl-2 family parameters was deemed independently prognostic for TTFT after inclusion in the model of Binet stage, CD38, or V_H gene mutation status.

Bcl-2 family expression and overall survival

Our cohort showed a reduced overall survival (OS) with advanced Binet stage (median survival for stage B/C, 6.9 years; stage A, 20.4 years; $P < .001$), LDT less than 12 months (median survival, 6.5 years vs 15.8 years; $P = .004$), CD38 expression more than or equal to 20% (median survival, 8.0 years vs not reached; $P < .001$), adverse cytogenetics ($11q^-/17p^-$) (median survival, 6.9 years vs 15.8 years; $P < .001$), unmutated V_H gene mutation status (median survival, 6.9 years vs 11.6 years; $P = .001$), and ZAP-70 expression more than or equal to 20% (median survival, 8.0 years vs 15.8 years; $P = .001$). Figure S3 shows Kaplan-Meier curves for OS (panels B, D, and F) for V_H gene mutation status, CD38 expression ($< / \geq 20\%$), and ZAP-70 expression ($< / \geq 20\%$). Figure 3B,D,F,H,J shows Kaplan-Meier curves for OS in subsets of Bcl-2 family proteins defined by median expression. Bcl-2 and Mcl-1 proteins were prognostic for OS (Bcl-2, $P = .01$; Mcl-1, $P = .008$). In addition, Bcl-2/Bax ratios and Mcl-1/Bax ratios were highly prognostic ($P = .001$ and $P = .001$, respectively). However, in multivariate analysis, none of the Bcl-2 family parameters retained independent prognostic value for OS after inclusion in the model of Binet stage, CD38, or V_H gene mutation status.

TTFT and OS for stage A patients only (n = 129)

In an attempt to shed further light on the role of the Bcl-2 family proteins in the progression of CLL, we examined TTFT and OS in the stage A patient subset (n = 129). Our cohort showed a reduced TTFT with LDT less than 12 months (median, 1.8 years vs 11.6 years; $P < .001$), CD38 expression more than or equal to 20% (median, 4.4 years vs not reached; $P < .001$), adverse cytogenetics ($11q^-/17p^-$) (median, 2.8 years vs 12.8 years; $P < .001$), and unmutated V_H gene mutation status (median, 3.0 years vs 14.7 years; $P < .001$). Interestingly, ZAP-70 status was not prognostic for TTFT in this subset of patients ($P = .54$). In terms of Bcl-2 family parameters, only Mcl-1 expression and Mcl-1/Bax ratios were prognostic for TTFT ($P = .002$ and $P = .001$, Figure 4E and I, respectively). Bcl-2, Bax, and Bcl-2/Bax ratios were not significantly associated with TTFT in this cohort ($P = .22$, $P = .46$, and $P = .06$, Figure 4A, C, and G, respectively). In multivariate analysis, Mcl-1/Bax ratios showed borderline prognostic value for TTFT ($P = .056$) in the presence of the most prognostic parameter, CD38 expression. In terms of overall survival, only Mcl-1/Bax ratios were prognostic in our stage A subset in univariate analysis ($P = .01$, Figure 4J). However, none of the Bcl-2 family parameters was deemed independently prognostic for OS after inclusion in the model of CD38 or V_H gene mutation status.

Combining Mcl-1/Bax ratios with V_H gene mutation status, CD38 expression, and ZAP-70 expression for the assessment of prognosis (whole cohort)

Given the importance of Mcl-1/Bax ratios in defining prognosis in our cohort, we next investigated whether Mcl-1/Bax ratios

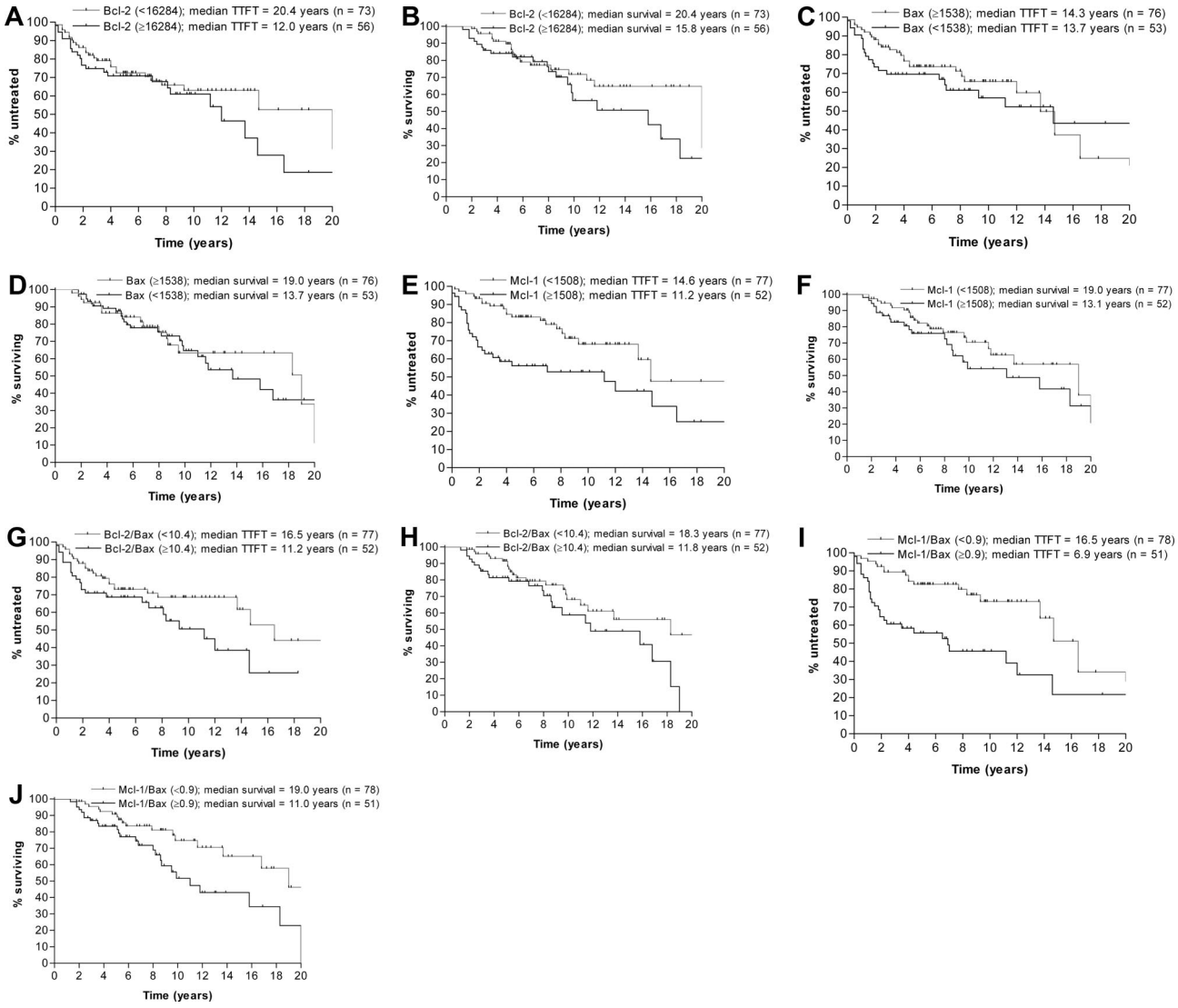

Figure 4. Kaplan-Meier curves for time to first treatment and overall survival defined by median expression of Bcl-2 family proteins. Curves are for stage A patient cohort only; n = 129. Bcl-2 (A), Bax (C), and Bcl-2/Bax ratios (G) were not prognostic for time to first treatment. In contrast, Mcl-1 (E) and Mcl-1/Bax ratios (I) were able to define distinct patient subsets at risk of requiring treatment. In terms of overall survival, only Mcl-1/Bax ratios were prognostic (J); all of the other Bcl-2 family parameters tested were not significant (B,D,F,H).

were able to further define subsets of CLL patients for TTFT and OS within known prognostic groups (Figure 5). The combination of Mcl-1/Bax ratios with V_H gene mutation status identified 4 distinct prognostic groups: 2 concordant and 2 discordant. The concordant poor prognostic group (unmutated V_H genes/high Mcl-1/Bax ratios) showed a remarkably short median TTFT (0.2 year), whereas the concordant good prognostic group (mutated V_H genes/low Mcl-1/Bax ratios) had a median TTFT of 16.5 years (Figure 5A). The combination of Mcl-1/Bax ratios with CD38 expression (Figure 5C) and ZAP-70 expression (Figure 5E) was even more remarkable, particularly with regard to defining good prognostic groups that did not reach their median TTFT. Furthermore, within the discordant subsets, Mcl-1/Bax ratios appeared to redefine the prognostic potential of CD38 and ZAP-70, that is, the more than or equal to 20% CD38/low Mcl-1/Bax ratios subset had a longer TTFT than the less than 20% CD38/high Mcl-1/Bax ratio group (9.3 years vs 3.5 years). The same was true for the more than or equal to 20% ZAP-70/low Mcl-1/Bax ratios subset and the less than 20% ZAP-70/high Mcl-1/Bax ratio group (7.7 years vs 1.9 years). In terms of OS, Figure 5B,D,F shows the Kaplan-Meier curves for the combination of Mcl-1/Bax ratios with V_H gene mutation status, CD38 expression, and ZAP-70 expression. The addition of Mcl-1/Bax ratios clearly identified a subset of patients with very low risk of CLL-related mortality (mutated/< 0.9 Mcl-1/Bax ratios; Figure 5B, $< 20\%$ CD38/< 0.9 Mcl-1/Bax ratios; Figure 4D and $< 20\%$ ZAP-70/< 0.9 Mcl-1/Bax ratios; Figure 5F).

Combining Mcl-1/Bax ratios with V_H gene mutation status, CD38 expression, and ZAP-70 expression for the assessment of prognosis (stage A patients only)

We next investigated whether Mcl-1/Bax ratios were able to further define subsets of CLL patients for TTFT and OS within the stage A patient group (Figure 6). The concordant poor prognostic group (unmutated V_H genes/high Mcl-1/Bax ratios) showed a very short median TTFT (1.9 years vs 3.0 years for unmutated V_H genes only). Similarly, the concordant good prognostic group (mutated V_H genes/low Mcl-1/Bax ratios) had a median TTFT of 16.5 years versus 14.7 years for mutated V_H genes only (Figure 6A). The

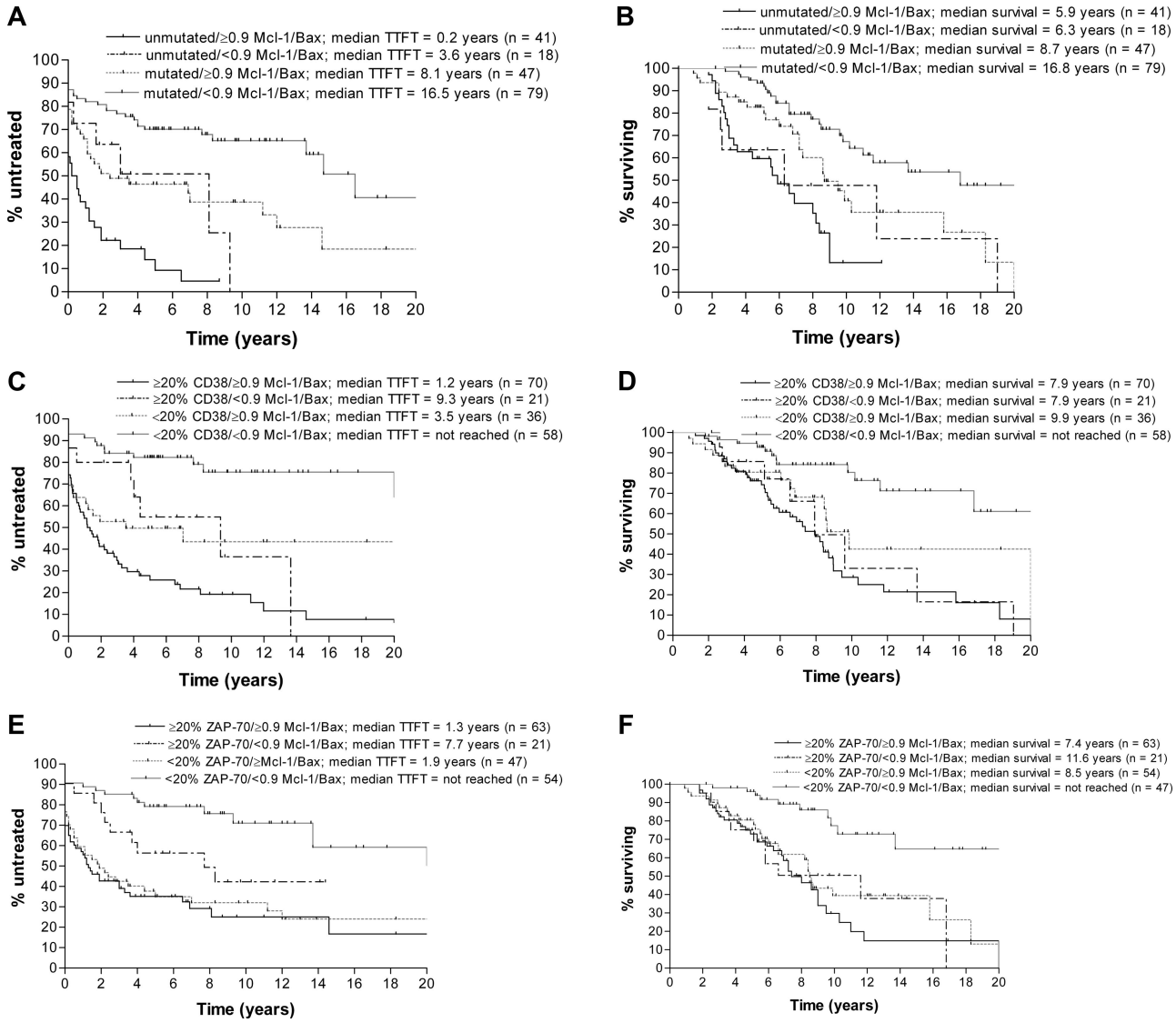

Figure 5. Kaplan-Meier curves for time to first treatment and overall survival defined by the combination of Mcl-1/Bax ratios and V_H gene mutation status, CD38 expression, and ZAP-70 expression (whole cohort). Mcl-1/Bax ratios in combination with V_H gene mutation status (A,B), CD38 expression (C,D), and ZAP-70 expression (E,F) increased the prognostic power of each parameter in defining time to first treatment. However, this was not the case for overall survival.

combination of Mcl-1/Bax ratios with CD38 expression (Figure 6C) also improved the ability of CD38 to define a patient subset at risk of requiring treatment (median TTFT, 3.0 years vs 4.4 years for CD38 alone). The same was true for ZAP-70 (median TTFT, 7.0 years vs 9.2 years for ZAP-70 only). It is of interest that within the stage A cohort Mcl-1/Bax ratios appeared to dominate in the ZAP-70 subsets, that is, both subsets with high Mcl-1/Bax ratios had similar TTFT regardless of ZAP-70 status and the 2 subsets with low Mcl-1/Bax ratios both failed to reach their median TTFT. In terms of overall survival, the addition of Mcl-1/Bax ratios failed to improve the prognostic power of unmutated V_H genes or high CD38 expression in the stage A cohort. In contrast, Mcl-1/Bax ratios improved the prognostic power within the high ZAP-70 expression subset (9.5 years vs 11.6 years for ZAP-70 alone).

Discussion

In this study, we showed, for the first time, that constitutive expression of Mcl-1 is associated with V_H gene mutation status, CD38 expression, and ZAP-70 expression. In addition, Mcl-1 and Mcl-1/Bax ratios were also shown to be prognostic for TTFT and OS in the whole cohort and for TTFT in the stage A cohort. In multivariate analysis, Mcl-1 and Mcl-1/Bax ratios were not deemed to have independent prognostic significance in the presence of Binet stage, V_H gene mutation status, or CD38 expression. However, given the close correlations between Mcl-1 and these parameters, it is perhaps not surprising that their significance was lost in the multivariate model. Our ability to analyze these Bcl-2 family proteins in freshly isolated CLL patient cells was undoubtedly a critical factor identifying these new insights. Previous studies have been hampered by having to analyze frozen material, and this has probably introduced significant variation particularly in the context of quantifying the labile protein Mcl-1.[47]

Interestingly, Bcl-2 and Bax protein expression was not associated with V_H gene mutation status ($P = .11$ and $P = .15$, respectively) or ZAP-70 expression ($P = .44$ and $P = .29$, respectively). However, Bcl-2 was significantly higher and Bax was significantly lower in the $CD38^+$ patients ($P = .001$ and $P < .001$, respectively), suggesting that these proteins may be regulated, at least in

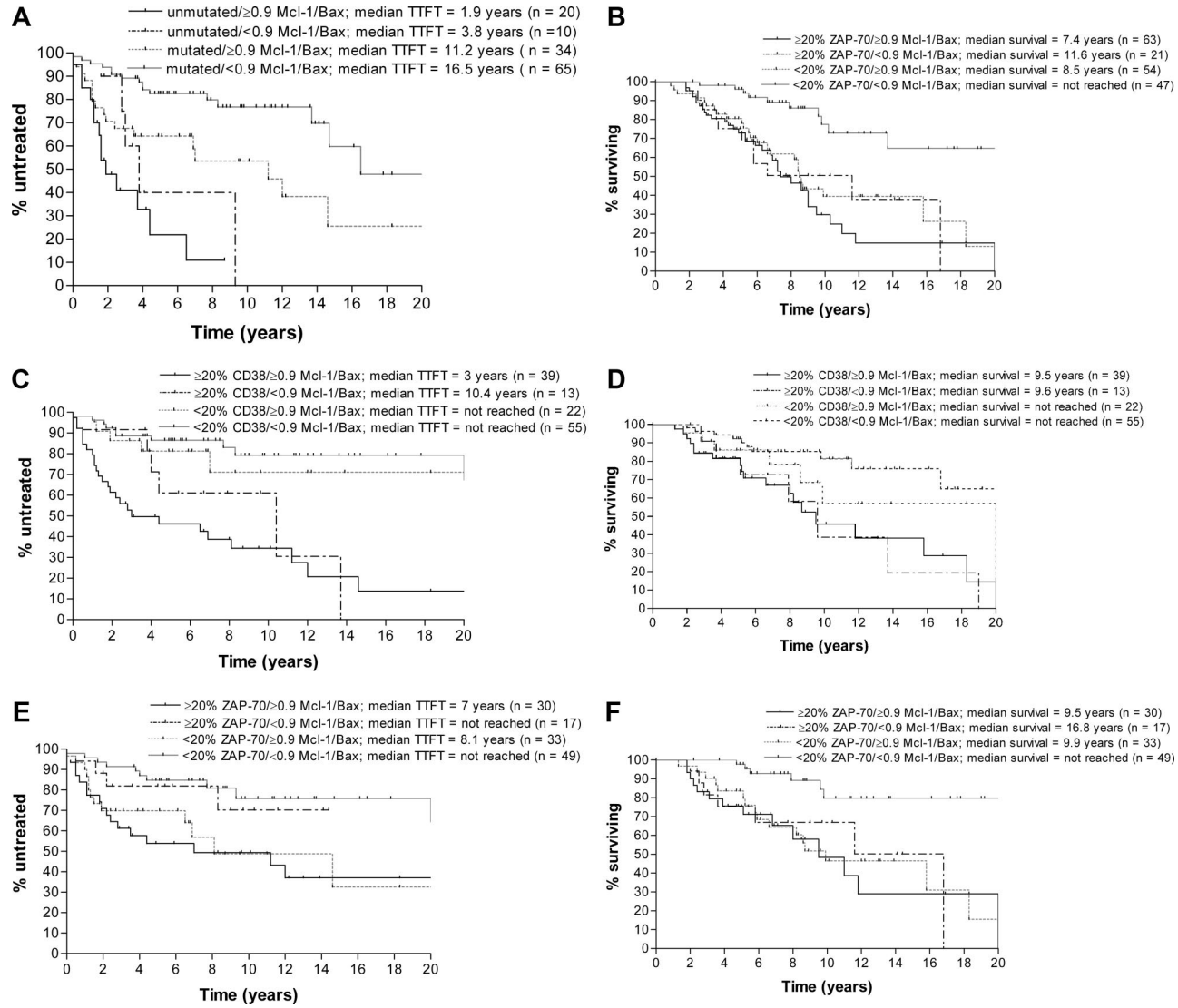

Figure 6. Kaplan-Meier curves for time to first treatment and overall survival defined by the combination of Mcl-1/Bax ratios and V_H gene mutation status, CD38 expression, and ZAP-70 expression (stage A patient cohort only; n = 129). Mcl-1/Bax ratios in combination with V_H gene mutation status (A,B), CD38 expression (C,D), and ZAP-70 expression (E,F) increased the prognostic power of each parameter in defining time to first treatment. However, in terms of overall survival, the addition of Mcl-1/Bax ratios only improved the prognostic power of ZAP-70 expression.

part, by CLL cell activation.[48] In terms of in vitro drug sensitivity, Bcl-2 expression did not correlate with fludarabine LD_{50} values. In contrast, Bax expression was inversely correlated and Mcl-1 expression positively correlated with in vitro resistance to fludarabine. It is noteworthy that the Bcl-2/Bax ratios and Mcl-1/Bax ratios showed even more significant correlations with fludarabine LD_{50} values. These data confirm previous studies that suggest that the relative expression of proapoptotic and antiapoptotic proteins, rather than the absolute expression of any one protein, is most informative of in vitro sensitivity to apoptotic stimuli.[24,30] However, it is also clear that other factors contribute to in vitro response to fludarabine as the r^2 values for the Bcl-2 family members evaluated were relatively low, for example, differences in Mcl-1/Bax ratios could only explain 14% of the variation in response to fludarabine.

Unlike Bcl-2 and Bax, high Mcl-1 expression was associated with V_H gene mutation status ($P < .001$), CD38 expression ($P < .001$), and ZAP-70 expression ($P = .003$). These findings are in keeping with the notion that Mcl-1 expression is regulated by CLL cell signaling pathways, including via the B-cell receptor,[41,49] whereas Bcl-2 and Bax are not so obviously modulated by these mechanisms. Intriguingly, although Mcl-1 appears to be regulated by multiple cellular signals, its expression remained remarkably constant in our longitudinal analysis (Figure S3). This indicates that Mcl-1 expression is tightly regulated in the tumor cells and does not appear to be significantly modulated over time by therapy.

The underlying reasons for the differential expression of Mcl-1 in prognostic subsets of CLL patients are probably complex. A recent study by Moshynska et al[50] reported nucleotide insertions in the promoter region of the Mcl-1 gene correlated with increased RNA and protein levels in CLL cells and influenced clinical outcome. Although this presented an attractive rationale for the relative overexpression of Mcl-1 found in some CLL patients, the findings of this study were refuted by a much larger study of 173 CLL patients.[51] This study found no association between Mcl-1 promoter insertion sequences and V_H gene status, Binet stage, or OS and concluded that they have no prognostic value in CLL.

An alternative explanation for elevated Mcl-1 expression may be found in the interaction between CLL cells and their microenvironment.

It was recently shown that phosphorylation of VEGF receptor led to increased Mcl-1 expression and protection against chlorambucil-induced apoptosis.[52] In addition, follicular dendritic cells are able to up-regulate Mcl-1 in CLL cells by direct contact through CD44.[33] The short half-life of Mcl-1 (1-2 hours) indicates that circulating CLL cells with high Mcl-1 expression most probably receive regular stimulation from circulating chemokines, such as VEGF to maintain expression levels. In this regard, we have previously shown that $CD38^+$ CLL cells have higher VEGF and Mcl-1 expression giving an autocrine positive loop.[53] Alternatively, the ability of CLL cells derived from patients with unmutated V_H genes to signal through the B-cell receptor may present another possible mechanism for controlling Mcl-1 expression levels. Petlickovski et al[41] reported that sustained signaling through the B-cell receptor induced Mcl-1 expression and promoted survival in CLL cells. In our study, we found a strong correlation between V_H gene mutational status and constitutive Mcl-1 expression, suggesting that CLL cells with unmutated V_H genes may be more sensitive to, or have a higher capacity for, processing, intrinsic survival signals resulting in elevated basal expression of Mcl-1. These signals may be mediated through the B-cell receptor and/or other signaling pathways implicated in the up-regulation of Mcl-1, including the phosphatidyl inositol-3 kinase/Akt pathway.[35]

Given the strong correlations between Mcl-1 and V_H gene mutation status, CD38 expression, and ZAP-70 expression, it was not surprising to find that Mcl-1 and Mcl-1/Bax ratios held prognostic significance in our cohort. However, it was remarkable that Mcl-1/Bax ratios were superior discriminators of TTFT in the whole cohort as evidenced by the highest hazard ratio (Table 2).

In conclusion, our data show that all 3 Bcl-2 family proteins assessed in this study play a role in the progression and outcome of CLL. Mcl-1 appears to have the most influence over in vitro drug sensitivity and in vivo survival. However, in keeping with our previous findings,[32] Bax increases in importance in patients who have received prior chemotherapy. Taken together, our data present an intriguing insight into the relevance of Bcl-2 family proteins in the pathogenesis and clinical outcome of CLL and reinforce the idea that these proteins represent legitimate molecular targets for the treatment of this incurable disease.

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Authorship

Contribution: C.P. designed the work, performed experiments, interpreted the data, and wrote the manuscript; T.T.L. performed experiments, interpreted the data, and revised the manuscript; G.P. contributed vital reagents and revised the manuscript; S.H. performed experiments and interpreted the data; P.B. interpreted the data and revised the manuscript; L.H. analyzed the data and revised the manuscript; R.H. analyzed the data; R.W., J.S., B.A., L.H., and T.S. performed experiments; and C.F. designed the work, interpreted the data, and wrote the manuscript.

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References

1. Wierda WG, O'Brien S, Wang X, et al. Prognostic nomogram and index for overall survival in previously untreated patients with chronic lymphocytic leukemia. Blood. 2007;109:4679-4685.
2. Hamblin T. Prognostic factors in chronic lymphocytic leukemia. Leuk Lymphoma. 2006;47:2261-2262.
3. Lee JS, Dixon DO, Kantarjian HM, Keating MJ, Talpaz M. Prognosis of chronic lymphocytic leukemia: a multivariate regression analysis of 325 untreated patients. Blood. 1987;69:929-936.
4. Geisler CH. Chronic lymphocytic leukaemia: newer prognostic approaches in relation to clinical stage. Dan Med Bull. 1993;40:190-207.
5. Binet JL, Auquier A, Dighiero G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer. 1981;48:198-206.
6. Montserrat E, Sanchez-Bisono J, Vinolas N, Rozman C. Lymphocyte doubling time in chronic lymphocytic leukaemia: analysis of its prognostic significance. Br J Haematol. 1986;62:567-575.
7. Dohner H, Stilgenbauer S, Dohner K, Bentz M, Lichter P. Chromosome aberrations in B-cell chronic lymphocytic leukemia: reassessment based on molecular cytogenetic analysis. J Mol Med. 1999;77:266-281.
8. Del Poeta G, Maurillo L, Venditti A, et al. Clinical significance of CD38 expression in chronic lymphocytic leukemia. Blood. 2001;98:2633-2639.
9. Ibrahim S, Keating M, Do KA, et al. CD38 expression as an important prognostic factor in B-cell chronic lymphocytic leukemia. Blood. 2001;98:181-186.
10. Rassenti LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. N Engl J Med. 2004;351:893-901.
11. Orchard JA, Ibbotson RE, Davis Z, et al. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. Lancet. 2004;363:105-111.
12. Oscier DG, Gardiner AC, Mould SJ, et al. Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. Blood. 2002;100:1177-1184.
13. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94:1848-1854.
14. McConkey DJ, Chandra J, Wright S, et al. Apoptosis sensitivity in chronic lymphocytic leukemia is determined by endogenous endonuclease content and relative expression of BCL-2 and BAX. J Immunol. 1996;156:2624-2630.
15. Thomas A, El Rouby S, Reed JC, et al. Drug-induced apoptosis in B-cell chronic lymphocytic leukemia: relationship between p53 gene mutation and bcl-2/bax proteins in drug resistance. Oncogene. 1996;12:1055-1062.
16. Robertson LE, Plunkett W, McConnell K, Keating MJ, McDonnell TJ. Bcl-2 expression in chronic lymphocytic leukemia and its correlation with the induction of apoptosis and clinical outcome. Leukemia. 1996;10:456-459.
17. Ghia P, Granziero L, Chilosi M, Caligaris-Cappio F. Chronic B cell malignancies and bone marrow microenvironment. Semin Cancer Biol. 2002;12:149-155.
18. Caligaris-Cappio F. Role of the microenvironment in chronic lymphocytic leukaemia. Br J Haematol. 2003;123:380-388.
19. Munk-Pedersen I, Reed J. Microenvironmental interactions and survival of CLL B-cells. Leuk Lymphoma. 2004;45:2365-2372.
20. Ghia P, Circosta P, Scielzo C, et al. Differential effects on CLL cell survival exerted by different microenvironmental elements. Curr Top Microbiol Immunol. 2005;294:135-145.
21. Schena M, Gottardi D, Ghia P, et al. The role of Bcl-2 in the pathogenesis of B chronic lymphocytic leukemia. Leuk Lymphoma. 1993;11:173-179.
22. Reed JC. Mechanisms of Bcl-2 family protein function and dysfunction in health and disease. Behring Inst Mitt. 1996:72-100.
23. Faderl S, Keating MJ, Do KA, et al. Expression profile of 11 proteins and their prognostic significance in patients with chronic lymphocytic leukemia (CLL). Leukemia. 2002;16:1045-1052.
24. Pepper C, Hoy T, Bentley DP. Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance. Br J Cancer. 1997;76:935-938.
25. Pepper C, Hoy T, Bentley P. Elevated Bcl-2/Bax are a consistent feature of apoptosis resistance in B-cell chronic lymphocytic leukaemia and are correlated with in vivo chemoresistance. Leuk Lymphoma. 1998;28:355-361.
26. Kitada S, Andersen J, Akar S, et al. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. Blood. 1998;91:3379-3389.
27. Hanada M, Delia D, Aiello A, Stadtmauer E, Reed JC. bcl-2 gene hypomethylation and high-level

expression in B-cell chronic lymphocytic leukemia. Blood. 1993;82:1820-1828.

28. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A. 2005;102:13944-13949.
29. Osorio LM, De Santiago A, Aguilar-Santelises M, Mellstedt H, Jondal M. CD6 ligation modulates the Bcl-2/Bax ratio and protects chronic lymphocytic leukemia B cells from apoptosis induced by anti-IgM. Blood. 1997;89:2833-2841.
30. Molica S, Dattilo A, Giulino C, Levato D, Levato L. Increased bcl-2/bax ratio in B-cell chronic lymphocytic leukemia is associated with a progressive pattern of disease. Haematologica. 1998;83:1122-1124.
31. Pepper C, Thomas A, Hoy T, Bentley P. Chlorambucil resistance in B-cell chronic lymphocytic leukaemia is mediated through failed Bax induction and selection of high Bcl-2-expressing subclones. Br J Haematol. 1999;104:581-588.
32. Starczynski J, Pepper C, Pratt G, et al. Common polymorphism G(-248)A in the promoter region of the bax gene results in significantly shorter survival in patients with chronic lymphocytic leukemia once treatment is initiated. J Clin Oncol. 2005;23:1514-1521.
33. Pedersen IM, Kitada S, Leoni LM, et al. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. Blood. 2002;100:1795-1801.
34. Smit LA, Hallaert DY, Spijker R, et al. Differential Noxa/Mcl-1 balance in peripheral vs lymph node chronic lymphocytic leukemia cells correlates with survival capacity. Blood. 2007;109:1660-1668.
35. Longo PG, Laurenti L, Gobessi S, et al. The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B cells. Blood. 2008;111:846-855.
36. Bannerji R, Kitada S, Flinn IW, et al. Apoptotic-regulatory and complement-protecting protein expression in chronic lymphocytic leukemia: relationship to in vivo rituximab resistance. J Clin Oncol. 2003;21:1466-1471.
37. Hussain SR, Cheney CM, Johnson AJ, et al. Mcl-1 is a relevant therapeutic target in acute and chronic lymphoid malignancies: down-regulation enhances rituximab-mediated apoptosis and complement-dependent cytotoxicity. Clin Cancer Res. 2007;13:2144-2150.
38. Derenne S, Monia B, Dean NM, et al. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. Blood. 2002;100:194-199.
39. Michels J, O'Neill JW, Dallman CL, et al. Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. Oncogene. 2004;23:4818-4827.
40. Ringshausen I, Schneller F, Bogner C, et al. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. Blood. 2002;100:3741-3748.
41. Petlickovski A, Laurenti L, Li X, et al. Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. Blood. 2005;105:4820-4827.
42. Willimott S, Baou M, Naresh K, Wagner SD. CD154 induces a switch in pro-survival Bcl-2 family members in chronic lymphocytic leukaemia. Br J Haematol. 2007;138:721-732.
43. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood. 1996;87:4990-4997.
44. Crespo M, Bosch F, Villamor N, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med. 2003;348:1764-1775.
45. Stankovic T, Stewart GS, Fegan C, et al. Ataxia telangiectasia mutated-deficient B-cell chronic lymphocytic leukemia occurs in pregerminal center cells and results in defective damage response and unrepaired chromosome damage. Blood. 2002;99:300-309.
46. Pepper C, Thomas A, Hoy T, et al. The vitamin D3 analog EB1089 induces apoptosis via a p53-independent mechanism involving p38 MAP kinase activation and suppression of ERK activity in B-cell chronic lymphocytic leukemia cells in vitro. Blood. 2003;101:2454-2460.
47. Grever MR, Lucas DM, Dewald GW, et al. Comprehensive assessment of genetic and molecular features predicting outcome in patients with chronic lymphocytic leukemia: results from the US Intergroup Phase III Trial E2997. J Clin Oncol. 2007;25:799-804.
48. Damle RN, Temburni S, Calissano C, et al. CD38 expression labels an activated subset within chronic lymphocytic leukemia clones enriched in proliferating B cells. Blood. 2007;110:3352-3359.
49. Ruiz-Vela A, Aggarwal M, de la Cueva P, et al. Lentiviral (HIV)-based RNA interference screen in human B-cell receptor regulatory networks reveals MCL1-induced oncogenic pathways. Blood. 2008;111:1665-1676.
50. Moshynska O, Sankaran K, Pahwa P, Saxena A. Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia. J Natl Cancer Inst. 2004;96:673-682.
51. Tobin G, Skogsberg A, Thunberg U, et al. Mcl-1 gene promoter insertions do not correlate with disease outcome, stage or VH gene mutation status in chronic lymphocytic leukaemia. Leukemia. 2005;19:871-873.
52. Lee YK, Bone ND, Strege AK, et al. VEGF receptor phosphorylation status and apoptosis is modulated by a green tea component, epigallocatechin-3-gallate (EGCG), in B-cell chronic lymphocytic leukemia. Blood. 2004;104:788-794.
53. Pepper C, Ward R, Lin TT, et al. Highly purified CD38(+) and CD38(-) sub-clones derived from the same chronic lymphocytic leukemia patient have distinct gene expression signatures despite their monoclonal origin. Leukemia. 2007;21:687-696.