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REVIEW

Expression profile of 11 proteins and their prognostic significance in patients with chronic lymphocytic leukemia (CLL)

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It has been suggested that the expansion of the leukemic cells in chronic lymphocytic leukemia (CLL) is due to dysregulation of pathways of programmed cell death (apoptosis) rather than cell proliferation, although differences may exist in early *vs* late and treated *vs* untreated patients. In the present study, we analyzed the expression of 11 proteins in CLL cells that are implicated in the control of apoptosis, proliferation, and differentiation, and correlated this expression profile with survival. Using a quantitative solid-phase radioimmunoassay (RIA), we measured the cellular protein levels of Bcl-2, cyclin D1, PCNA, ATM, Fas, Bax, retinoic acid receptor alpha (RARα), retinoic acid receptor beta (RXRβ), Flt1, VEGF, and cellular β_2-microglobulin in 230 samples of CLL. Univariate analysis using the Cox proportional hazard model showed a correlation with survival of only the following proteins: Bcl-2 ($P < 0.001$), cyclin D1 ($P = 0.027$), Fas ($P = 0.055$), PCNA ($P < 0.001$), and ATM ($P = 0.028$). In a multivariate analysis using classification and regression tree analysis (CART), five groups of patients (nodes) could be generated with significant differences of survival expectation ($P < 0.0001$) based on levels of expression of the above proteins. Based on CART analysis, Bcl-2 levels emerge as the most important protein in predicting survival between all 11 proteins studied. Patients with marked elevation in Bcl-2 levels had the worst outcome while patients with intermediate levels, but with high levels of PCNA and cyclin D1 or abnormal ATM expression had intermediate survival. These data indicate that intracellular levels of proteins such as Bcl-2, ATM, cyclin D1, and PCNA can be used as markers to predict clinical behavior and survival in patients with CLL. The pathways in which these proteins are involved may also represent possible targets for future therapeutic trials in CLL.
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Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent form of adult leukemia in the Western world where it accounts for about 25% of all leukemias. The disease may be characterized by a rather indolent course with good long-term prognosis in the absence of therapeutic intervention, or it may take on an accelerated course requiring treatment immediately.[1,2] However, patients with early stage disease may sometimes progress more rapidly than would be expected on the basis of their clinical stage. Identification of other prognostic factors such as β_2-microglobulin and soluble CD23 has allowed a better stratification of early-stage disease patients, but whether initiation of treatment based on these parameters changes outcome favorably or delays progression is still unknown.[3,4]

Recent advances in molecular biology and the development of biochips and RNA arrays made it possible to evaluate the expression profile of many genes and correlate these RNA profiles with the clinical outcome.[5] High throughput analysis of protein expression profiles is at early stages in its development. Protein expression profiling of a tumor may provide information on post-transcriptional regulation and modification including stability, phosphorylation, and alternative splicing. Most of the current high throughput protein analysis uses mass spectrometry and 2-D gel analysis and recognizes changes in protein expression patterns.

Here, we evaluated the expression profile of 11 proteins in patients with CLL and correlated the pattern of expression with clinical behavior and outcome. We evaluated proteins involved in programmed cell death or apoptosis. Failure of CLL cells to undergo apoptosis is frequently considered the primary lesion in CLL that provides the leukemic cell population with its growth advantage over their normal B and T lymphocyte counterparts.[6] High protein levels of Bcl-2 are found in CLL cells even in the absence of the classical t(14;18) translocation that is detected frequently in follicular lymphomas.[7] Bax, a proapoptotic antagonist to Bcl-2, has been found to determine prognosis in patients with CLL such that a higher Bcl-2/Bax protein ratio is associated with more progressive disease and shorter survival.[8] Bcl-2 and Bax are only two members of a multitude of pro- and anti-apoptotic proteins. At the effector stage of apoptosis, caspase enzymes mediate cleavage and breakdown of cytosolic and nuclear proteins ultimately accounting for the typical cellular changes associated with apoptosis.[9] Expression of caspase 3 has been demonstrated in leukemic cells of patients with CLL but not much is known about its level of dysregulation in these cells and its pathophysiologic implications.[10] Levels of the proliferating cell nuclear antigen (PCNA) correlate with cell proliferation, clinical stage and lymphocyte doubling time.[11] Higher levels of PCNA have been observed in more advanced stage disease.[12] The ATM gene (mutated in ataxia telangiectasia (AT)), located on human chromosome 11q22-q23, was found to be deleted in up to 14% of cases with CLL and the levels of its protein product reduced in up to 34%.[13] Mutations of the ATM locus predispose AT patients to lymphoid malignancies and mutations of the ATM gene or deficiency of its protein product have been associated with extensive lymph node involvement and poor survival in patients with B cell CLL.[14] Increased levels of the cellular angiogenic factor VEGF (vascular endothelial growth factor) have been found to correlate with outcome in patients with CLL, but not much is known yet about the pathophysiologic role of angiogenic factors and their receptors in CLL.[15]

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In the current study, we analyzed the cellular protein levels of the proliferating cell nuclear antigen (PCNA), Bcl-2, Bax, Fas, ATM (mutated in AT patients), retinoic receptor α (RARα), retinoic receptor β (RXRβ), vascular endothelial growth factor (VEGF), the cellular receptor for VEGF, Flt-1, β_2-microglobulin, and cyclin D1 in patients with CLL and determined their interaction with each other and their impact on survival.

Materials and methods

Patients and clinical samples

Bone marrow and/or peripheral blood samples of 230 consecutive patients with CLL were analyzed (Table 1). Samples were obtained with the informed consent of the patients and the procedures were in compliance with guidelines established by the Institutional Review Board for clinical studies at the University of Texas MD Anderson Cancer Center.

The diagnosis of CLL was made according to standard diagnostic criteria.[16] Patients were required to have a peripheral blood lymphocytosis of more than 10×10^9/l or more than 5×10^9/l if associated with an unequivocal immunophenotype, and more than 30% lymphocytes or prolymphocyte in the bone marrow. By morphology, cells had to be small, round, and mature-appearing. The diagnostic evaluations were complemented by determination of the immunophenotype of the leukemic cell population by flow cytometry. The cut-off point for positivity was 20%. The CLL cells of all patients had coexpression for CD5 and CD19, were positive for CD23, showed dim staining with CD20, and were negative for FMC-7. Patients with CLL variants such as *de novo* prolymphocytic leukemia and hairy cell leukemia, and patients with transformed stages of CLL (Richter's transformation) were excluded from the study. The median follow-up of the studied patients was 22.4 months and all patients were treated on Fludarabine-based protocols. Cytogenetic studies were performed in 169 patients. Only one patient had t(14;18) translocation and this patient had a Bcl-2 level of 2.1. Three patients had t(11;14) translocation CD23 positivity, FMC-7 negativity and morphology consistent with CLL rather than mantle cell lymphoma. All analyzed samples contained more than 70% leukemic cells (lymphocytes or prolymphocytes).

Table 1 Clinical and laboratory characteristics of the study group

Characteristic	*n (%)*	*Median (range)*
Number of patients	230	
Rai stage		
0	38 (16)	
1	91 (40)	
2	32 (14)	
3	18 (8)	
4	44 (19)	
N/A	7 (3)	
Treatment status		
Untreated	171 (74)	
Treated	56 (26)	
Age (years)		58 (21–86)
Hemoglobin (g/dl)		13.1 (4–18)
Splenomegaly	80 (35)	
Male	147 (64)	
Platelets ($\times 10^9$/l)		158 (5–541)
White blood cells ($\times 10^9$/l)		49 (3–652)
% of lymphocytes/prolymphocytes		85 (73–99)
β_2-microgobulin (mg/l)		2.8 (1.1–15.4)

Cytogenetic studies were performed using standard techniques. Polymerase chain reaction (PCR) for Bcl-2 rearrangement was performed on DNA samples using conventional methods. PCR amplification was used to detect both MBR and mcr breakpoints. PCR products were resolved on 2% NuSieve gel and then transferred to a nylon membrane. Membranes were hybridized with probes for MBR and mcr at 63°C (MBR) or 42°C (mcr) overnight.

Western blot analysis

All antibodies used in this study were first tested by Western blot to ensure specificity. The following antibodies were used: mouse anti-RARα/RARβ (Catalog No.: MA1–810 and MA3-812; Affinity BioReagents, Neshanic Station, NJ, USA), goat anti-VEGF (Catalog No.: AF-293-NA; R&D Systems, Minneapolis, MN, USA), mouse anti-Bcl-2 and rabbit anti-β_2 microglobulin (Catalog No.: M0887 and A0072; Dako Corporation, Carpinteria, CA, USA), mouse anti-cyclin D1 (Catalog No.: M033575; Pharmingen, San Diego, CA, USA), mouse anti-Bax (Catalog No.: PC66; CalBiochem, San Diego, CA, USA) mouse anti-Fas (Catalog No.: 65 311A; Pharmingen) and mouse anti-PCNA (Catalog No.: SC-56; Santa Cruz, Santa Cruz, CA, USA), and rabbit anti-ATM (Catalog No.: PC116, CalBiochem). Western blots were performed as previously described.[17] Three hundred micrograms of cell extract were used in the analysis of ATM protein (Figure 1). Protein was electrophoretically separated on a 5.5% SDS-PAGE gel, transferred to nitrocellulose paper and filters were incubated overnight at 4°C with rabbit anti-ATM polyclonal antibody. The membrane was washed, then incubated with 1:2000 diluted anti-rabbit immunoglobin linked to horseradish peroxidase (Amersham, Arlington Heights, IL, USA) in PBS containing 1% nonfat milk and 0.1% Tween 20. Immunoreactive bands were developed using the ECL detection system (Amersham). After ECL detection, the membrane was stripped from the primary and secondary antibodies under conditions recommended by Amersham, and then blocked and probed with an anti-actin (Amersham) to check for equal loading of protein in each lane. All cases that we had adequate protein for immunoblot analyzed had detectable levels of ATM.

All Western blot results were correlated with the solid-phase radioimmunoassay (RIA). Correlation between Western blot and RIA was performed on a case by case basis and whenever it was possible. For example, cyclin D1 is not detectable in the majority of CLL cases on Western blot but is detectable by RIA with significant variation between cases. In contrast, most of the Bcl-2 results were correlated by comparing intensity of bands on Western blot with RIA values as a whole ensuring that a case does not have higher levels by RIA but a lower intensity band on Western blot as compared with other cases. In normal controls, we were able to correlate intensity of Western blot bands and RIA values for PCNA, Bcl-2, ATM, and cellular β_2-microglobulin only. Correlation between Western and RIA in normal controls for the other proteins was not possible because they were not detectable in the majority of samples. Using the sign test to test the null hypothesis we found no significant difference between the two methods for PCNA, Bcl-2, ATM, and cellular β_2-microglobulin (P values: 0.09, 0.1, 0.2 and 0.07, respectively).

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Solid-phase radioimmunoassay (RIA)

RIA plates (Fisher Scientific, Pittsburgh, PA, USA) were coated overnight at 4°C with 5 μg of protein extracted from samples of patients with CLL and normal individuals in 50 μl of PBS. The plates were then washed with PBS and blocked with 100 μl of 1% BSA (Amersham) in PBS for 1 h at 37°C. The plates were incubated overnight at 4°C with 50 μl of antibody diluted 1:500 in PBS containing 1% BSA. Then, plates were washed with PBS and amplified with goat anti-rabbit, rabbit anti-mouse, or rabbit anti-goat antisera diluted in 1:500 in 0.1% BSA in PBS for 2 h at 37°C. After washing, plates were developed with excess ^{125}I-labelled protein G (200 000 c.p.m. (50 IU) of 0.1% BSA in PBS per well) (Amersham) for 2 h at room temperature, washed with PBS, separated into individual wells, and the counts in each well were counted with a gamma counter (LKB Biotechnology, Uppsala, Sweden). The assays were performed in triplicate and the results were corrected for the nonspecific binding (1–2%) detected in control wells that were not coated with a test antigen but blocked with BSA. In addition, samples that showed no evidence of ATM protein on Western blot were also included every time a new patch of samples was analyzed along with low and high samples to ensure reproducibility. The assay was also performed with anti-actin antibodies to confirm complete and uniform coating of the surfaces of the plates. Since each well on the plate has a limited capacity for binding protein (approximately 2.5 μg of protein) and excess protein is used in saturating all wells, no significant variation between wells or plates is seen as confirmed by the actin which showed a variance of 0.006. All samples were analyzed using a master mix under the same conditions and the normal controls were used in each assay. Actin was used to confirm the use of equal loading for protein. There was no correlation between actin and any of the protein studied (r values between –0.1 and +0.1) and the variance in the actin measurement was calculated to ensure low levels. The linearity of the assays was confirmed using dilutions. Example of the demonstration of linearity is shown in Figure 2, in which dilutions of recombinant Bcl-2 (Pharmingen) were analyzed and demonstrated complete direct correlation.

Statistical methods

Pearson and Spearman correlations were calculated for pairs of continuous covariates or when at least one covariate was ordered. Associations between categorical variables were tested using chi-square statistics. The sign test was used to test the null hypothesis (ie lack of significant difference between two measurements). Not significant P value in the sign test indicates no significant difference. Survival curves were calculated from the date of initiation of treatment to death or the last date of follow-up according to the actuarial method of Kaplan and Meier.[18] The log-rank test of Peto and Peto was used to assess the significance of differences between patient groups.[19] The ability of each variable alone to predict survival was tested by a univariate Cox analysis with martingale residual plots as model-fitting diagnostics.[20]

A new and important aspect of our work is the use of a 'tree-structured' survival analysis. The method, also termed 'recursive partitioning', is based on the original work by Breiman *et al*[21] on classification and regression trees with extensions to a survival framework by others.

CART is a computer-intensive non-parametric tool used in medical applications as a classification or regression tool. In contrast to its parametric competitors such as Fisher's linear discriminant analysis, logistic regression, or linear regression, the CART methodology does not depend on any underlying distributional assumption.[22] CART allows for non-linear relations between predictive factors and outcomes and for mixed data types (numerical and categorical), isolates outliers, and incorporates a pruning process using cross-validation as an alternative to testing for unbiasedness with a second data set.

Some examples of the usage of this methodology in the medical arena include papers by ourselves and others.[23–26]

The tree is derived by recursive partitioning, beginning with the total sample population and all variables. At each step the program determines for each possible predictor variable (covariate) a cut-point which optimally splits the population into pre-specified subgroups, and then selects the variable which performs best (according to some criterion based on minimizing impurities of the subgroups or on reducing the overall error rate). If an observation has no data for this variable, the program uses the next most important variable (the surrogate). It then takes the resulting subpopulations and repeats the process until no further partitioning is warranted: either that a subpopulation contains only one class of the observed response variable or the subpopulation is too small to subdivide further. A 'pruning' procedure then recombines subgroups using a minimal cost complexity measure that takes into account the size of the tree to avoid any overfilling for a selected complexity parameter. The result of the pruning procedure is a nested subset of trees starting from the largest tree grown and continuing until only one node of the tree remains. Cross-validation or test sample is used to provide estimates of future prediction error for each sub-tree. During the partitioning process, the program keeps track of how well each predictor performs on each split and thus can evaluate its overall discriminating or explanatory ability relative to the other factors in the analysis measured by variable importance rankings. The final result is a decision tree. Assessment of the predictive power for each variable is given by the variable importance ranking, rather than the level where it appears on the final tree. The optimal tree is the one that yields the lowest cross-validated error rate.

For a survival endpoint, the CART analysis was implemented using a method suggested by Therneau *et al*.[27] In this method, the censored survival data are transformed into a single uncensored data value (the so-called 'null martingale residual'), which is used as input into a standard regression tree algorithm.[28] This *ad hoc* method has been shown to perform reasonably well for censored time-to-event data.[29] In addition to the default tree generated by the CART algorithm, we examined alternative initial splits using systematic inspection.[30] The frequency of alternative splits was assessed by bootstrap simulations.[31]

Results

Heterogeneity in protein expression

The intracellular levels of Bcl-2, cyclin D1, PCNA, ATM, Fas (CD95), Bax, RARα, RXRβ, Flt1, VEGF, and cellular β_2-microglobulin were determined by Western blot analysis (Figure 1). Solid-phase radioimmunoassay (RIA) was used for quantification. RIAs are reproducible and the linearity of all assays was confirmed using dilutions (Figure 2). We analyzed

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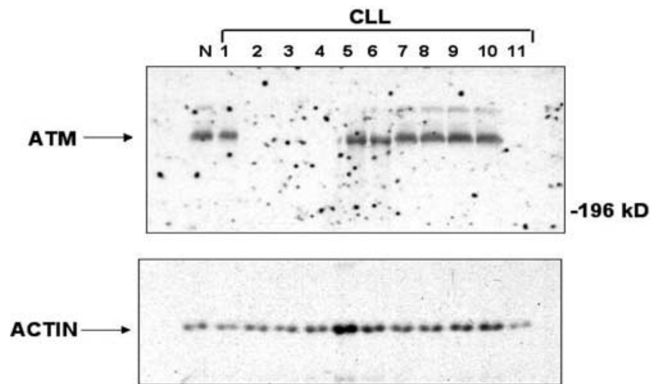

Figure 1 Western blot analysis of ATM protein. A sample from normal control (N) is shown. The median level of protein expression in normal control individuals was assigned a value of 1 and the values in patients were normalized to the median of the normal control. Patients with levels 0.7 (2, 3, 4, and 11) showed no detectable ATM protein on Western blot (0.3, 0.4, 0.6, and 0.1 by RIA, respectively). The filter was stripped and reprobed with actin to confirm adequate protein loading.

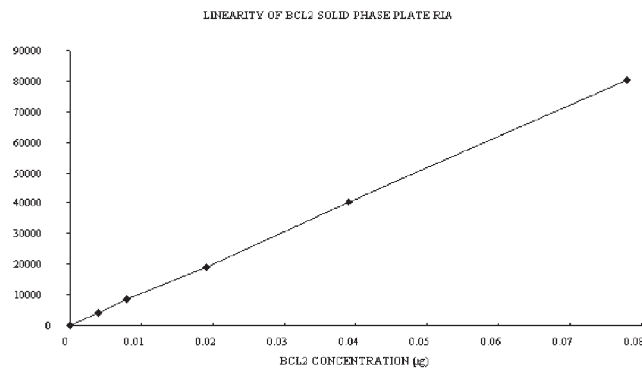

Figure 2 Linearity of the solid-phase RIA. Dilutions of recombinant Bcl-2 were analyzed to demonstrate the linearity of the assay.

Table 2 Variation in protein expression in CLL

Protein	*Median*	*Range*	*s.d.*	*Variance in normal controls*
ATM	0.85	0.0–3.15	0.52	0.03
Cyclin D1	1.5	0.7–8.83	1.07	0.03
Bcl-2	2.7	0.5–13.19	2.05	0.06
Fas	2.19	0.75–8.16	1.57	0.01
PCNA	1.89	0.45–12.97	1.35	0.003
Bax	3.9	0.4–18.1	4.09	0.03
RARα	3.5	0.37–104.00	7.66	0.13
RARβ	2.5	0.3–6.5	1.61	0.34
Flt1	4.33	0.11–6.98	2.35	0.01
VEGF	5.69	1.37–16.64	2.91	0.002
β_2M	2.54	1.02–10.04	1.31	0.003
Actin	1.01	0.68–1.14	0.08	0.006

s.d., standard deviation.
All values were normalized to the median of the normal control, which was assigned a value of 1.

the level of expression of these proteins in mononuclear cells of 23 healthy individuals and used the median to normalize the values obtained from the patients' samples. This median was assigned a value of 1. Various characteristics of the studied patients are listed in Table 1. Variation in levels between samples was noted (Table 2).

Correlation between the various proteins

As shown in Table 3, increasing levels of Bcl-2 correlated with most of the poor prognostic factors such as high white blood cell (WBC) count, peripheral blood lymphocytosis, advanced Rai or Binet stage, decreased hemoglobin, low platelet counts, and high levels of serum β_2-microglobulin. Higher levels of Bcl-2 correlated with higher levels of Bax possibly representing a compensatory increase. In addition, increasing levels of Bcl-2 correlated with increasing levels of PCNA representing the proliferative potential of the leukemic cell population. This observation suggests that increased proliferation may accompany increased resistance to apoptosis in clinically advanced cases of CLL. PCNA levels further demonstrated a positive correlation with levels for Fas (CD95) and Bax (Figure 3). Cyclin D1 levels correlated with β_2-microglobulin, reflecting its prognostic value, and increasing Bcl-2 and PCNA levels. This may reflect the complex interaction between cell cycle regulation and apoptosis. VEGF levels correlated with FLT-1. In addition, VEGF levels correlated with increased proliferation (PCNA) which may suggest a role of VEGF as a growth factor. RARα levels correlated negatively with cyclin D1 and BAX , but did not correlate with Bcl-2 or PCNA. In contrast, RXRβ correlated positively with the Bcl-2, ATM, PCNA, BAX and FAS in addition to RARα, most likely reflecting that the retinoic acid receptors may play a role in the regulation of the various genes studied here.

Except for cyclin D1 and RARα, age did not correlate with levels of the other proteins that we analyzed suggesting that the biological characteristics of the disease are similar in all patients regardless of age.

The cellular β_2-microgobulin levels did not correlate with the serum levels of β_2-microglobulin, nor positively with most of the characteristics that reflect more aggressive disease. In general, correlation among proteins was more significant within the same biological pathway, such as between Fas (CD95), Bcl-2, and Bax (apoptosis).

Survival analysis

In a univariate survival analysis using the Cox proportional hazard model with the various protein levels as continuous variables, cyclin D1 ($P=0.027$), Bcl-2 ($P<0.001$), Fas (CD95) ($P=0.055$), PCNA ($P<0.001$), and ATM ($P=0.028$) emerged as the only proteins that correlated with survival (Table 4). In contrast, RARα, RXRβ, Flt1, VEGF, and cellular β_2-microglobulin showed no correlation with survival when patients of all stages are considered. RARα, RXRβ, Flt1, VEGF, and cellular β_2-microglobulin did not predict survival when analyzed as continuous variables or using median or upper or lower quartiles as cut-off points. To ensure proper fit for the Cox regression model, we used transformed logarithmic variables when appropriate to ensure that the model is not heavily influenced by outliers. We found that the relationship of survival and ATM was quadratic, so when we fitted a model for ATM, we included both the linear term ATM and a quadratic

Table 3 Correlation between various proteins and clinical characteristics (Spearman rank order correlations)

	Bcl-2	*CD1*	*ATM*	*VEGF*	*PCNA*	*FLT-1*	*BAX*	*FAS*	*cβ_2M*	*RARα*	*RXRβ*
Bcl-2		<0.001	0.49	0.2	<0.001	0.1 (–)	<0.001	0.02	<0.001 (–)	0.4 (–)	0.004
CD1	<0.001		0.02 (–)	0.03	<0.001	0.01	0.02	<0.001	0.05 (–)	<0.001 (–)	0.08 (–)
ATM	0.49	0.02 (–)		<0.001	0.9	<0.001	0.6	0.02	0.006	0.002	<0.001
VEGF	0.2	0.467	<0.001		0.003	<0.001	0.6 (–)	0.01	0.1	0.03	<0.001
PCNA	<0.001	<0.001	0.9	0.003		0.3	<0.001	<0.001	<0.001 (–)	0.06 (–)	0.01
FLT-1	0.1	<0.001	<0.001	<0.001	0.3		0.02	<0.001	0.2	0.7	0.001
BAX	0.0000	0.02	0.06	0.6 (–)	<0.001	0.02		0.7	<0.001 (–)	<0.001 (–)	<0.001
FAS	0.01	<0.001	0.02	0.01	<0.001	<0.001	<0.001		<0.001 (–)	0.06 (–)	<0.001
cβ_2M	0.0004	0.05	<0.001	0.19	<0.001 (–)	0.2	<0.001 (–)	<0.001 (–)		0.005 (–)	
RARα	0.4	<0.001 (–)	0.002	0.03	0.06 (–)	0.7	<0.001 (–)	0.06 (–)	<0.001		<0.001
RARβ	0.004	0.08 (–)	<0.001	0.0003	0.01	0.001	<0.001	0.0001	0.005 (–)	<0.001	
Rai	<0.001	0.3	0.1 (–)	0.8 (–)	0.3	0.5 (–)	0.004	0.3	0.8	0.02 (–)	0.5
Binet	0.02	0.7	0.05 (–)	0.1	0.3	0.6	0.04	0.5	0.6	0.02 (–)	0.5
β_2M	0.01	0.007	0.4 (–)	0.8	0.11	0.6 (–)	0.2	0.1	0.8	0.1 (–)	0.9
HGB	<0.001 (–)	<0.001	0.09	0.5	0.002 (–)	0.8 (–)	0.01 (–)	0.06 (–)	0.3	0.1	0.08 (–)
PLT	0.05 (–)	0.6	0.7 (–)	0.9	0.9 (–)	0.9 (–)	0.05 (–)	0.9 (–)	0.3 (–)	0.08	0.6
WBC	<0.001	0.12	0.4 (–)	0.09 (–)	0.003	0.002 (–)	0.01	0.6	0.4	0.2	0.9
ALC	<0.001	0.15	0.7 (–)	0.08 (–)	0.004	<0.001 (–)	0.02	0.9	0.4	0.2	0.8
Age	0.8	0.02	0.1 (–)	0.7 (–)	0.6	0.3 (–)	0.4	0.1	0.4 (–)	0.03 (–)	0.1
BM cell	0.02	0.6	0.5 (–)	0.6 (–)	0.1	0.6 (–)	0.9	0.1	0.1	0.7	0.7

(–), indicates reversed correlation with negative R-value; ALC, absolute lymphocyte count; CD1, cyclin D1; BM, bone marrow; cβ_2M, cellular beta-2-microglobulin.

term ATM-squared (ATM^2). Using Kaplan–Meier analysis, Bcl-2, PCNA, and Fas showed that patients with higher expression had significantly shorter survival (Figure 4). As expected, reduced ATM protein correlated with shorter survival as we have previously reported.[13] In a multivariate analysis using a classification and regression tree (CART), we derived a decision tree that included only the variables found significant in univariate analysis (Table 5). The tree is derived by recursive partitioning, beginning with the total sample population and Bcl-2, ATM, Cyclin D1, PCNA, and Fas (CD95) as covariates. The program determined for each possible predictor variable (covariate) a cut-point, which optimally splits the population into pre-specified subgroups, and then selected the variable, which performs best. It then took the resulting subpopulations and repeats the process. This analysis generated five groups of patients (nodes) that are significantly different with respect to survival (Figure 5). Markedly high levels (up to eight-fold the levels detected in normal cells) of Bcl-2 were associated with a significantly shorter (2 to 7 months) survival probability. Cytogenetic studies were performed on four of the five patients in this group and none of them had t(14;18) translocation. Two of the patients had trisomy 12 and two had no analyzable metaphases. All patients in this group were previously treated and had Rai stage IV disease. None of the patients had a t(14;18) by polymerase chain reaction. The probability of survival was worse in patients with high levels of Bcl-2 (3.1–8.58) and CD1 when compared to patients with similar levels of Bcl-2 and low levels of cyclin D1 (32.5 months *vs* 45 months). Survival was longest in patients with low levels of Bcl-2 (<3.1) and normal levels of ATM (1–2) (Table 4). The survival was longer than in patients with similar levels of Bcl-2, but with abnormal levels of ATM (<1 or >2). The difference between these groups was significant ($P < 0.0001$). In order to validate this system, we analyzed an additional 59 CLL patients for Bcl-2, cyclin D1, PCNA, and ATM and tested the above model for survival. Patients with very high Bcl-2 (<8.5) had significantly shorter survival ($P < 0.001$) and patients with very low (<0.8) or very high ATM (>2) had significantly shorter survival ($P = 0.01$), while patients with high cyclin D1 had marginally shorter survival ($P = 0.04$), confirming the CART analysis. However, the number of cases was too small to run the entire CART analysis and reproduce the same tree.

Discussion

CLL, for the most part, is an indolent disease that may remain clinically silent for many years and can frequently be observed without immediate therapeutic intervention in its earlier clinical stages. On a molecular basis, the gradual accumulation of leukemic cells is thought to be due to a dysregulation of apoptotic pathways and abrogation of programmed cell death, mainly through upregulation of the anti-apoptotic protein Bcl-2.[7,32] On the other hand, clinically more advanced stages of CLL as well as CLL in transformation to a diffuse large cell lymphoma type process (Richter's transformation) may also demonstrate abnormalities in proteins involved in cell cycle regulation and regulation of proliferation.[6] Finally, even patients with CLL in early clinical stages may express markers or a combination thereof that predict a more aggressive clinical course than expected on clinical grounds alone and may be useful for prognostic purposes and to decide whether to postpone therapy or not.[33]

In the current study, we analyzed levels of 11 proteins in leukemic cells of patients with CLL and tried to establish a profile of markers that may serve as prognostic indicators, elucidate pathways of CLL pathophysiology, and eventually provide novel targets for therapy. First we studied the levels of these proteins as continuous variables in all patients regardless of the stage of the disease. By not using a cut-off point, we focused on the relevance of a continuous increase or decrease of the levels of these proteins. The possibility of effects of any increase *vs* no increase (grouping) was not tested in this analysis. Using this approach, only ATM, Bcl-2, PCNA, cyclin D1 and Fas (CD95) levels correlated with survival. These proteins are involved in cell cycle regulation and apoptosis pathways. In a multivariate analysis, Bcl-2 protein

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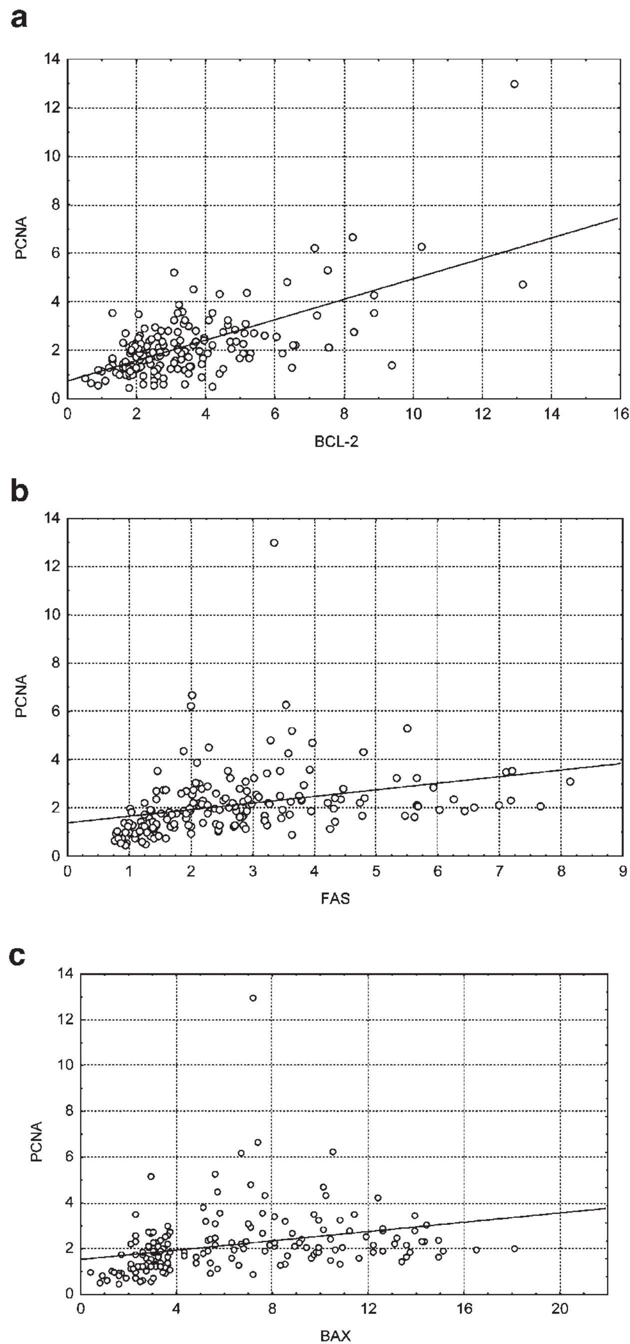

Figure 3 Correlation of levels of Bcl-2 (a), Fas (b), and Bax (c) with PCNA.

levels were the most important predictors of overall survival. Bcl-2 levels positively correlated with levels of Fas (CD95), Bax, and PCNA. Likewise, high levels of PCNA predicted for short survival, whereas low levels of PCNA, as well as cyclin D1, associated with median levels of Bcl-2 were predictive of the longest survival among the patients studied. Strikingly, as shown in Figure 5, a major difference in survival is noted between patients in node 3 and patients in node 4 and the only difference between the two groups is increased expression of cyclin D1 in node 4 patients. This suggests that cyclin D1 is a powerful predictor of survival in CLL patients despite the fact that its levels in CLL are significantly lower

Table 4 Univariate analysis (Cox proportional hazard model) and correlation with survival

Variable	*Coefficient*	*CI of risk ratio*	*P value*
Cyclin D1	0.269	(1.03, 1.66)	0.028
Log (CD1)	0.652	(1.08, 3.43)	0.027
Bcl-2	0.315	(1.21, 1.55)	<0.001
Log (Bcl-2)	0.952	(1.54, 4.35)	<0.001
Fas	0.169	(0.997, 1.41)	0.055
PCNA	0.285	(1.13, 1.56)	<0.001
Log (PCNA)	0.686	(1.13, 3.51)	0.018
ATM	−1.421	(0.068, 0.857)	0.028
ATM^2	0.453	(1.001, 2.474)	0.050

CI, confidence interval.

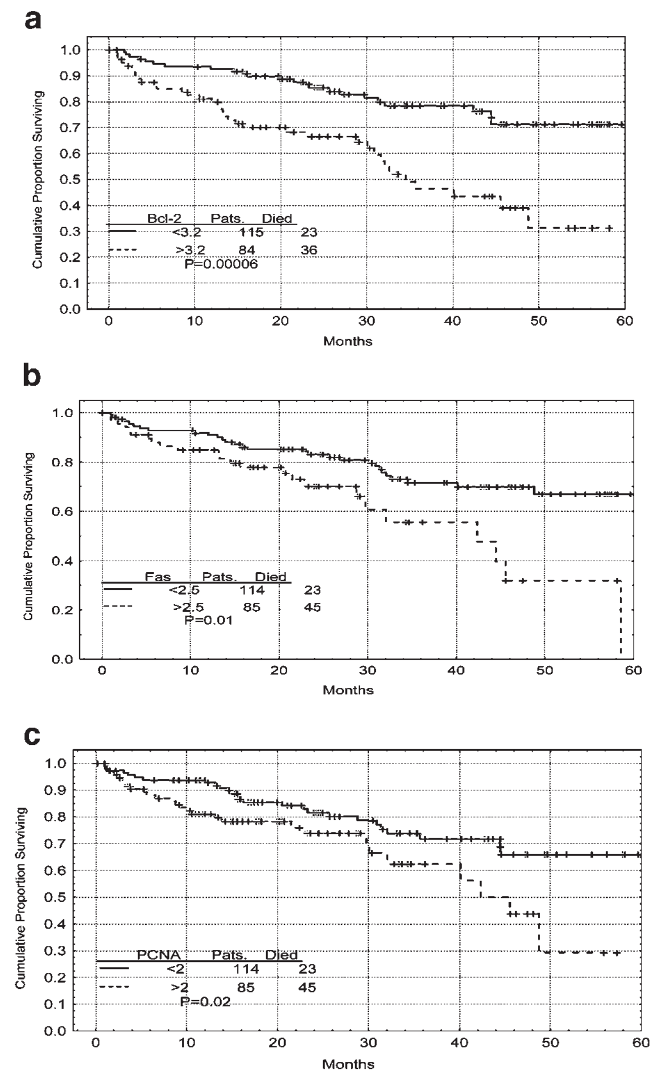

Figure 4 Kaplan–Meier survival curves for CLL patients for Bcl-2 (a), Fas (b), and PCNA (c).

that those in mantle cell lymphoma. We could not establish any association of Flt-1 and VEGF levels in our analysis, although we have demonstrated that these proteins play a role in a subset of patients.[15] We also observed that the levels of Flt-1 decreased as the disease duration increased, the clinical significance of which is unclear. We have reported cyclin D1

Table 5 Classification and regression tree analysis (CART)

Node	Variable				Survival probability ($P < 0.0001$)
	Bcl-2	ATM	PCNA	CD1	
1	≤3.1	1–2			86% (28 patients; MST, 45 months)
2	≤3.1	<1, >2			66% (68 patients; MST, 45 months)
3	3.1–8.58		>1	≤1	100% (4 patients; MST, 45 months)
4	3.1–8.58		>1	>1	27% (23 patients; MST, 32.5 months)
5	>8.582				17% (1 patient; MST, 2.7 months)

CD1, cyclin D1.
The protein levels are expressed here as normalized to the median levels detected in the normal control, which was assigned a value of 1. MST refers to median survival time.

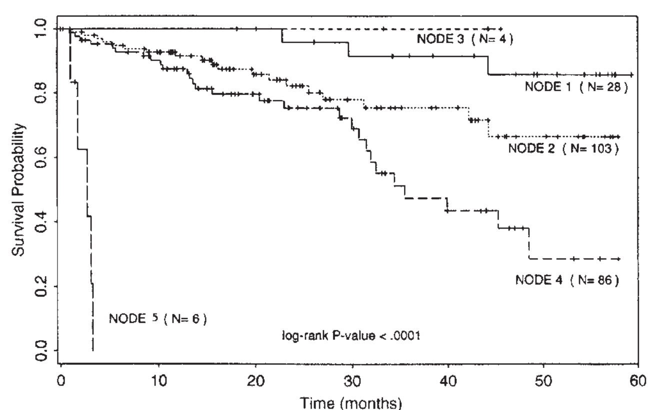

Figure 5 Survival probability of patients with CLL according to nodes (defined in Table 5).

as a prognostic factor although its levels are significantly lower in patients with CLL than in patients with mantle cell lymphoma or leukemia.[34]

Bcl-2 is frequently overexpressed in CLL although abnormalities of chromosome 18q21 are rare, leaving as other possible causes gene hypomethylation and *trans*-acting regulatory factors.[7] Bcl-2 levels reflect decreased apoptotic potential and increased accumulation of leukemic cells. High levels of Bcl-2 have been associated with shorter survival in previously treated patients and increased chemoresistance to treatment with fludarabine.[35,36] Overexpression of PCNA has been recognized as a prognostic indicator in several previous studies.[11,12] In a study of 40 patients with CLL, significantly lower levels of PCNA were observed in earlier stage CLL when compared with more advanced disease.[11] Likewise, PCNA expression correlated with other markers of cell proliferative rate such as lymphocyte doubling time. Similar to higher levels of the anti-apoptotic protein Bcl-2, high levels of PCNA predict for shorter survival and allow identification of a subgroup of patients with unfavorable prognosis. In this group of patients, high levels of PCNA may reflect a proliferative rather than apoptotic tendency of the CLL cells. The profile of levels of Bcl-2 and PCNA in our study supports these results and permits further risk-oriented subgrouping depending on their levels in association with each other. Expression of Fas (CD95/APO-1) is thought to lead to apoptosis if attached to its ligand. Fas (CD95) ligand levels may vary between patients and even within the same patient dependent on the stage of the disease. The current data raise the possibility that Fas ligand may play a role in the outcome of CLL. Expression of Fas (CD95) is rarely observed on the surface of CLL cells by flow cytometry, but can be modulated when exposed to cytokines such as interleukin-2, -4, -12, or interferon-alpha.[36–38] Whereas interleukin-2 and interleukin-12 as well as interferon-alpha were capable of upregulating Fas (CD95) and, in the case of interferon-alpha and interleukin-12, promoted Fas-ligand mediated lysis of CLL cells, interleukin-4 inhibited Fas-mediated killing of CLL cells and exposure to interleukin-2 was associated with *ex vivo* drug resistance. Thus, exposure to cytokines may elicit differential responses of Fas-bearing cells. Our results suggest a positive correlation of Fas (CD95) with Bcl-2 and a prognostic impact of both in patients with CLL. Panayiotidis and colleagues[37] suggested that the combination of high levels of Bcl-2 and resistance to anti-Fas mediated cytotoxicity contributed to dysregulation of apoptosis and prolonged survival in patients with CLL. As for ATM, we have previously reported that ATM protein levels can be decreased in a subset of patients with CLL where lower levels of ATM are associated with shorter survival.[39] Deficiency of the ATM protein, located on human chromosome 11q22–23 is found in up to one-third of CLL cases and has been associated with significantly shorter survival times and more aggressive disease.[13,14,40] In the present study, very low and very high ATM levels in association with high levels of Bcl-2 were significantly associated with shorter survival. This suggests that very low levels of ATM, which we believe reflects mutation or deletion is associated with more aggressive tumor due to lack of the ATM tumor suppressor function. However, very high levels may reflect the unsuccessful response of the ATM to mechanisms that deregulate the cell cycle and perhaps the higher the levels, the more the disturbance of the cell cycle.

Most of the proven important proteins in this study show correlation with each other and suggest abnormalities in common pathways. For example, there was significant correlation between PCNA and cyclin D1 suggesting changes in cell cycle. Similarly there was significant correlation between Bcl-2, Bax, and WBC supporting abnormalities in the apoptosis pathway.

The results of the present study emphasize that the expression of several proteins involved in the regulation of differentiation, proliferation, and apoptosis, influence the biological behavior of CLL and, subsequently, its clinical prognosis. The identification of a profile of markers and associations among them may prove even more crucial in predicting clinical outcome, contribute to a better understanding of the molecular basis of the disease, and may perhaps help in the design of new therapeutic approaches.

Editor's note

We are very indebted to Dr Peter Daniel who recruited and evaluated all the Reviews published in this Spotlight. Authors who are interested in contributing a Review for this Spotlight are invited to contact the Editor-in-Chief, Dr Muller Bérat.

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