Int. J. Cancer: **118,** 2329–2336 (2006)
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Silencing of APAF-1 in B-CLL results in poor prognosis in the case of concomitant p53 mutation

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Apoptosis protease-activating factor 1 (APAF-1), a transcriptional target of p53, is a cytosolic adaptor protein that links the mitochondrial apoptosis pathway to the caspase cascade. Here, we aimed to study the impact of APAF-1 expression levels on cell death induced by anticancer drugs or ionizing irradiation (IR) and disease prognosis in B-type chronic lymphocytic leukemia (B-CLL) patients. Samples from 138 patients with B-CLL were investigated for APAF-1 expression and p53 mutations. The results were related to survival data, *in vitro* cytotoxicity of various cytotoxic drugs and IR and clinico-pathological data. Variable APAF-1 expression was observed in all investigated B-CLL samples. Reduction in APAF-1 expression was observed at both mRNA and protein level indicating transcriptional silencing whereas mutation of p53 or the immunoglobulin heavy chain variable genes (IgH_V) had no impact on APAF-1 expression. Surprisingly, APAF-1 loss did not result in resistance to cytotoxic therapies. Likewise, APAF-1 downregulation on its own showed no impact on disease prognosis. Nevertheless, a poor prognosis was observed in patients with loss of APAF-1 expression and additional p53 mutation. Thus, loss of APAF-1 may become relevant when additional core apoptosis signaling components are disrupted.
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Key words: APAF-1; p53 mutation; apoptosis; drug sensitivity; B-CLL

Chronic lymphocytic leukemia (CLL) is the most frequent B non-Hodgkin's lymphoma (B-NHL) in western countries. Clinically, CLL is characterized by a smouldering disease course, over years in most cases, leading to the accumulation of small, mostly nondividing lymphocytes in various organs including lymphoid tissues and bone marrow. So far, the disease is incurable by conventional cytotoxic therapies. By cell morphology, clinical course and general incurability by drugs or irradiation CLL may be considered a prototypic disease of intrinsic apoptosis deficiency.

We and others have shown in various models that cytotoxic drugs kill *via* the induction of apoptosis. Consequently, resistance to apoptosis leads to resistance to therapy. The vast majority of intrinsic and extrinsic cell death stimuli rely on the activation of the mitochondrial pathway of apoptosis. Triggering mitochondria for apoptosis results in the release of various pro-apoptotic factors including cytochrome c that binds to the cytosolic adaptor protein apoptosis protease-activating factor 1 (APAF-1). This initiates formation of the apoptosome in a (d)ATP dependent manner and recruitment of procaspase-9 to the apoptosome complex. Induced proximity in the apoptosome facilitates autocatalysis of procaspase-9 and this initiates activation of the caspase cascade and execution of cell death.[1–4]

Recently, APAF-1 was shown to be a transcriptional target of p53.[5,6] Moreover, enforced expression of APAF-1 was shown to sensitize for p53-mediated apoptosis.[7] In cell-line models, APAF-1 deficiency has been shown to correlate with resistance to apoptosis.[8–10] Notably, APAF-1 might play a role in tumorigenesis, as loss of APAF-1 facilitates *myc*-driven transformation in murine embryonal fibroblasts[11] even while recent evidence challenges the potency of APAF-1 loss to replace p53 inactivation in oncogenic transformation. The first report to demonstrate that APAF-1 plays a role in human cancer came from the observation that malignant melanoma shows downregulation of APAF-1 expression by epigenetic modification, *i.e.* hypermethylation.[12] Further studies in melanoma affirmed a loss of APAF-1 expression in this entity,[13,14] and allelic imbalance of the APAF-1 associated gene locus 12q22-23 was correlated with bad clinical outcome in 1 study.[15] Nevertheless, these findings are under scrutiny and have recently been challenged.[16,17]

Thus, there is so far no unequivocal evidence whether APAF-1 is clinically relevant for disease prognosis and the clinical response to anticancer therapy. We previously demonstrated that disruption of the p53/Bax pathway results in resistance to anticancer therapy and a poor prognosis in B-CLL.[18,19] In view of the role of APAF-1 and the apoptosome in cell death regulation and tumorigenesis, we therefore investigated the impact of APAF-1 expression on disease prognosis and response to anticancer drugs or ionizing irradiation in 138 patients with B-CLL.

Material and methods

Patients

Peripheral blood from 138 B-CLL patients was analyzed for drug sensitivity and γ-irradiation, using fresh cells. The same samples were analyzed for APAF-1 protein expression and for mutations in the p53 DNA binding domain, using snap-frozen cells. Clinicopathological data of the 138 patients are given in Table I. Of the 138 patients, 80 were pretreated (58%) with 1–6 drug regimens (median of 2 treatments). Samples were obtained for analysis in median 7.34 months after diagnosis of B-CLL. Diagnosis of B-CLL was confirmed according to the NCI guidelines,[20] by morphology, a white cell count of $>15 \times 10^9$ cells/l and cell markers including coexpression of CD5 and B-cell markers (CD19, CD20 and CD23) on leukemic cells. Median white cell count was 120.8×10^9 cells/l. Patients with low leukocyte counts were not tested in this study to avoid false positive/negative results due to a higher contamination with nonmalignant leukocytes in such "low counter" samples. This study was performed in accordance with local ethical standards and the declaration of Helsinki.

APAF-1 protein expression

Lymphocytes frozen in liquid nitrogen were thawed, protein was extracted and endogenous expression of APAF-1 protein was measured by immunoblotting as described.[18,21] The protein concentration from each sample was measured with the BCA protein assay (Pierce; Rockford, IL), and 15 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis on 6% polyacrylamide gels. Proteins were transferred to nitro-cellulose membranes,

Grant sponsor: Schwerpunktprogramm Apoptose by the Deutsche Krebshilfe and by the Deutsche José Carreras Leukämie Stiftung e.V.
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Received 18 October 2004; Accepted after revision 4 July 2005
DOI 10.1002/ijc.21535
Published online 5 December 2005 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – PATIENT CHARACTERISTICS

	All patients (n = 138)	APAF-1 low (n = 67)	APAF-1 high (n = 67)	p
Gender				
Male	99	48	47	
Female	39	19	20	n.s.[1]
Binet stage[2]				
A	29	16	12	
B	24	10	12	
A/B[3]	15	8	7	
C	62	27	34	n.s.[1]
Age (mean ± SEM) (years)	63.25 ± 0.8	63.6 ± 1.3	63.0 ± 1.1	n.s.[4]
IgH_V gene hyper-mutation status				
No (n = 81)	1.78 ± 0.113	35	46	
Yes (n = 28)	1.8 ± 0.165	12	16	n.s.[1]
Previous treatments				
None	58	25	31	
≥1	80	42	36	n.s.[1]

[1]χ^2-test.–[2]Data available for 130 patients.–[3]In 15 cases, the assignment to stage A or B was not possible. This group is referred to as stage A/B.–[4]t-test.

which were stained using 0.1% Ponceau S in 1% acetic acid to verify equal loading of protein. After blocking, membranes were incubated with primary antibody for 1 hr (anti-APAF-1 polyclonal rabbit antibody (Zymed Laboratories, San Francisco, 1:150 (3.3 μg/ml) and anti-Actin polyclonal rabbit antibody, Sigma-Aldrich, Saint Louis, 1:500 (3.5 μg/ml)) and secondary anti-rabbit IgG HRP (Promega, 1:5000 (0.2 μg/ml)) at room temperature. Bands were detected using the enhanced chemiluminescence (ECL) system from Amersham (Braunschweig, Germany). Densitometric videoscanning using a Gel Doc 2000 apparatus and Quantity One software (BioRad, München, Germany) was performed. For standardization, 15 μg of protein extract from the human Burkitt-like lymphoma cell line BJAB was included on every blot. To enable comparison between membranes, the individual APAF-1 measurements were normalized with respect to those of the BJAB controls on the same membrane. Linearity of APAF-1 protein expression was determined to be in the range of 10–20 μg protein per lane.

APAF-1 quantitative cDNA analysis by real-time PCR

RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany), and 250 ng of total RNA was reverse transcribed using the RT-PCR kit (Applied Biosystems, Foster City, California, USA). Quantification was done with the real-time PCR approach.[22]

PCR was performed in a 25 μl reaction mixture on a ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, California, USA). Primers and probes were designed for APAF-1 with Primer Express version 2.0 (Applied Biosystems). The primers and probes used are as follows: 6FAM-MGB probe 5′-CAAAGGCTTGGCTCAT-3′, sense primer 5′-CCACCGGCCTGGAGTCT-3′ and antisense primer 5′-AGATCTTTC TCTCTC TGAGCT GTC AA-3′. All reactions were done in triplicate. 18S rRNA was used as an internal reference. Quantitative mRNA was calculated using the following formula: $\Delta C_T = C_T(\text{APAF-1}) - C_T(\text{18SrRNA})$ (C_T: threshold cycle). This value represents the PCR cycle at which a significant increase in fluorescence, *i.e.* PCR product, was determined.

Analysis of p53 mutation and IgH_V hypermutation

DNA was extracted, and mutations in the p53 exons 5–8 were analyzed by genomic SSCP-PCR analysis as described.[19] Samples with aberrant bands were sequenced, as described. The mutation status of the immunoglobulin heavy chain variable region genes (IgH_V) was known in 109 cases. IgH_V gene mutation was analyzed as described,[23] and hypermutation status was assigned as described.[24]

Chemosensitivity assay

Drug-induced overall cell death (apoptosis and necrosis) was determined by the use of a morphometric test, as described.[25] In brief, fresh peripheral blood lymphocytes (8×10^4) were incubated in duplicate in 0.6 ml polypropylene tubes in supplemented RPMI medium or UltraCulture (BioWhittaker, Walkersville, MD). Final volume of 90 μl included 10% phosphate buffered saline (PBS) either without (control samples) or with added cytotoxic drugs: alkylating agents (chlorambucil and mafosfamide); fludarabine phosphate; methylprednisolone; doxorubicin and vincristine, or cells were exposed to 2 Gy γ-irradiation using a cobalt source. After incubation for 92 hr, as described previously,[26] a mixture of fast green and nigrosin dyes in PBS, including fixed duck erythrocytes (50,000) as an internal standard, were added to stain dead cells black before cytocentrifugation onto microscope slides. The slides were air-dried and counter-stained with Romanowsky stain. Subsequent morphological evaluation gave the percentage of live lymphocytes in the treated samples of live lymphocytes in control cultures.

Data analysis

Normalized APAF-1 protein expression values were dichotomized using the median protein expression as cut-off ("low APAF-1 expression" <1.432, "high APAF-1 expression" ≥1.432). To assure that other cutoffs yield comparable results, we additionally tested the 10th, 25th and 75th percentile, *i.e.* more extreme cutoff-points for APAF-1 loss as compared with the median. Similar results were obtained, and data for the median are presented. For intervariable assessment, the t-test or the Mann-Whitney U-test and the χ^2-test or Fisher's exact test—where appropriate—were applied. $p \leq 0.05$ was regarded significant. Overall survival was estimated by the Kaplan-Meier product-limit method, starting from the time of sample collection. The survival curves were compared by the Log-rank-Mantel-Cox test. For the *in vitro* chemosensitivity data, logistic curves were fitted to the cell survival data; the logit of survival probability was taken to be linear with respect to the logarithm of drug concentration.[27] LC_{90} doses, *i.e.* the concentration of drug to produce a 90% reduction in cell survival compared with control cells, were determined by calculating the log-dose at which the fitted survival probability was equal to 0.1. As LC_{90} results are log-normal, all LC_{90} values were logged (base 10) before calculation of mean and SEM, as described.

Results

Patients and specimens

Patient characteristics of the 138 B-CLL specimens[18,19] are shown in Table I. Of the specimens 99 were from male patients and 39 were from female patients. Of the 138 patients 80 had received prior anticancer therapy whereas 58 patients were "chemonaïve". Of the 80 pretreated patients 62 had received an alkylating agent (either chlorambucil or cyclophosphamide). The cohort of patients was typical of those encountered in routine clinics.

APAF-1 protein expression

APAF-1 protein expression was measured by standardized Western blot analysis. We previously demonstrated that this method reliably allows quantification of Bax, Bcl-2 and caspase-3 protein expression levels in leukemic cell samples from patients with B-CLL or acute lymphoblastic leukemia (ALL).[18,21] Measurement took place in the linear range of the standard curve. Western blot analysis could be performed in 134 of the 138 samples. APAF-1 protein was detectable at a mean (±SEM) value of 1.669 (±0.082) arbitrary units (median 1.432, minimum 0.258 and maximum 4.934). Information of APAF-1 expression in relation to clinicopathological data is given in Table I. There was no correlation between APAF-1 levels and clinical Binet stages or other variables including IgH_V gene hypermutation.

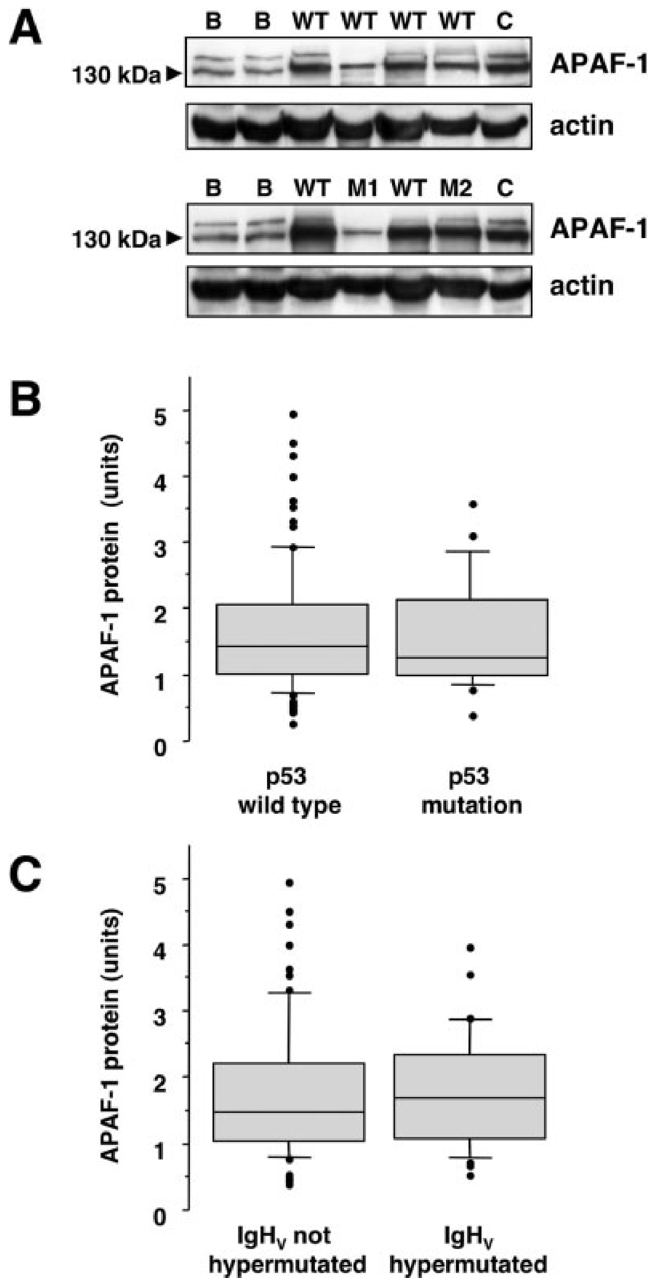

Figure 1 – APAF-1 expression and p53 mutation. (*a*) Western blot for APAF-1 and β-actin. WT, wild type p53 and M, p53 gene mutation. M1: Ins A at pos. 807 (X02469) → Stop at pos. 811. M2: ATG → ATA, Met → Ile. pos 711. B and C: internal control cell lines, B: BJAB, C: HEK293. (*b*) Box plot analysis: APAF-1 protein expression and p53 gene status. p53 gene aberration $n = 23$, p53 wild type $n = 111$, U-test: $p = 0.69$. (*c*) Box plot analysis: APAF-1 protein expression and IgH_V gene hypermutation status. IgH_V gene hypermutation $n = 28$, IgH_V gene not hypermutated $n = 81$, U-test: $p = 0.70$.

In view of p53 being a transcriptional activator of the APAF-1 gene, we determined APAF-1 protein expression levels in relation to the p53 mutation status (Fig. 1*a,b*). Of the 138 samples 23 (16.7%) were known to be mutated in the DNA-binding domain of p53.[19] There was no significant difference between p53 wild type and mutated samples in the level of APAF-1 expression (Mann-Whitney U-test, $p = 0.69$) (only a trend for lower APAF-1 in p53 mutated samples) (Fig. 1*b*). This indicates that inactivation of p53 contributes to impaired APAF-1 expression but that additional factors must be involved in mediating APAF-1 protein expression. A similar lack of correlation was observed previously in various tumor entities including B-CLL when expression levels of Bax, a well established p53 target, were correlated to the p53 mutational status.[18,28–32] In analogy to p53, hypermutation of the IgH_V gene locus had no impact on the level of APAF-1 expression (Fig. 1*c*, Mann-Whitney U-test, $p = 0.70$). Thus, the negative impact of p53 or unmutated IgH_V gene loci on disease prognosis is not a consequence of impaired APAF-1 expression.

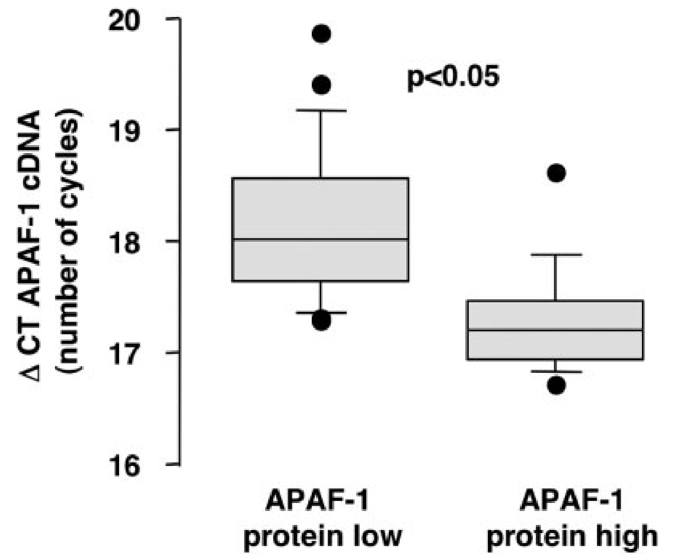

Figure 2 – APAF-1 mRNA expression in relation to APAF-1 protein expression. Box plot analysis of quantitative RT-PCR results for 32 patient samples (given as number of cycles necessary to reach a predefined threshold amount of PCR-product) in relation to the APAF-1 protein expression category: left column: APAF-1 low and right column: APAF-1 high (U-test: $p = 0.028$).

APAF-1 mRNA expression

To determine whether the downregulation of APAF-1 protein expression occurs at the transcriptional or the posttranscriptional level, quantitative RT-PCR was employed. For real-time PCR analysis, 40 samples were selected: the 20 samples with highest APAF-1 protein expression and the 20 samples with lowest APAF-1 protein expression. RNA of sufficient quality was extracted from 32 specimens: in 19/20 samples with low APAF-1 protein expression, and in 13/20 samples with high APAF-1 expression. The results obtained demonstrate a good correlation of the mRNA expression data with the level of protein expression (Fig. 2).

Impact of APAF-1 expression on patient survival

Median overall survival was 30.1 months. 17 (12%) of the patients were censored "alive" (median follow-up 97.9 months). As previously described,[19] the 23 patients with p53 alterations showed a significantly shorter survival and all had died at the time of analysis (median survival 11.7 versus 37.5 months in the wild type group ($p < 0.0001$, Fig. 3*a*). In contrast, APAF-1 expression alone had no significant impact on overall survival: median survival for APAF-1 low was 28.4 ± 2.0 months versus 35.0 ± 9.7 months for APAF-1 protein high ($p = 0.15$, Fig. 3*b*).

In previous analyses of p53 pathway components, we demonstrated that the combined disruption of p53 and Bax results in a rather poor prognosis whereas the analysis of single genes frequently failed to identify patients at high risk for rapid disease progression and death. Thus, the combined inactivation of both p53 and Bax identified patients with extremely poor prognosis in gastric and colorectal cancer.[29,33] In B-CLL, loss of Bax has no

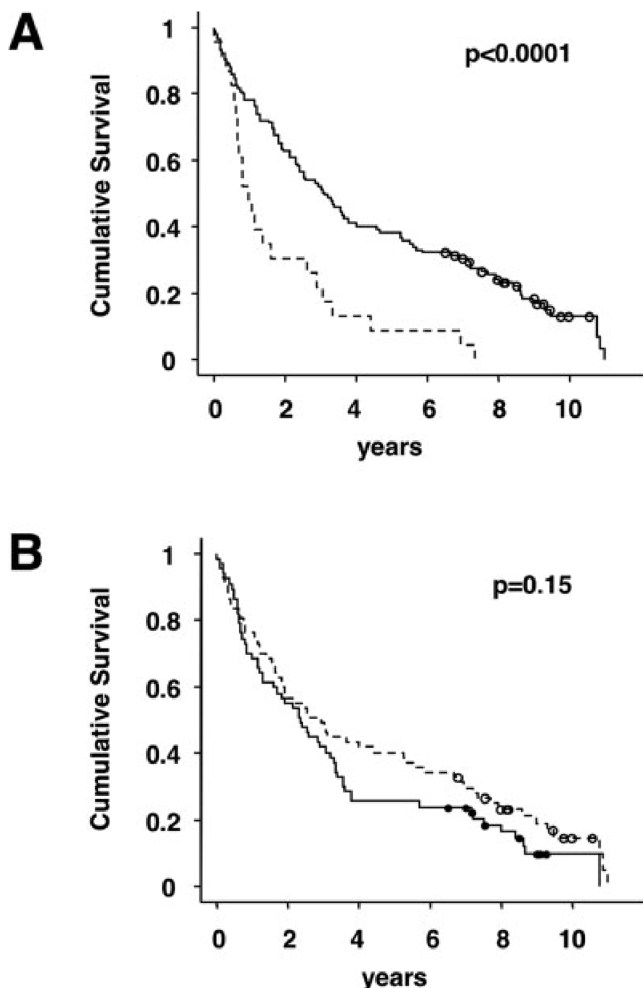

FIGURE 3 – Impact of p53 mutation and APAF-1 expression on disease prognosis. (*a*) P53 status and overall survival ($p < 0.0001$). Continuous line: p53 wild type ($n = 115$); dashed line, p53 mutation ($n = 23$). Open circles: censored patients. (*b*) APAF-1 status and overall survival ($p = 0.15$). Continuous line, closed circles: APAF-1 low protein expression ($n = 67$); dashed line, open circles: APAF-1 high protein expression ($n = 67$). Circles indicate censored patients.

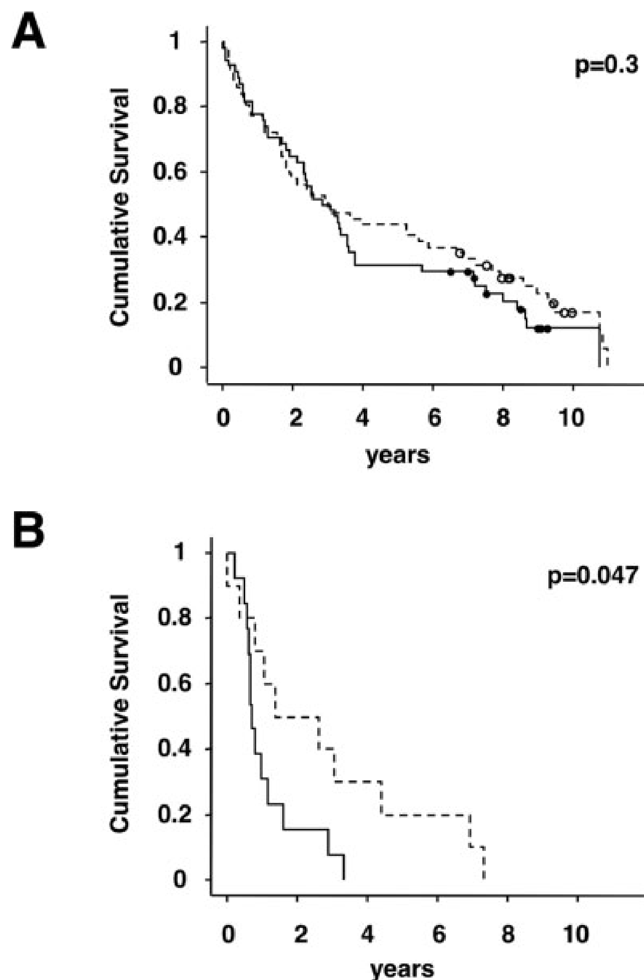

FIGURE 4 – APAF-1 expression and survival in relation to p53 disruption. (*a*) Patients with p53 wild type B-CLL ($n = 111$): APAF-1 expression is not related to survival ($p = 0.3$). Continuous line, closed circles: APAF-1 low ($n = 54$); dashed line, open circles: APAF-1 high ($n = 57$). Circles indicate censored patients. (*b*) Patients with p53 mutated B-CLL ($n = 23$): low APAF-1 expression is associated with poor survival ($p = 0.047$). Continuous line: APAF-1 low ($n = 13$); dashed line: APAF-1 high ($n = 10$). There are no censored patients in this group.

prognostic relevance.[18] Therefore, we asked whether APAF-1 loss becomes relevant in the p53 mutation context.

For the p53 wild type patients, APAF-1 is not a relevant prognostic factor: median survival was 35.9 ± 4.6 months for p53 wild type/APAF-1 high and 34.0 ± 10.8 months for p53 wild type/APAF-1 low patients ($p = 0.3$, Fig. 4*a*). In contrast, APAF-1 shows prognostic relevance in the small subset of p53-mutated patients. There, patients with high APAF-1 expression had a significant better survival than those with low APAF-1 expression (median survival 16.3 ± 14.6 months for p53 mutated/APAF-1 high versus 8.4 ± 1.2 months for p53 mutated/APAF-1 low, $p = 0.047$, Fig. 4*b*).

Impact of p53 and APAF-1 on resistance to anticancer therapy

Next, we analyzed the relevance of APAF-1 or the combined p53/APAF-1 status on resistance of B-CLL cells to anticancer therapy by the *ex vivo* apoptotic differential staining assay.[25] The chemotherapeutic drugs tested in all 138 samples were fludarabine, chlorambucil and doxorubicin. Subsets of patient samples were tested with methylprednisolone ($n = 137$), cyclophosphamide (as 4-OH metabolite analog mafosfamide; $n = 133$), the vinca alkaloid vincristine ($n = 104$) and ionizing irradiation (IR, 2 Gy by a ^{137}Cs source; $n = 83$). All treatment methods induced cell death in a dose-dependent manner. To establish whether APAF-1 expression is related to resistance to chemotherapy, we compared the LC_{90} doses in relation to the p53 mutation and APAF-1 expression status (Table II).

Resistance to cell death induced by irradiation, alkylating agents and fludarabine was observed in p53-mutated B-CLL samples. Intronic or silent sequence aberrations did not have an impact on drug sensitivity profiles as described previously.[19] In contrast to p53, neither APAF-1 by itself nor the combined analysis of p53 and APAF-1 had an impact on sensitivities to anticancer drugs or irradiation (Table II). This was also true when other cut-off points for loss of APAF-1 expression were applied. This indicates that the role of APAF-1 in cell death execution following exposure to cytotoxic drugs or ionising irradiation is rather limited. This is well in line with recent cell biological data showing that mitochondria release a plethora of proapoptotic factors that, in contrast to cytochrome c, do not rely on APAF-1 to initiate execution of apoptosis.[34] This is well reflected by the cell death data as obtained by use of the DiSC assay that measures overall cell death, *i.e.* apoptosis and necrosis. Notably, recent work indicates

TABLE II – RESISTANCE TO IONISING IRRADIATION AND CYTOTOXIC DRUGS IN RELATION TO THE P53/APAF-1 STATUS

	p53					APAF-1			p53WT/APAF-1			p53 mutated[1]/ APAF-1		
	Intronic or silent aberration		p		p			p			p			p
	Wild type	Mutated				Low	High		Low	High		Low	High	
γ–Irradiation (% surviving cells)	29.6 ± 3.5 (n = 69)	60.6 ± 11.5 (n = 9)	0.0075	34.5 ± 11.2 (n = 5)	0.58	29.4 ± 4.6 (n = 44)	37.6 ± 4.9 (n = 39)	0.21	26.0 ± 4.9 (n = 36)	33.4 ± 5.0 (n = 33)	0.28	52.2 ± 16.2 (n = 5)	71.1 ± 17.1 (n = 4)	0.46
Log_{10} LC_{90} chlorambucil	0.61 ± 0.06 (n = 15)	1.17 ± 0.11 (n = 17)	0.0003	0.81 ± 0.22 (n = 6)	0.38	0.66 ± 0.08 (n = 67)	0.72 ± 0.074 (n = 67)	0.39	0.58 ± 0.08 (n = 54)	0.64 ± 0.08 (n = 57)	0.51	1.04 ± 0.19 (n = 9)	1.31 ± 0.09 (n = 8)	0.26
Log_{10} LC_{90} mafosfamide	0.27 ± 0.04 (n = 111)	0.45 ± 0.09 (n = 16)	0.095	0.41 ± 0.13 (n = 6)	0.41	0.32 ± 0.05 (n = 65)	0.30 ± 0.05 (n = 65)	0.92	0.28 ± 0.05 (n = 53)	0.28 ± 0.10 (n = 55)	0.98	0.51 ± 0.15 (n = 8)	0.39 ± 0.10 (n = 8)	0.67
Log_{10} LC_{90} fludarabine	0.06 ± 0.07 (n = 115)	0.44 ± 0.19 (n = 17)	0.037	0.00 ± 0.27 (n = 6)	0.79	0.18 ± 0.08 (n = 67)	0.05 ± 0.1 (n = 67)	0.13	0.16 ± 0.09 (n = 54)	−0.02 ± 0.1 (n = 57)	0.094	0.27 ± 0.18 (n = 9)	0.64 ± 0.36 (n = 8)	0.53
Log_{10} LC_{90} vincristine	−0.15 ± 0.08 (n = 83)	−0.11 ± 0.17 (n = 17)	0.76	−0.14 ± 0.37 (n = 4)	0.84	−0.12 ± 0.09 (n = 51)	−0.15 ± 0.11 (n = 50)	0.63	−0.15 ± 0.11 (n = 39)	−0.14 ± 0.12 (n = 41)	0.92	−0.03 ± 0.16 (n = 9)	−0.19 ± 0.32 (n = 8)	0.41
Log_{10} LC_{90} doxorubicin	−0.58 ± 0.03 (n = 115)	−0.52 ± 0.09 (n = 17)	0.60	−0.58 ± 0.16 (n = 6)	0.97	−0.55 ± 0.04 (n = 67)	−0.57 ± 0.05 (n = 67)	0.96	−0.56 ± 0.04 (n = 54)	−0.58 ± 0.06 (n = 57)	0.81	−0.47 ± 0.12 (n = 9)	−0.57 ± 0.15 (n = 8)	0.56
Log_{10} LC_{90} methylprednisolone	1.02 ± 0.11 (n = 114)	1.01 ± 0.31 (n = 17)	0.98	0.88 ± 0.40 (n = 6)	0.76	1.04 ± 0.15 (n = 67)	1.0 ± 0.15 (n = 67)	0.73	1.02 ± 0.18 (n = 54)	1.03 ± 0.16 (n = 57)	0.96	1.15 ± 0.42 (n = 9)	0.84 ± 0.5 (n = 8)	0.70

Comparison of $\log_{10}$ LC_{90} concentrations (for the cytotoxic drugs) or percentage of surviving cells (for the irradiated cells after a fixed γ-irradiation with 2 Gy) for chlorambucil, mafosfamide, fludarabine, γ-irradiation, vincristine, doxorubicin or methylprednisolone.–[1]p53 gene mutated: point mutation, insertion or deletion leading to amino acid exchange as described. Mean ± SEM is given. Statistical significance was calculated by means of the 2-sided Mann-Whitney U-test.

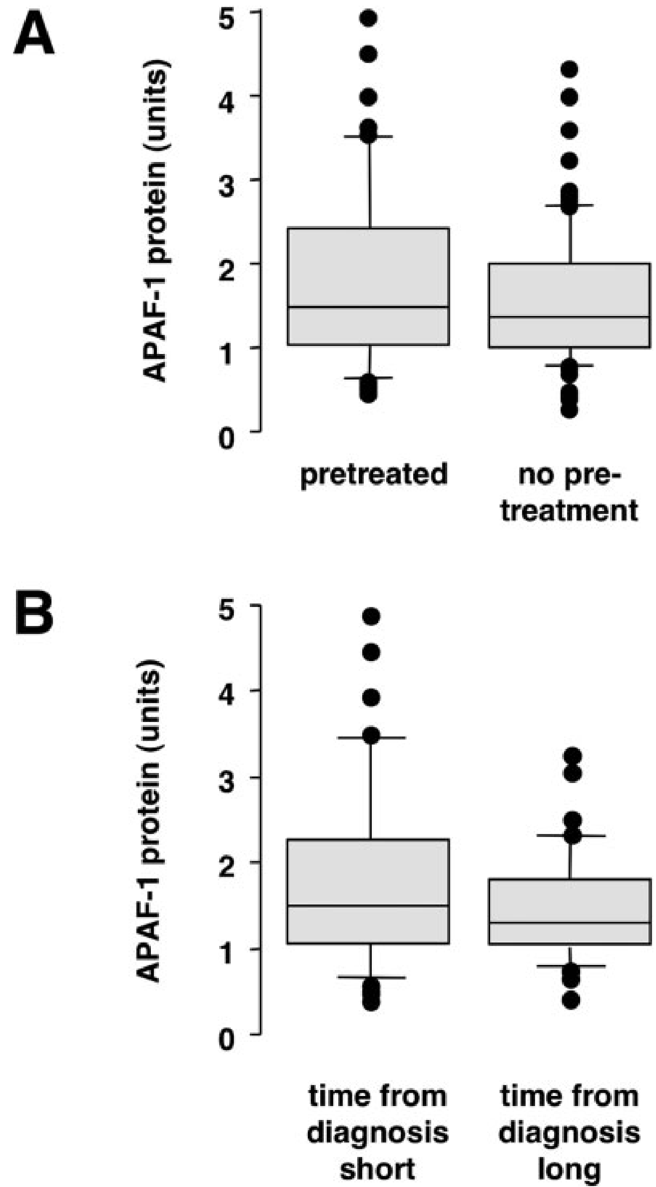

FIGURE 5 – APAF-1 expression in relation to pretreatment status. (*a*) Box plot analysis of APAF-1 protein expression and prior chemotherapy. Pretreated patients: $n = 78$; chemonaïve patients: $n = 56$; U-test: $p = 0.3$. (*b*) Box plot analysis of APAF-1 protein expression and the interval of sampling date from date of diagnosis. Cut-off for "short" and "long" was the median (7.4 months). Data available for $n = 78$, U-test: $p = 0.23$.

that anticancer drugs may trigger a regulated form of cell death that can be inhibited by Bcl-2 but occurs independently from the APAF-1/caspase-9 apoptosome.[35,36]

This finding of APAF-1 being not relevant for responses to anticancer therapy in B-CLL is corroborated by the lack of correlation between APAF-1 levels, pretreatment status and disease progression. Our previous analyses demonstrated that pretreatment with alkylating agents is strongly correlated with the occurrence of p53 mutations. This makes sense given the strong selection pressure of such anticancer therapies toward therapy resistance and the role of p53 in drug-induced apoptosis. In contrast to the correlation of p53 mutation and pretreatment status, no loss of APAF-1 was observed in the samples obtained from the pretreated patients. Range and median of APAF-1 levels were slightly but not

significantly lower in B-CLL cells from the pretreated patients (Fig. 5*a*). Thus, probability for a role of APAF-1 loss in the development of resistant leukemia subclones is low.

Impact of the natural disease course on APAF-1 expression and p53 mutational status

As described earlier, not all samples were drawn at the time of diagnosis of B-CLL. The time course of the disease was known in 80 patients. Samples were obtained for analyses in median 7.34 months after diagnosis of B-CLL. To determine a potential impact of the natural course of the disease on the level of APAF-1 protein expression, patients were dichotomized according to time from diagnosis to sampling date at the median into "short time from diagnosis" versus "long time from diagnosis" (Fig. 5*b*). There was, however, no significant difference in expression (Mann-Whitney U-test, $p = 0.23$). In contrast, p53 mutations did accumulate in the course of disease, with 2/11 mutations diagnosed early in the disease course and 9/11 mutations diagnosed later (after 7.4 months from time of diagnosis; Fishers exact test $p = 0.048$). This is in accordance with our previous report that p53 mutations might be induced by treatment.[19]

Discussion

The p53 pathway is a frequent target for inactivation, *e.g.* by mutation, in a variety of tumors.[37] Loss of p53 function or inactivation of p53 target genes results in an impaired response to DNA damaging therapies, *i.e.* apoptosis induced by anticancer drugs or ionizing irradiation.[38] Moreover, disruption of p53 is a frequent event that is involved in both tumor development and development of resistance to anticancer therapies.[39,40] We previously observed that the inactivation of the p53 gene or loss of its downstream pro-apoptotic effector Bax is related to chemoresistance in B-CLL.[18,19] Recent evidence identified APAF-1, a key component of the apoptosome,[41,42] to be transcriptionally induced by p53.[5,6] In the present work, we therefore addressed the role of APAF-1 on disease prognosis and the response to anticancer therapies in B-CLL.

APAF-1 serves as an adaptor protein that mediates activation of the caspase cascade upon release of cytochrome c by the mitochondria during apoptosis.[43] Binding of cytochrome c to APAF-1 facilitates recruitment and autocatalytic activation of procaspase-9 presumably through induced proximity of at least 2 procaspase-9 molecules.[44] This results in the initiation of the caspase cascade that ultimately leads to execution of apoptosis.

In cell line models, including leukemia cell lines, APAF-1 deficiency has been shown to correlate with resistance to apoptosis.[8–10,45,46] In addition, enforced expression of APAF-1 was shown to sensitize for p53-mediated apoptosis.[47] Evidence for a role of APAF-1 in tumorigenesis and resistance to anticancer therapy came from the observation that APAF-1 is downregulated in human malignant melanomas in consequence of DNA hypermethylation.[12] This finding was interpreted to be the basis of the well-known clinical resistance of melanoma to various cytotoxic drugs. Another report by the same group demonstrated that loss of APAF-1 can complement for inactivation of p53 during *myc*-induced malignant transformation. These data demonstrated a key role of APAF-1 disruption in tumor biology and resistance to anticancer therapy.

In accordance with these findings, we observed that reduction of APAF-1 protein expression may result in a poor prognosis in B-CLL. Interestingly, this was the case only in a subset of patients carrying a p53-mutated leukemia. This rather limited role of APAF-1 in B-CLL as compared with other p53 target genes such as Bax[30,32] or p21$^{CIP/WAF-1}$ [48] is well in accordance with 2 recent reports[49,50] that opposes the initial view of APAF-1 being critically involved in malignant transformation.[11] This report carefully investigated malignant transformation in both lymphomagenesis in the Eμ-myc lymphoma murine model and oncogene-driven tranformation of mouse embryonal fibroblasts (MEF). Notably, no differences were seen in rate, incidence or severity of lymphoma or MEF transformation with loss of APAF-1.

This impact of APAF-1 loss in patients with p53-mutated B-CLL might have been related to the transcriptional activation of the APAF-1 gene by p53.[5,6] Nevertheless, APAF-1 protein levels were not decreased in the p53-mutated B-CLL. This indicates, similar to the situation of the p53 target Bax,[30,32,48] that additional genes are involved in the regulation of APAF-1 expression and it remains to be established whether these so far upstream regulators play a role in the response to anticancer therapies. In addition, the finding of APAF-1 being relevant in B-CLL with a disrupted p53 is well in line with our findings in other cancer entities. In gastrointestinal carcinoma of the colorectum[29] and the stomach,[33] we demonstrated that the combined inactivation of p53 and Bax identified patients at high risk for early death whereas the clinical significance of single gene defects was rather limited. This finding is well in line with the high redundancy found at every level of cell death signaling. Thus, our data suggest that multiple defects in consecutively involved signaling modules must accumulate to yield a clinically relevant poor prognosis genotype. Nevertheless, our data do not allow the reverse that the genetic context of p53 gene mutations and IgHv mutations are the only genetic backgrounds of potential interest for the clinical relevance of APAF-1.

Finally, we demonstrated that a reduced APAF-1 protein level does not result in resistance to cytotoxic anticancer therapies. This is a rather surprising finding that is, however, well in accordance with the cell biology of APAF-1. Drug sensitivities were assessed by the use of the DiSC assay, a morphometric assay that detects both caspase dependent and independent cell death. Recent data showed that mitochondria release not only cytochrome c but also other pro-apoptotic factors that do not rely on APAF-1 to exert their apoptosis promoting activities. These include SMAC, the second mitochondrial activator of caspases, the apoptosis inducing factor AIF, the serine protease Omi/HtrA2, heat shock protein HSP10 and endonuclease G.[34] This may explain the limited relevance of APAF-1 in our system as APAF-1 deficiency may be circumvented by these factors. In the same vein, a recent report that studied the role of APAF-1 loss in Eμ-myc-induced murine lymphoma cells did not observe any impact on chemosensitivities.[49] In line with these data and our findings is a recent report in melanoma cells with variable APAF-1 status indicating independence of DNA damaging therapies from APAF-1.[50] Our findings are also supported by data obtained from the analysis of ovarian cancer cell lines. There, loss of APAF-1 correlated with drug resistance in some but not all of the cell lines,[9] a finding fully concordant with our dataset. In line with these findings, we did not observe downregulation of APAF-1 in patients who were pretreated. In contrast, such patients showed a highly significant clustering of p53 mutations whereas only a few of the "chemonaïve" patients exhibited a mutated p53.[19] Thus, loss of APAF-1 (or caspase-9) does not appear to be *per se* a key event during disease progression and development of chemoresistant genotypes but may determine more basic features of tumor biology, such as tumor cell turnover and genetic stability. Nevertheless, neither the above mentioned cell line studies nor the data derived from the Eμ-myc model[49] take into account combined inactivation of both APAF-1 and p53. Thus, unlike drug-induced cell death, the analysis of such combined inactivation of APAF-1 and additional core cell death signaling modules might yield novel insights into apoptosome function in tumorigenesis and tumor biology as evidenced by our current patient data.

Acknowledgements

We thank Jana Rossius, Alison Burlton, Philip Bell and David Head for expert technical assistance. The following drug companies supplied drugs: Asta (mafosfamide) and Schering (fludarabine).

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