Antigen receptor triggering and apotopic pathways in neoplastic B cells
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CHAPTER 5

Comprehensive profiling of apoptosis regulators in B-CLL reveals overexpression of Bmf and Noxa and links drug-induced Puma upregulation with IgV_H status

SUBMITTED FOR PUBLICATION

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Chapter 5

ABSTRACT
At present, an integrated understanding of dysregulated apoptosis in B cell chronic lymphocytic leukemia (B-CLL) is lacking. In order to comprehensively quantify expression of virtually all direct regulators of apoptosis, we applied a novel multiplex assay that targets 34 genes, and includes the Bcl-2 and IAP families and miscellaneous regulators such as Flip, PI-9, AIF and Apaf. Using this technique the apoptosis gene expression profile of B-CLL cells was investigated in relation to (1) normal tonsilar B cell subsets, (2) IgVH mutation status, and (3) incubation with cytostatic drugs. In accord with the non-cycling, anti-apoptotic status of CLL cells in vivo, they displayed high constitutive expression of Bcl-2 and Flip, while Survivin, Bid and Bik were absent. Paradoxically, next to these protective changes B-CLL cells showed increased expression of apoptogenic BH3-only members Bmf and Noxa. Upon cytostatic drug treatment in vitro, only the p53-responsive Puma was prominently induced. Moreover, the degree of Puma induction was more profound in cells of mutated IgVH B-CLL (15±8-fold in 8 unmutated and 41±24-fold in 10 mutated IgVH cases; P=0.0062).

Thus, disturbed apoptosis in B-CLL is the net result of both protective and sensitising aberrations, and this delicate balance may be tipped via a p53-response. Our results suggest that the clinical distinction between B-CLL subgroups may be linked to differences in the p53-responsive, Puma-mediated apoptosis pathway.

INTRODUCTION
B cell chronic lymphocytic leukemia (B-CLL) is considered a prime example of a malignancy resulting from deregulated apoptosis. B-CLL cells are arrested in the G0/G1-phase of the cell cycle and do not display a high proliferative capacity. Two subgroups of patients can be identified based on mutations in the rearranged immunoglobulin heavy chain variable (IgVH) genes. Patients with leukemic cells bearing mutated IgVH genes have a significantly better overall survival rate than patients with unmutated IgVH genes. Recent DNA micro array analyses have revealed distinct gene expression profiles for the two B-CLL subgroups, but yielded no specific clues regarding the mechanisms underlying the prognostic differences nor the deregulated apoptotic program.

Apoptosis is ultimately mediated by a cascade of proteases called caspsases that cleave essential cellular proteins. Two major pathways have been described (reviewed in 12). The 'extrinsic' pathway is triggered via death receptors such as CD95 which activate caspase 8. The 'intrinsic' pathway of apoptosis responds to a wide variety of stress signals and culminates in release of, amongst others, cytochrome C (cytC) from mitochondria. Once released, cytC complexes with cytoplasmic Apaf to recruit and activate caspase 9. A connection between these pathways is formed when caspase 8 cleaves Bid into truncated Bid which translocates to mitochondria and induces cytC release. B-CLL cells generally express low levels of CD95 and increased levels of the surrogate caspase Flip, and CD95-triggering does not lead to apoptosis. In contrast, the intrinsic pathway of apoptosis can be readily activated in CLL cells in vitro by incubation with cytostatic drugs, which ultimately trigger cytC release. Mitochondrial integrity is controlled by members of the Bcl-2 family of proteins. These can be divided into anti-apoptotic Bcl-2-like proteins, and pro-apoptotic Bax-like proteins (reviewed in 23). A third group within the
Bcl-2 family consists of so-called BH3-only proteins which includes e.g. Bid and Puma. By forming heterodimeric complexes at the mitochondria, the interplay of these Bcl-2 family members determines mitochondrial integrity and either promotes or suppresses apoptosis. A next level of apoptosis control can be provided by the cytosolic IAP family of proteins, which in a complex interplay with additional factors released from mitochondria such as Smac/Diablo regulate caspase activation downstream of mitochondria.

Various reports have addressed the deregulated apoptotic program in B-CLL, mainly focusing on regulation and expression of anti-apoptotic proteins. It is generally agreed that B-CLL cells contain high levels of Bcl-2, which has been associated with aggressive disease and refractoriness to chemotherapy. But not all studies have found this association, implying that other apoptosis regulators might be involved. In vitro incubation of leukemic cells with purine analogues or alkylating agents revealed alterations in expression levels of some apoptosis regulators such as inversion of the Bcl-2:Bax ratio, conformational changes of pro-apoptotic Bax and Bak, and reduction of Mcl-1 and the inhibitor of apoptosis (IAP) family member XIAP.

The tumor suppressor gene p53 plays a central role in coupling of DNA damage or cellular stress to activation of apoptosis via the mitochondrial apoptosis pathway. Expression of several of the pro-apoptotic Bcl-2 family members such as Bax, Noxa and Puma has been reported to be controlled by p53. Mutations in the p53 gene occur in half of all human cancers. Inactivation of p53 occurs in 15 - 25% of B-CLL patients which is caused either by p53 mutation or by inactivation of the gene encoding ATM, a kinase that regulates p53. In B-CLL, p53 dysfunction is associated with an adverse clinical outcome and poor response to therapy.

At present, an integrated understanding of dysregulated apoptosis in B-CLL is lacking. We therefore aimed to quantify expression of virtually all direct regulators of apoptosis. In this study, we applied a novel reverse transcriptase multiplex ligation-dependent probe amplification assay (RT-MLPA). The probe set targets 34 apoptosis-related genes and includes the complete Bcl-2 and IAP family and miscellaneous proteins such as Flip, PI-9, AIF and Apaf. Using this technique the apoptosis gene expression profile of B-CLL cells was investigated in relation to (1) normal tonsilar B cells, (2) IgVH mutation status, and (3) changes induced upon incubation of leukemic cells with cytostatic drugs. Our results indicate that, in comparison to normal tonsilar B cells, the apoptotic balance in B-CLL is grossly disturbed. CLL cells display major aberrations in anti- as well as pro-apoptotic genes involved in both the death receptor-mediated and mitochondrial apoptosis pathways. Upon cytostatic drug administration and apoptosis induction, only the p53-responsive Puma transcript was strongly augmented. Moreover, Puma expression was more prominently induced in cells expressing mutated IgVH genes.

PATIENTS, MATERIAL AND METHODS

B-CLL patients and isolation of leukemic and normal B cells
Peripheral blood was taken after informed consent from 18 B-CLL patients from the outpatient clinic of the department of Hematology of the Academic Medical Center, Amsterdam, The Netherlands, as approved by the local Ethical Review Board. Clinical characteristics of patients...
are presented in Table 1. To isolate normal B cell subsets tonsils were obtained from children during routine tonsillectomies performed at the St. Lucas Hospital, Amsterdam, The Netherlands. All isolation steps were performed on ice. B-CLL cells and normal B cells were enriched from PBMCs, obtained via density gradient centrifugation, via negative depletion as described previously. In brief, T cells, monocytes and granulocytes were depleted using α-CD3, α-CD14 and α-CD16 immunomagnetic beads and a magnetic particle concentrator (both Dynal A.S., Oslo, Norway). Purified leukemic cells contained >97% of CD5⁺CD19⁺ B cells. Tonsilar B cells were separated into fractions of >95% purity after staining with saturating amounts of CD19-PerCP-Cy5, CD38-PE (both Becton Dickinson, San Jose, CA) and α-lgD-FITC mAbs (Dako A/S, Glostrup, Denmark) on a MoFlo (Dako Cytomation, Ford Collins, CO) into the following B cell subsets: naïve (CD19⁺CD38⁻lgD⁻), germinal center (CD19⁺CD38⁺lgD⁺) and memory B cells (CD19⁺CD38⁺lgD⁺).

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<td>(+)</td>
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</table>

Table 1. Patient's characteristics
Of 18 patients the following characteristics were determined at time of inclusion in the study; sex (M=male, F=female); age (in years); White Blood Cell (WBC)-count (x10⁹/L); Rai stage: 0 (good prognosis), I-II (intermediate prognosis), and III-IV (poor prognosis); therapy (- = no therapy, + = therapy, (+) = prior received therapy which stopped at least 3 months before inclusion); IgVH mutation status (- = unmutated IgVH genes, + = mutated IgVH genes).

VH gene analysis
Total cellular RNA was isolated from 10x10⁶ purified B-CLL cells using Trizol (Gibco, Life Technology, Paisley, UK) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesised out of 2 μg RNA using oligo-dT primer and Superscript II RT (Gibco). Of each patient the VH gene mutation status was determined by PCR- and sequence-analysis as described previously. Sequencing of the clonal VH family PCR product was performed with an ABI sequencer (Perkin Elmer Corp., Norwalk, CT) using the dye-terminator cycle-sequencing kit (Perkin Elmer Corp.). The sequences found were compared with published germline sequences, using the Vbase database and DNAplot on the Internet (http://www.mrc-
Expression profiling of apoptosis regulators in B-CLL

cpe.cam.ac.uk/DNAplot) to identify mutations. Patients were considered to express unmutated IgV<sub>H</sub> genes when <2%<sup>55,56</sup> of the V<sub>H</sub> family sequence was mutated when compared to the most homologous germline sequence.

### Analysis of apoptosis

Cell viability was analyzed in duplicate samples via detection of phosphatidyl serine on the outer membrane of apoptotic cells as described previously<sup>143</sup>. In brief, cells were incubated with Annexin-V-FITC (APOPTEST-FITC, ImmunoQuality Products, Groningen, The Netherlands) (diluted 1:200 in HEPES buffer) for 15 minutes and washed twice. Propidium iodide (PI) (Sigma, St. Louis, MO) was added (final concentration 5 μg/ml) to distinguish necrotic cells from apoptotic cells.

In cell stimulation experiments only freshly isolated, purified B-CLL cells were used. Cells were cultured in complete IMDM medium (Gibco) at a concentration of 1.0x10<sup>6</sup> cells/ml in 25-cm<sup>2</sup> culture flasks. Cell viability was detected directly after isolation (t<sub>0</sub>) and upon 24 or 48 hrs of culture. Cells were incubated with different concentrations of cytostatic drugs for 48 hrs after which the lethal concentration by which 50% of cells died by apoptosis was determined (LC<sub>50</sub>). For MLPA experiments, cells were cultured with a concentration of twice the LC<sub>50</sub> of etoposide (25 μM); chlorambucil (100 μM); or fludarabine (80 μM) (all Sigma), or CCCP (10 μM) for 24 hrs. For the induction of CD95-mediated apoptosis 2.5x10<sup>5</sup> cells/ml were stimulated with an agonistic anti-CD95 mAb (kind gift of Prof. Dr. L. Aarden, Sanquin Research at CLB) (5 μg/ml) for 48 hrs.

### MLPA probes and oligonucleotides

MLPA probes consist of two oligonucleotides that anneal to adjacent sites on a target sequence and are then ligated by a heat stable ligase. Each ligated probe gives rise to an amplification product of unique length, and the resulting DNA mixture is analysed by capillary sequencer and standard software for identification and relative quantification of the amplicons<sup>236</sup>. Each MLPA probe consists of one short synthetic oligonucleotide (Biolegio, Malden, The Netherlands) and one phage M13 derived long probe oligonucleotide. Preparation of the M13 derived MLPA probe oligonucleotides and all probes used in the apoptosis gene probe set, have been described elsewhere<sup>236,237</sup>. All apoptosis genes for which probes are included are presented in Table 2. In order to avoid detection of contaminating DNA fragments, all target sequences have an exon boundary close to the probe ligation site. For each probe target sequence a specific Reverse Transcription primer was designed that is complementary to the RNA sequence immediately downstream of the probe target sequence. Tm of all RT primers was between 55 and 60 °C. Information from the public databases was used, and sequences of oligonucleotides are available upon request.

### MLPA reaction

Detailed reaction conditions for RT-MLPA have been described elsewhere<sup>237</sup>. In brief, RNA samples (40-60 ng of total RNA) were first reverse transcribed using a gene-specific probe mix. The resulting cDNA was annealed overnight at 60°C to the MLPA probe mix (1-4 fmol of each synthetic oligonucleotide and M13-derived oligonucleotide in TE). Ligation of annealed oligonucleotides was performed at 54°C by adding one unit Ligase-65, and incubation for 15 min. After inactivation of ligase enzyme by heating at 98°C for 5 min, ligation products were amplified by PCR. Samples were amplified (33 cycles, 30 s at 95°C; 30 s at 60°C and 1 min at 72°C) with one unlabeled and one FAM labeled primer (10 pMol), and were analysed on an Applied Biosystems 3100 capillary sequencer (Applied Biosystems, Warrington, UK).

### Analysis of MLPA data

After the PCR stage, aliquots of samples were mixed with Genescan-500 ROX size standards and run on an ABI 3100 capillary sequencer in Genescan mode. Data were analyzed with
Table 2. Apoptosis genes targeted by MLPA assay

<table>
<thead>
<tr>
<th>Bcl-2 family</th>
<th>IAP family</th>
<th>Miscellaneous</th>
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<tbody>
<tr>
<td>Bcl-2-like anti-apoptotic</td>
<td>Bax-like pro-apoptotic</td>
<td>BH3-only pro-apoptotic</td>
</tr>
<tr>
<td>A1/Bfl-1</td>
<td>Bak</td>
<td>Bad</td>
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<tr>
<td>Bcl-W</td>
<td>Bax</td>
<td>Bid</td>
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<tr>
<td>Bcl-xL (2 variants)</td>
<td>Bik</td>
<td></td>
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<td>Bcl-2</td>
<td>Bcl-GS</td>
<td>Bm</td>
</tr>
<tr>
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<td>Bcl-Rambo</td>
<td>BNIP3/NIP3</td>
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<tr>
<td>Mcl-1-S</td>
<td>Harakiri MAP-1</td>
<td></td>
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<tr>
<td></td>
<td>Noxa</td>
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<td></td>
<td>Puma</td>
<td></td>
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<tr>
<td>Apollon</td>
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<tr>
<td>Livin</td>
<td>Smac/Diablo</td>
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<tr>
<td>NIAP</td>
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<tr>
<td>Survivin</td>
<td>Xiap</td>
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Genes and categories for the apoptosis probeset. Pro-apoptotic Bax is targeted by probes discriminating two splice variants (www.ncbi.nlm.nih.gov/LocusLink). The transcript encoding Apaf-xL was proposed to have anti-apoptotic function\(^1\) and is for that reason tentatively presented as such. Various probes were designed and tested for IAP2, but for unknown reasons these never gave a significant signal. Signals for Bcl-GS, Livin (also called ML-IAP) were generally very low and/or rare, in accord with their restricted expression. Housekeeping genes included in the apoptosis probeset are β-2-microglobulin, ferritin light chain and poly A-specific ribonuclease.

Genescan and Genotyper software packages (ABI), successively. Category tables containing the area for each assigned peak (scored in arbitrary units) were compiled in Genotyper and exported for further analysis with Microsoft Excel spreadsheet software. The sum of all peak data was set at 100% to correct for fluctuations in total signal between samples, and individual peaks were calculated relative to the 100% value. Signals below the detection limit in medium were assigned a value corresponding to the threshold value for noise cut-off in Genescan.

Western blotting

Western blotting was performed as described previously\(^2\), after separation of protein samples on 13% SDS-PAGE gels. Mabs against Noxa were purchased from Biocarta (Carlsbad, CA), against Bmf from Alexis (Leiden, The Netherlands) and against tubulin from Sigma.

Statistical analyses

Kruskal Wallis test was used for analysis of differences between >2 groups after which two-sided Mann-Whitney U test was used to further identify differences between 2 groups. The Wilcoxon signed rank test was used to compare the differences between paired samples. P-values < 0.05 were considered statistically significant.
RESULTS

Profiling of apoptosis genes in B-CLL and normal B cell subsets by RT-MLPA

The RT-MLPA procedure provides an efficient means to accurately quantify the relative expression of up to 45 transcripts in a one-tube assay. The apoptosis probe set targets essentially all direct regulators of apoptosis (Table 2), and also three housekeeping genes\(^{257}\). Using this novel assay we performed expression profiling of purified B cells from 18 B-CLL patients, in comparison to normal tonsilar B cell subsets. The purified B cell fractions were >95% pure as determined by flow cytometry (data not shown). After the RT-MLPA procedure, peak areas generated by GeneScan software were converted to percentages of total signal, thus normalizing all samples for fluctuations in total signal strength. An example of a GeneScan peak profile is displayed in Fig. 1A. Averaged results calculated for all normal tonsil B cell and CLL samples are summarized in Fig. 1B&C. Each cell type displayed a specific expression profile with respect to a limited set of genes. Levels of housekeeping were comparable in normal and malignant B cells. The leukemic cells showed a small but significant increased expression of ferritin light chain (FLT) which is part of a main iron-storage protein and reported to be increased in B cell malignancy\(^{239}\). We conclude that RT-MLPA provides a relatively simple means to comprehensively quantify 34 apoptosis-related transcripts, and will further focus on specific differences observed between normal and malignant cells, and among normal B cell subsets.

Blockage of the death receptor-mediated apoptosis pathway

B-CLL cells are known to be generally refractory to CD95-mediated apoptosis\(^{112}\). Consistent with this, incubation of CLL cells for 48 hrs with an agonistic monoclonal antibody against CD95 did not increase apoptosis compared to culture in medium only (control: 51 ± 3% of apoptotic cells; anti-CD95: 50 ± 3% ; n = 13). RT-MLPA analysis of B-CLL cells revealed aberrant expression of two genes involved in the death receptor pathway. B-CLL cells showed a ~2-3-fold increase of the mRNA encoding Flip (Fig. 2A). In contrast, expression of the apoptogenic BH3-only family member Bid was hardly detectable. (Fig. 2B). Together, these data might explain the CD95-unresponsiveness of B-CLL cells.

Expression of cell cycle regulator Survivin is absent in B-CLL

Survivin has been reported to function as an inhibitor of apoptosis as well as a promotor of cell cycle progression\(^{240}\). Although in lymph nodes Survivin has been detected in a population of B-CLL cells held responsible for the clonal expansion of the disease\(^{241}\), in the present study Survivin expression could not be detected in peripheral blood B-CLL cells (Fig. 2C). Survivin was however clearly detectable in normal tonsilar B cell subsets; high expression in germinal center B cells distinguished this subset from naïve and memory B cells. The absence in non-cycling CLL B cells and presence in proliferating normal B cells support that Survivin is a mediator of cell cycle progression\(^{242}\).
Figure 1. Overall apoptosis gene expression profile of normal tonsilar and leukemic B cells

(A). Example of peak profile and data obtained via RT-MLPA. Shown is a representative trace of GeneScan peaks for a B-CLL patient. Scalebar on top indicates basepairs, and labels below peaks indicate the designated identities of the transcripts based on calculated and expected length of PCR fragments. Scale on the right indicates peak height in arbitrary units.

(B). Overall representation of the total apoptosis gene expression profile of the naïve (black bars; n=3), germinal center (white bars; n=5) and memory (grey bars; n=5) B cells. Bars represent peak areas of GeneScan profiles (mean ± SD), normalized to
Expression profiling of apoptosis regulators in B-CLL

percentages of total signal per sample. Significant differences among B cell subsets were observed for Bcl2 (P= 0.0097), Bik (P=0.0132) and Survivin (P=0.0176).

(C). Differences in the apoptosis gene expression profile of B-CLL cells (n=18): immediately after isolation of cells (t 0, black bars), after 24 hr culture of cells in medium (t 24, white bars), or after incubation of cells with fludarabine (fluda, grey bars). Expression of housekeeping genes β-2-microglobulin, ferritin light chain and poly A-specific ribonuclease remained quite similar after the different treatments. Survivin and Bik were completely absent in B-CLL. Highest basal signal was attributed to Noxa which declined during apoptosis induction as also observed for Mcl-1, whereas only Bax and Puma expression was upregulated after treatment with fludarabine.

Figure 2. Aberrant expression of anti- and pro-apoptotic genes in B-CLL
RT-MLPA was performed on naïve (■), germinal center (▲), memory (◆) B cell subsets form normal tonsils and B-CLL cells (○) immediately after isolation as described under methods. Each symbol represents one sample analysed. Relative percentage of gene expression is presented on the Y-axis.

Concerning the death receptor pathway, B-CLL cells show overexpression of anti-apoptotic Flip (A) and absence of pro-apoptotic Bid (B). Survivin is absent in non-cycling B-CLL cells (C). As to the mitochondrial apoptosis pathway, B-CLL cells show upregulation of Bcl-2 (D), minimal expression of A1 (E) and absence of Bik (F). Germinal center B cells are distinguished from naïve and memory B cells by low expression of Bcl-2 (D) and high expression of Bik (F). The P-values for all genes tested comparing B-CLL cells with tonsil B cells were P< 0.0001, except for Flip which was P=0.0002.

Aberrant expression of genes regulating the mitochondrial apoptosis pathway
A restricted set of both anti- and pro-apoptotic regulators of mitochondrial cytC release was aberrantly expressed in B-CLL cells. Increased expression of Bcl-2 in B-CLL has been reported earlier, and was confirmed by RT-MLPA analysis (Fig. 2D). In comparison to naïve and memory B cells, expression of Bcl-2 was 6-9-fold increased in B-CLL cells. Notably, in germinal center B cells Bcl-2 mRNA levels did not exceed the detection limit, thereby distinguishing this subset from naïve and memory B cells (P = 0.0097). Another aberrantly expressed anti-apoptotic Bcl-2 family member was
Chapter 5

A1, which is normally induced in B cells upon CD40 ligation via NF-κB\textsuperscript{39,40}. Although A1 was detectable in some B-CLL samples, expression was at least 10-fold lower in comparison to normal B cell subsets (Fig. 2E).

The apoptogenic BH3-only protein Bik was reported to be induced and activated in B104 B lymphoma cells upon triggering of the antigen receptor (BCR)\textsuperscript{206}. In all B-CLL samples examined, Bik expression was completely absent (Fig. 2F). BCR-triggering of leukemic cells did not increase Bik expression to detectable levels (data not shown). This is consistent with the observation that CLL cells are refractory to apoptosis by BCR ligation\textsuperscript{102,243}. In contrast, high levels of Bik transcript were detected in germinal center B cells, again distinguishing these cells from naïve and memory B cells (Fig. 2F). These results indicate that expression of both pro- and anti-apoptosis genes involved in mitochondrial apoptosis activation are modulated during the germinal center reaction. Moreover, expression of these genes is altered in B-CLL cells in such a way that mitochondrial integrity is ensured.

**Overexpression of pro-apoptotic BH3-only genes Bmf and Noxa**

In strong contrast to the protective changes mentioned above, significant overexpression was observed in B-CLL cells of two BH3-only apoptogenic genes, Bmf and Noxa. While Bmf expression was low in normal B cell subsets, in B-CLL cells Bmf was abundantly expressed with levels ranging from 1.5 to 7.8% of total signal (Fig. 3A). The second BH3-only gene overexpressed in B-CLL cells was the putative p53-inducible gene Noxa. In normal B cell subsets, Noxa signals were already quite high, with levels ranging from 2.3 to 9.5%, but in CLL cells this was even further enhanced (Fig. 3B).

![Figure 3. BH3-only genes Bmf and Noxa are upregulated in B-CLL](image)

RT-MLPA was performed on naïve (■), germinal center (▲), memory (●) and leukemic B cells (○) immediately after isolation as described under methods. Each symbol represents one sample analysed. Relative percentage of gene expression is presented on the Y-axis.

Expression of pro-apoptotic BH3-only genes Bmf (P<0.0002) (A) and Noxa (P=0.0082) (B) was strongly upregulated in CLL B cells in comparison to normal B cell subsets.
The increase in Bmf and Noxa transcripts could be validated at the protein level. In Fig. 4 (top panel) a protein doublet at 20-25 kD detected by a mAb against Bmf is clearly apparent in all 5 B-CLL samples, and is absent in a tonsil sample. In the article describing Bmf244, the protein is sometimes also visible as a doublet on blots. In addition, the 5'-end of the gene is not well characterized, and some heterogeneity and nearby partial ESTs are reported in the databases (www.ncbi.nlm.nih.gov evidence viewer, and www.genome.ucsc.edu). The double bands therefore most probably represent splice variants of Bmf. The blot was reprobed for presence of Noxa (Fig. 4 middle panel), which was also increased in patients relative to the tonsil sample.

Both Bmf244 and Noxa231 have been reported to bind to anti-apoptotic Bcl-2 family members, amongst others Mcl-1. Mcl-1 was also abundantly expressed in B-CLL cells, although not significantly different from normal tonsilar B cell subsets (Fig. 1). Further analysis of leukemic samples did not point to a correlation between the expression levels of Bmf and Noxa with one of the anti-apoptotic Bcl-2 family members (data not shown). In addition, no correlation with Rai stage or discrimination based on IgVH mutation status between patient groups was observed for the aberrantly expressed apoptosis genes described above.

![Figure 4. Increase in Bmf and Noxa protein in B-CLL](image)

Protein samples from purified, unfractionated tonsil B and B-CLL cells containing 50 μg of protein were separated on 13% gels and subsequently probed for presence of Bmf (top panel), Noxa (middle panel) and tubulin as loading control (bottom panel). Three IgVH unmutated and two mutated CLL samples (UM-CLL and M-CLL) were used, as indicated. As an additional control, lysate from Ramos Burkitt lymphoma cells was added, which also showed increased Bmf and Noxa signals by MLPA (data not shown).

Cytostatic drug-induced upregulation of Puma is more profound in B-CLL patients expressing mutated IgVH genes

To identify changes in expression profile related to induction of apoptosis, B-CLL cells were cultured in vitro for 24 hrs in either the absence or presence of cytostatic drugs. Consistent with previous reports108,225, in vitro culture of CLL cells caused spontaneous apoptosis. Incubation of cells with either the purine analogue fludarabine, the inhibitor of topoisomerase II etoposide or the alkylating agent chlorambucil considerably enhanced programmed cell death (see Fig. 5). These drugs induce DNA damage resulting in apoptosis either via inhibition of DNA synthesis
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Figure 5. Induction of apoptosis of B-CLL cells cultured in vitro in the absence or presence of cytostatic drugs
Viability of B-CLL cells was determined by Annexin-V-FITC/PI staining immediately after isolation (t 0, □) and upon in vitro culture for 24 hrs in medium only (ctrl, ▲) or upon incubation of cells with cytostatic drugs such as fludarabine (◇), etoposide (♦), chlorambucil (●). Each symbol represents one patient analysed. In vitro culture of B-CLL cells for 24 hrs results in apoptosis (P<0.0001, indicated with one asterisk, *) which is enhanced upon cytostatic drug treatment (P<0.01 as indicated with two asterisks, **).

(fludarabine) or by generating lethal DNA lesions (etoposide and chlorambucil). As a control, the general caspase inhibitor zVAD was added to cultured cells of two patients, which as expected blocked apoptosis execution (not shown). No significant difference in percentage of apoptotic cells was observed between IgV\(_H^\) unmutated (n=8) and mutated (n=10) B-CLL groups (not shown).

The overall apoptosis gene expression profile of CLL cells after in vitro culture in the absence or presence of cytostatic drugs was analogous to that observed immediately after isolation (Fig. 1B&C). Drug treatment resulted in significant upregulation of only two apoptogenic genes: Bax and Puma. Both isoforms of Bax showed a ~ 2-fold induction in expression (P = 0.0003).

Most striking was the drastic induction of the p53-responsive BH3-only gene Puma (Fig. 1C & 6). Already after culture in medium for 24 hrs, expression of this apoptogenic gene was induced 4-fold (Fig. 6). Treatment of cells with fludarabine, etoposide or chlorambucil even further enhanced Puma expression in all samples compared to cells in medium only (Fig. 6), in association with apoptosis induction (Fig. 5). Importantly, during in vitro culture of cells in the presence of z-VAD, Puma upregulation in both spontaneous and drug-induced apoptosis was maintained, demonstrating that it occurred independently of caspase activation and apoptosis execution.

After 24 hrs of culture in medium, higher levels of Puma induction were observed in cells containing mutated IgV\(_H^\) genes (P = 0.0360) (Fig. 7). This became more prominent after incubation of cells with fludarabine as the relative Puma induction was 15 ± 8-fold (mean ± SD) in unmutated IgV\(_H^\) samples and 41 ± 24-fold in mutated samples (P = 0.0062) (Fig. 7). The same trend was observed upon incubation of leukemic cells with the other cytostatic drugs (Fig. 7), but due to a limited number of samples this did not reach statistical significance. These results indicate that drug-induced apoptosis in B-CLL is mediated primarily via strong upregulation of the pro-apoptotic Puma gene, perhaps facilitated by a more modest increase in Bax. Secondly, of all genes assessed in the RT-MLPA analysis Puma was the only gene differentially expressed in relation to IgV\(_H^\) mutation status.
Figure 6. Apoptosis-related induction of the p53-responsive gene Puma in B-CLL
Cells were cultured for 24 hrs in vitro in the presence or absence of cytostatic drugs and expression of Puma was assessed by RT-MLPA as described under methods. The number of patients (n) included in the apoptosis responses is depicted underneath each bar. Data represent mean ± SD. Expression of Puma was strongly enhanced upon in vitro culture in medium for 24 hrs (ctrl) when compared to non-cultured cells (t 0) (P=0.0006, indicated with one asterisk *). Cytostatic drug treatment with either fludarabine, etoposide or chlorambucil further augmented the level of Puma expression in B-CLL cells (P<0.01, indicated with two asterisks, **). During culture of cells in the presence of z-VAD Puma upregulation in both spontaneous or fludarabine-induced apoptosis was maintained.

Figure 7. Mutated IgVH B-CLL patients show a more profound apoptosis-related induction of Puma
Cells were cultured for 24 hrs in vitro in the presence or absence of cytostatic drugs and expression of Puma was assessed by RT-MLPA as described under methods. A discrimination was made between IgVH unmutated (black bars) and mutated (white bars) B-CLL patients. The number of patients (n) included is depicted underneath each bar. Data represent mean ± SD. After 24 hrs of culture in medium (ctrl) induction of Puma expression was more prominent in cells of patients expressing mutated IgVH genes when compared to patients expressing unmutated IgVH genes (P=0.0360, indicated with one asterisk, *). Incubation of cells with cytostatic drugs even further increased this difference in Puma expression amongst B-CLL patients (P=0.0062, indicated with two asterisks, **).
DISCUSSION

In the present study we used a novel multiplex technique to quantify and compare the overall apoptosis gene expression profile of normal tonsilar B cells and B-CLL cells. The data allow a comprehensive overview and uncovered novel aspects of dysfunctional apoptosis in B-CLL. High constitutive expression of Bcl-2 and Flip, together with the absence of Bid and Bik may well account for inhibition of death-receptor and BCR-mediated apoptosis pathways in B-CLL cells. Unexpectedly, next to these protective changes the majority of CLL cases showed increased expression of apoptogenic BH3-only members Bmf and Noxa. Among 34 apoptosis regulators monitored, only prominent upregulation of Puma coincided with spontaneous and drug-induced apoptosis. Moreover, the degree of Puma expression correlated with IgVH mutation status, which may provide a functional clue to the differential prognosis in B-CLL.

Previous expression profiling of B-CLL using micro arrays \(^\text{58,59}\) did not involve responses of cells after in vitro culture and drug administration, and therefore did not address the important contribution of the p53-Puma pathway to apoptosis induction. In addition, by employing RT-MLPA several clear differences between B-CLL and subsets of normal tonsilar B cells were observed that were not noted before, such as the absence of Bid and Bik and the overexpression of Bmf and Noxa. This can at least partly be explained by the fact that not all genes targeted by the RT-MLPA probe set are represented on the micro arrays used previously. Also, the focus on a gene set involved in a defined cellular response such as apoptosis may afford better discrimination than large scale automated analyses. Conversely, general expression patterns uncovered by micro arrays which allow the classification of B-CLL as resembling memory B cells \(^\text{58}\) or possessing a ‘BCR activation’ signature \(^\text{59}\), were not reflected using the apoptosis probe set. Still, based on apoptosis regulatory genes alone, germinal center B cells could be distinguished from both naive and memory B cells by the absence of Bcl-2 and the augmented expression of Bik and Survivin (Fig. 2). These data are consistent with events during the germinal center reaction. Upon interaction with T cells, B cells are either positively selected and expand rapidly, explaining the high levels of cell cycle-associated Survivin, or negatively selected by apoptosis \(^\text{245}\), which correlates well with the absence of Bcl-2 and presence of Bik. Our results obtained with RT-MLPA agree well with recent micro array data showing that B cells during differentiation from naïve to a germinal center stage switch from an anti- to a pro-apoptotic program, with upregulation of Bik and downregulation of Bcl-2. When cells further mature into memory cells the pro-apoptotic program is turned off again \(^\text{57}\).

RT-MLPA analysis of B-CLL cells identified aberrant expression of Flip and Bid, two important regulators of the death receptor pathway. The increase in Flip mRNA suggests that CD95-induced apoptosis is inhibited at the level of the death-inducing signaling complex via prevention of caspase 8 activation, as has been reported for TRAIL-induced apoptosis \(^\text{224}\). Caspase 8 can also be activated independent of CD95 signaling via an amplification loop induced by active caspase 3 \(^\text{246}\). Since RT-MLPA analysis demonstrated absence of Bid mRNA in B-CLL cells, this indicates that further downstream signaling of caspase 8 via Bid cannot take place. Thus, blockage of the death receptor-signaling pathway in B-CLL at least involves aberrations at two different levels.

Crosslinking of CD40 on B cells normally induces expression of anti-apoptotic A1, and protects from BCR-mediated apoptosis \(^\text{39,40}\). In comparison to tonsilar B cell
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subsets, A1 was minimally expressed, implying that the bulk of B-CLL cells had not been in recent contact with T cells expressing CD40L. Yet, in vitro CD40 crosslinking of leukemic cells leads to upregulation of A1 (A.P. Kater & E. Eldering, unpublished observation), and shows that this pathway is still intact. Together, these observations suggest that T cell-mediated help is not a dominant factor in in vivo survival of circulating B-CLL cells.

An important finding of our study is the strongly augmented expression of BH3-only members Bmf and Noxa, which by itself seemed insufficient for apoptosis induction as cell viability measured immediately after isolation was generally >90%. However, during in vitro culture B-CLL cells quickly underwent spontaneous and drug-induced apoptosis, in association with drastic upregulation of Puma. This leads us to propose that in B-CLL (1) induction of cell death is constantly blocked by anti-apoptosis regulators and (2) additional pro-apoptosis regulators are required to execute apoptosis. The first point is supported by augmented levels of Bcl-2 and Mcl-1, which most probably function to sequester Bmf and Noxa and thus prevents mitochondrial apoptosis. Overexpression of Bcl-2 is a known feature in B-CLL, and was confirmed by RT-MLPA analysis. High Mcl-1 protein expression in CLL has been correlated with failure to achieve complete remission after single-agent therapy. Although high Mcl-1 mRNA levels were detected in CLL samples in our study, they did not differ from normal B cell subsets (Fig. 1), indicating that Mcl-1 is not specifically deregulated in B-CLL.

The second point follows from the striking observation that apoptosis of B-CLL cells was accompanied exclusively by strong upregulation of Puma. In model systems, Puma has recently been identified as a direct mediator of p53 which results in rapid apoptosis. In addition, Puma can be induced upon serum deprivation in a caspase-independent manner. As the culture medium in our studies did not contain autologous serum, serum deprivation or low level p53 activation might cause Puma upregulation in case of spontaneous apoptosis observed after in vitro culture of B-CLL cells. Addition of cytostatic drugs known to activate p53 strongly enhanced Puma expression, in a caspase-independent manner, and further augmented apoptosis. Together, these results demonstrate for the first time that also in a primary malignancy, extensive p53-mediated upregulation of Puma can initiate execution of apoptosis.

BH3-only proteins are essential for the induction of apoptosis but their exact mechanism of action is not known. They are regulated at the transcriptional level or by post-translational modifications that result in conformational changes and/or cause release from an inactive complex. Subsequent binding to Bcl-2 family members may induce a switch from anti- to pro-apoptotic capacity of the heterodimer. Based on different affinities for interactions with Bcl-2, an alternative model was proposed in which BH3-only proteins are divided in either "sensitizing" proteins (e.g. Bad) which occupy the binding pocket of anti-apoptotic Bcl-2 family members, or "activating" proteins (e.g. Bid) which can directly promote Bax- and Bak-mediated apoptosis induction.

Noxa and Puma are reported to localize at the mitochondria making it unlikely that posttranslational modifications are needed for activity. In contrast, Bmf has to be released from the cytoskeleton where it is sequestered to myosin V motor complexes via association to dynein light chain 2 complexes. Certain damage signals, such as cell detachment, induce release of Bmf whereupon it associates with Bcl-2. Interestingly, B-CLL is characterised by modifications in the cytoskeleton organisation
such as the formation of F-actin-containing podosomes and a substantial lack of microfilaments which have been related to the malignant transformation of B-CLL cells\textsuperscript{247}. In addition, routine microscopy analysis of B-CLL bloodsmears shows cells which appear ruptured indicating their fragile state. Future studies will determine if in B-CLL cells Bmf remains bound to the cytoskeleton, or perhaps dissociates \textit{ex vivo} and saturates Bcl-2, thereby promoting apoptosis. Bmf, Noxa and Puma have all three been reported to interact with anti-apoptotic but not with pro-apoptotic Bcl-2-family members\textsuperscript{231-233,244}, which suggests that they should act as sensitizing BH3-only proteins\textsuperscript{204}. Most of these interactions have been studied by the yeast-two-hybrid system or by co-immunoprecipitation of overexpressed proteins\textsuperscript{231-233,244}, which may not always represent the endogenous situation. However, if these results are genuine and the ‘sensitiser-activator’ model is correct, the question arises which other lethal BH3-only protein(s) is liberated to induce apoptosis. On the other hand, since binding of Puma to Bax has not formally been excluded as yet, Puma might directly associate with Bax to induce cytC release.

Mitochondrial translocation and oligomerization of Bax and/or Bak is essential for apoptosis induction, also in case of B-CLL cells\textsuperscript{187,225}. Puma induces mitochondrial translocation and multimerization of Bax and is fully dependent on Bax for its apoptotic function\textsuperscript{248}. Cytostatic drug-mediated p53 activation induced only a \textasciitilde{}2-fold rise of Bax mRNA in B-CLL. Moreover, no significant induction of either Noxa or Bid was observed, although both were reported to be p53-responsive\textsuperscript{231,249}. These data seem to exclude these three genes as direct targets of p53, at least in B-CLL cells.

Noteworthy is that Puma was the only gene differentially expressed and which was also associated with IgV\textsubscript{H} mutation status. Patients expressing mutated IgV\textsubscript{H} genes clearly displayed a more profound upregulation of Puma compared to unmutated IgV\textsubscript{H} patients. The separation between the two patient groups was not mirrored in differences in apoptosis read-out under the experimental conditions used. This may be interpreted in support of the proposals above concerning the delicate balance between pro- and anti-apoptotic regulators in B-CLL. The mere synthesis of additional apoptogenic proteins such as Puma is apparently sufficient to shift the balance towards apoptosis. Since our cohort of 18 samples all displayed Puma upregulation, which can be considered as good a surrogate marker for p53 activity as p21\textsuperscript{233}, this would imply that all these patients have an intact p53 response. It cannot, however, be excluded that Puma is under control of additional pathways, as is illustrated by the serum-deprivation response mentioned above. Together, our data imply that clinical outcome of B-CLL patients may be related to a different capacity to activate the p53-mediated pathway. Recently, poor prognosis of CLL patients was associated with ATM/p53 dysfunction. Both 17p deletion resulting in p53 dysfunction and IgV\textsubscript{H} mutation status have separately been recognized as prognostic markers of B-CLL\textsuperscript{72-74}, and our data suggest there may in fact be a functional link. Alternatively, the distinct p53-responses may merely reflect independent differences intrinsic to the developmental stage in which the cells became malignant. Regardless, these considerations provide an exciting opportunity for further studies into the relation between BCR signaling, p53-mediated Puma upregulation and IgV\textsubscript{H} mutation status in B-CLL.
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