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High Bad and Bax mRNA expression correlate with negative outcome in acute myeloid leukemia (AML)

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The search for molecular markers in AML that allow prediction of outcome has recently focused on genes involved in the regulation of programmed cell death (PCD). The aim of our study was to determine whether mRNA levels of Mdm-2, Bcl-2, Bcl-x_L, Bad, and Bax are independent prognostic parameters for outcome. Transcript levels were analyzed by real-time quantitative RT-PCR in 232 samples collected either at diagnosis or following induction chemotherapy (ICT). Multivariate COX regression analysis adjusted for chemotherapy protocol, *de novo vs* secondary AML, and *de novo vs* relapsed AML indicated: (1) At diagnosis, high expression of Bad ($P = 0.015$) and even more so high Bax and Bad levels ($P = 0.018$) predicted adverse outcome, regardless of the response to ICT. In patients who subsequently failed to enter complete remission (CR), high levels of Bad, Bax and Bax high/Bad high were associated with an increased relative risk (RR) to die from tumor (RR = 5.0 for Bad, 3.4 for Bax and 6.14 for Bax high/Bad high). (2) Following ICT, high expression of Bax ($P = 0.005$) and high Bcl-2/Bax ratios ($P = 0.004$) were independent predictors of unfavorable outcome, regardless of response to ICT. We conclude that high levels of Bax and Bad correlate with poor outcome, particularly in patients who do not enter CR and may serve as prognostic markers in AML.
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Introduction

Inspite of considerable advances in combination chemotherapy, a significant number of malignant tumors become resistant to treatment. One major cause of either intrinsic or acquired resistance is the selective activation of a number of highly effective cellular defense mechanisms which have evolutionarily developed to protect the cell from exogenous toxic compounds. The first defense barrier to a great variety of cytotoxic drugs is formed by prominent members of the ATP-binding cassette membrane transporter family, such as P-glycoprotein and the multidrug resistance-associated protein (MRP)[1] which may lower intracellular drug concentrations to sublethal levels by active transport mechanisms.[2] Agents which escape the primary efflux mechanisms are generally believed to effect tumor cell killing by inducing apoptosis.[2] A second mechanism associated with primary resistance to chemotherapy-induced cell death may be the failure to activate the apoptotic machinery. Therefore, the search for new prognostic markers which may reflect tumor prognosis has recently focused on genes involved in the regulation of PCD. Experimental evidence has accumulated that PCD genes may be dysregulated in several types of human malignancies.[3–16] Particularly, members of the expanding Bcl-2 superfamily have been extensively studied. Current opinion holds that, if proapoptotic family members (eg Bax, Bad, Bak, Bid, Bcl-x_S) are in excess, cells execute a death command, but if anti-apoptotic members (eg Bcl-2, Bcl-x_L, Mcl-1) dominate, this program is inhibited.[17,18] Death ligands such as Bad, represent an evolving set of proapoptotic molecules which appear to reside more proximal to Bcl-2 in the apoptotic pathway and connect upstream signal transduction paths with the Bcl-2 family thus modulating apoptosis.[18] There is evidence that Bax is transcriptionally activated by the tumor suppressor p53 and may be a key regulator of apoptosis.[18] p53 itself is controlled by murine double minute-2 (Mdm-2) which promotes inactivation of p53 by rapid degradation or by masking the p53 transactivation domain.[19,20] We recently showed that low levels of Mdm-2 mRNA are associated with poor prognosis in soft tissue sarcoma.[21]

Several attempts have been made to correlate expression levels of resistance genes with clinical outcome of leukemia.[14,22–25] Only a few studies have assessed the association between candidate gene expression and survival in acute myelogenous leukemia (AML), most of them with rather small patient numbers, and sometimes with conflicting results.[9,22,26,27] Therefore, the potential prognostic value of apoptosis-related genes in AML remains to be elucidated. In this retrospective analysis, we used a semi-automated high-throughput methodology to study the expression of apoptosis-related genes (Mdm-2, Bcl-2, Bcl-x_L, Bad, Bax) in 232 AML specimens and correlated expression with response to ICT and survival.

Materials and methods

Patients, samples, and treatment

Two-hundred and thirty-two peripheral blood or bone marrow (BM) aspirates were collected from adult AML patients at diagnosis or after treatment with the first or second ICT cycle (71% paired samples) according to either the low-dose (AML'93) or the intermediate-dose (AML'96) protocols of the East-German Hematology and Oncology Study Group (OSHO) (Table 1). The AML'93 ICT protocol comprised two cycles of cytarabine (ARA-C) 120 mg/m^2 i.v. every 12 h (days 1–7), idarubicin (IDA) 12 mg/m^2 i.v. (days 1–3), followed by a first and second consolidation therapy. The AML'96 ICT protocol consisted either of ARA-C 2 g/m^2 as a 8 h infusion (days 1, 3, 5, 7) and IDA 12 mg/m^2 (days 1–3) or ARA-C 1 g/m^2 every 12 h as a 3 h infusion (days 1, 3, 5, 7) and IDA 12 mg/m^2 (days 1–3).

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Table 1 AML study group, patient characteristics and composition of samples either obtained at the time of diagnosis or following ICT treatment

Parameter	*Characteristics*	*At diagnosis*	*After ICT*
Samples	total	140	92
	de novo	85	48
	secondary	25	19
	relapse	30	25
Sex	male/female	74/66	52/40
Age (years)	median: male/female	58.5/54	53.5/51
	range: male/female	17–77/26–77	19–75/26–75
FAB classification	M0/M1/M2/M4/M5/M6/M7	1/22/57/27/22/3/3	2/14/43/13/12/3/-
	NA	5	5
$CD34^+$	characterized patients	77	59
	NA	63	33
Karyotype[52]	standard	51	32
	favorable	3	3
	unfavorable	39	18
	NA	47	39
Treatment	low dose (AML'93)	95	65
	intermediate dose (AML'96)	45	27
Response	complete remission	102	66
	no response	37	25
	NA	1	1

As not otherwise indicated the numbers in the table correspond to the number of samples.
FAB, French–American–British classification; CD, cell differentiation marker; NA, not available.

Complete remission was defined as ⩽5% marrow blasts by morphology. All other patients were classified as non-responders (NR). At diagnosis, the mean percentage of blasts in samples were 65 ± 22% (median: 70%). After ICT, the NR group showed 46 ± 31% blasts (median: 48%) and the CR group 4 (1% blasts (median: 4%). Patients with acute promyelocytic leukemia (FAB-M3) and patients who died from toxicity were excluded from the analysis.

FACS analysis

$CD34^+$ cells were counted with a FACscan flow cytometer following incubation with the phycoerythrin-labeled HPCA-2 monoclonal antibody (Becton Dickinson, San Jose, CA, USA).

Clinical sample preparation

Mononuclear cells from 5–10 ml blood (9% of samples) or BM samples (91% of samples) were collected after separation on sterile Histopaque-1077 lymphocyte separation medium (Sigma, St Louis, MO, USA), washed three times with phosphate-buffered saline, and homogenized with 2 ml of RNAzol 'B' (Tel-Test, Friendswood, TX, USA) or 1 ml of Reagent 14 (Integrated Separation Systems, Natick, MA, USA). Homogenates were stored at −20°C until RNA preparation was performed.

RNA isolation and reverse transcription (RT)

Total RNA was isolated manually or in an Autogen 540 nucleic acid extraction robot (Integrated Separation Systems). cDNA was synthesized from 1 μg aliquots in a 20-μl standard reaction mixture containing AMV reverse transcriptase buffer (250 mM Tris/HCl; pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 50 mM dithiothreitol, 2.5 mM spermidine), 5 U AMV reverse transcriptase, 0.5 mM of each dNTP (Promega, Madison, WI, USA), 10 U recombinant RNase inhibitor (AGS, Heidelberg, Germany), and 200 ng oligo (dT) (Amersham Pharmacia Biotech, Uppsala, Sweden) at 42°C for 1 h. RNA and cDNA samples were stored at −80°C until use.

High-throughput transcript analysis by quantitative real-time fluorescence PCR

Mdm-2 (splice variants A, B, C, D; not E), Bcl-2, Bcl-x_L, Bax (splice variants alpha, beta, delta, sigma, zeta; not gamma and epsilon), Bad, and glyceraldehyde-3-phosphate dehydrogenase GAPDH) transcripts were measured from cDNA in duplicate experiments by ready-to-use PCR testkits (Roboscreen Gesellschaft für molekulare Biotechnologie, Leipzig, Germany). Briefly, conventional 96-well bases were loaded with one eight-well ready-to-use reference-DNA strip which was storage-stable coated with eight different amounts of respective reference-DNA for quantitation of the desired transcripts, forward and reverse primer, and the TaqMan probe. The probes were either 5′-labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) or 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE) for GAPDH, and the common 3′-fluorescent quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) in order to generate the respective standard reference curves for each run. The remaining free positions were loaded with the required number of sample tubes containing only the respective TaqMan oligonucleotide sets. Reaction premixes containing PCR buffer, the passive fluorescence dye 6-carboxy-tetramethyl-rhodamin (ROX), dNTPs, and 0.5–1 U of AmpliTaq 'GOLD' (Applied

Table 2 Univariate analysis on prognostic factors in AML at diagnosis and following ICT

Prognostic factor	*At diagnosis*		*After ICT*	
	n	*P value*	*n*	*P value*
Treatment protocol	140	0.014	92	0.058
Secondary *vs de novo* AML	110	0.082	67	0.139
Relapsed *vs de novo* AML	115	0.096	72	0.034
Response (NR *vs* CR)	138	0.004	90	0.021
Mdm-2	134	0.786	88	0.075
Bcl-2	133	0.256	88	0.446
Bcl-x_L	133	0.232	88	0.829
Bax	134	0.095	88	0.040
Bad	134	0.026	88	0.238
High Bax + Bad	134	0.014	88	0.215
Bcl-2/Bax	124	0.566	81	0.044

Conventional parameters to predict AML 5-year survival and correlation of high *vs* low transcript levels and high *vs* low transcript ratios with survival. All indicated *P* values correspond to high expression levels or high transcript ratios, respectively. Sex, age, karyotype, CD34, and the Bcl-x_L/Bad ratios were without predictive value.
N, number of samples, differences between the total number and the indicated number of samples are due to missing patient data or sample RNA degradation.

Biosystems, Weiterstadt, Germany) were mixed according to the manufacturer's guidelines and supplemented with H_2O to a final volume of 25 μl. Aliquots of the mixes were added to each reaction tube using a BIOMEK 2000 laboratory automation workstation (Beckman Instruments, Fullerton, CA, USA). Sample reactions were completed by the addition of 2-μl aliquots of cDNA. PCR amplification and detection was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Amounts were calculated from a reference curve obtained from the simultaneously processed reference DNA strips. Mdm-2, Bcl-2, Bcl-x_L, Bax, and Bad data were expressed as zeptomoles [zmol, 10^{-21} mole] cDNA per attomole [amol, 10^{-18} mole] of GAPDH cDNA which was calculated from the same cDNA sample.

Statistical analyses

Statistics were performed using the SPSS 9.0 software program (SPSS, Chicago, IL, USA). Univariate survival analysis was performed using the Kaplan–Meier method and log-rank test. A multivariate Cox regression model was used to estimate the influence of individual gene expression parameters on prognosis, adjusted for the covariates AML sub-type and chemotherapy protocol. Changes of likelihood interactions between Bax and Bad gene expression were analyzed using an interaction term.[28] High Bax and Bad were classified as '1', low Bax or Bad values as '0'. *P* values of less than 0.05 were considered statistically significant.

Table 3 Medians and ranges of apoptosis-related transcripts

Transcript	*At diagnosis*		*After ICT*	
	Median	*Range*	*Median*	*Range*
Mdm-2	0.633	0–2465	0.568	0–402
Bcl-2	0.071	0–103	0.141	0–41.4
Bcl-x_L	0.064	0–95.6	0.316	0–81.4
Bax	2.473	0–333	1.902	0–147
Bad	4.104	0–470	3.850	0–1707
Bcl-2/Bax ratio	0.036	0–258	0.051	0–657
Bcl-x_L/Bad ratio	0.016	0–2.24	0.129	0–23.5

Values calculated separately either at the time of initial diagnosis or following ICT. All data are expressed as zmol/amol GAPDH transcripts measured in the same cDNA sample.

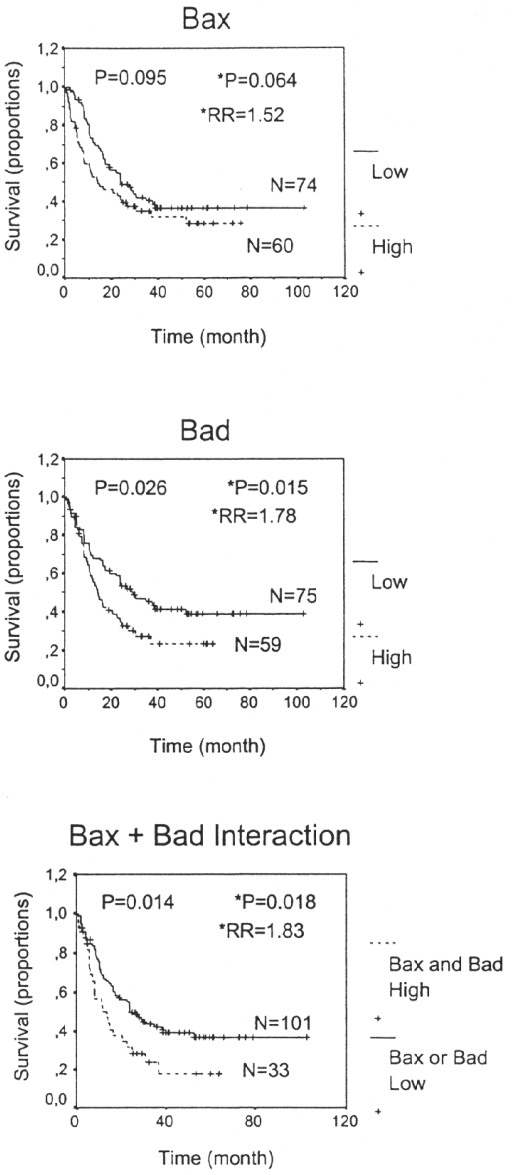

Figure 1 Kaplan–Meier plots of Bax, Bad, and interacting Bax as well as Bad levels measured at diagnosis. High expression is defined as greater than the median, and low expression is defined as less than or equal to the median value. Statistical analysis performed by the log-rank test, corresponding *P* values indicated in the figure. Symbols: '+': censored data, *P* and RR values labeled by asterisk are obtained from multivariate Cox regression analysis, N, number of samples.

Results

Validation of quantitative RT-PCR

The expression of the Bcl-2 family genes Bcl-2, Bcl-x_L, Bad, Bax, and the p53 antagonist Mdm-2 were assessed by recently developed ready-to-use fluorescence RT-PCR assays based on the TaqMan technology. The repeatability of the Roboscreen assays was good: the coefficients of variation of the mean reference curves slopes were usually below 5% which indicates reliable standard stability.[21,29] The precision of the assays was tested by analysis of cDNAs prepared from the K562 and CCRF ADR5000 cell lines for Bcl-2 and GAPDH expression, respectively. Input analyte amounts at the attomole detection level, ie typical for GAPDH reference transcripts, were detected within a 10% precision rate, whereas Bcl-2 transcripts which are usually at a low zeptomole detection level were detected within a precision of ±28% (eight repeated analyses performed for each cell line, data not shown). All values were adjusted for the number of GAPDH transcripts, which have been reported to be constant in various tumors, tissues and cell lines.[30–32]

Univariate and multivariate correlation between transcript levels and outcome at the time of diagnosis

The most important conventional prognostic parameters are shown in Table 2. Univariate Kaplan-Meier regression analysis and log-rank test showed that poor prognosis was strongly associated with the low-dose therapy protocol compared to the intermediate-dose protocol ($P = 0.014$) and with later NR compared to CR ($P = 0.004$). A trend towards unfavorable prognosis was found for relapsed and secondary AML compared to *de novo* AML ($P = 0.097$) and for unfavorable karyotypes compared with standard or favorable karyotypes ($P = 0.186$) confirming earlier data from the literature.[33,34] There was no significant effect of age, sex, or CD34$^+$ expression on survival.

To assess the influence of individual transcript amounts on prognosis, medians were calculated for each transcript separately at diagnosis and following ICT in order to group patients into high and low expressing individuals (Table 3). For this analysis, AMLs at presentation and newly diagnosed relapsed leukemias were analyzed together. Patients were divided according to their expression level of the respective transcript into high (expression above median) and low (expression below or equal to median value) expressing groups, and survival was compared between the two groups. In univariate Kaplan–Meier analysis, the most important predictors of survival at diagnosis were Bad ($P = 0.026$) and the combination of high Bad and high Bax levels ($P = 0.014$), whereas Bax

Table 4 Gene expression and relative risk (RR) of tumor-related death

Prognostic factor	*At diagnosis*		*After ICT*	
	P value	*RR*	*P value*	*RR*
Treatment protocol	0.036[a]	2.27[a]	0.081[a]	2.21[a]
Relapsed *vs de novo*	0.009[a]	2.94[a]	0.019[a]	4.16[a]
Secondary *vs de novo*	0.194[a]	1.36[a]	0.091[a]	2.11[a]
Mdm-2	0.983	1.01	0.098	1.68
Bax	0.064	1.52	0.005	2.40
Bad	0.015	1.78	0.151	1.56
High Bax + Bad	0.018	1.83	0.122	1.68
Bcl-2/Bax ratio	0.664	1.10	0.004	2.62

Parameters characterized by *P* values <0.1 as revealed by Kaplan–Meier analysis were subjected to a multivariate Cox regression model adjusted for the covariates treatment protocol and AML type. Gene expression levels and RR are separately examined at the time of initial diagnosis and following ICT. The indicated P and RR values correspond to high expression levels or high transcript ratios of the indicated genes, respectively.
[a]Mean values obtained from the Cox analysis of each gene expression parameter assessed separately for its impact on prognosis.

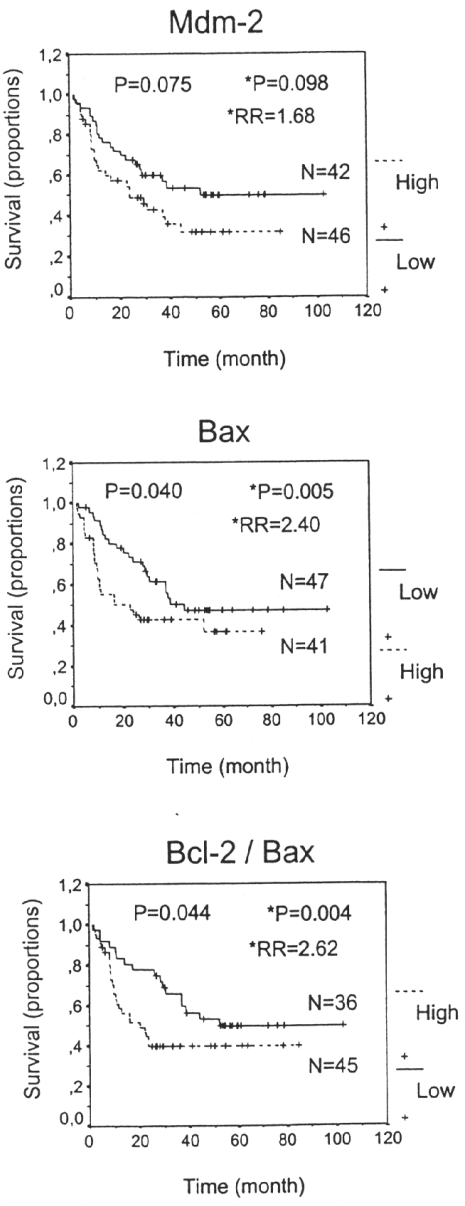

Figure 2 Kaplan–Meier plots of Mdm-2, mdr-1, and Bax expression, and Bcl-2/Bax ratios after ICT treatment. High and low expression is defined as described previously. Statistical analysis is performed by the log-rank test (*P*), *data confirmed by multivariate Cox regression analysis.

alone showed only a marginal impact on survival ($P = 0.095$) (Table 2, Figure 1). These univariate findings remained stable when Bad and Bax expression were analyzed by a multivariate COX model adjusted for (1) chemotherapy protocol (AML 93 *vs* AML 96), (2) *de novo vs* secondary AML, and (3) *de novo vs* relapsed AML (Table 4). Cox analysis further revealed that Bad and combined Bax/Bad levels above the median values are independent negative prognostic parameters for survival ($P = 0.015$ and $P = 0.018$, respectively) at the time of initial diagnosis, whereas Bax alone remained borderline significant ($P = 0.064$) confirming the univariate analysis. Furthermore, the data indicate that AML patients characterized by Bad, Bax or Bax as well as Bad levels above median showed a 1.78-fold, 1.52-fold and 1.83-fold higher relative risk of tumor-related death compared to patients with levels equal to or below the median (Table 4, Figure 1). All other transcripts were without predictive value at diagnosis.

Univariate and multivariate correlation between transcript levels and outcome after ICT

After ICT (regardless of response), the initially observed correlation between survival and expression of Bad disappeared, whereas a poor prognosis was now associated with high Bax levels ($P = 0.040$) and high Bcl-2/Bax ratios ($P = 0.044$, Table 2, Figure 2). Analysis by the COX model showed that Bax expression above the median, high levels of Bax and Bad, and high Bcl-2/Bax ratios were independent prognostic factors of adverse outcome and correspond to a 2.40-fold, 2.80-fold and 2.62-fold RR, respectively, of tumor-related death (Table 4, Figure 2). Mdm-2 mRNA levels had only borderline impact on survival after treatment ($P = 0.098$, Figure 2).

Since the response to ICT is an important prognostic factor in itself, the groups of complete responders and non-responders were analyzed separately. Bax and Bad expression influenced survival only within the NR group. NR expressing high levels of Bax, Bad or high levels of Bax and Bad had a much worse prognosis than patients with low expression, both at diagnosis ($P = 0.001$, $P = 0.01$ and $P = 0.002$, respectively) and after ICT ($P = 0.001$, $P = 0.045$ and $P = 0.004$). The corresponding RR values are 4.97, 3.42 and 6.14 respectively, at the time of diagnosis and 9.62, 3.46 and 10.82, respectively, after ICT (Table 5, Figure 3). Except in one patient, failure to achieve CR together with high expression of Bax at diagnosis predicted survival of less than 12 months, whereas none of the group characterized by high Bax and Bad levels survived the first 9 months after ICT start (Figure 3). In contrast, a similar tendency but no statistical significance was observed in patients who achieved CR (Figure 3). Consequently, the strongest parameter for predicting poor prognosis in NR is the combination of increased Bax and Bad levels.

In patients achieving CR Bad and Bax levels were not correlated with outcome. However, patients with high Bcl-2/Bax and Bcl-x_L/Bad ratios after treatment were characterized by a 2.3-fold and 2.5-fold increased RR to die from their leukemia (Table 5).

Discussion

The aim of our study was to determine whether mRNA levels of the apoptosis-regulators Mdm-2, Bcl-2, Bcl-x_L, Bad, and Bax could serve as independent parameters to predict clinical outcome in AML. The semi-automated high-throughput methodology used in our study allows for a much more rapid quantitation (up to 200 samples per day and method) of expressic of apoptosis-related genes than would be possible by traditional techniques such as quantification at the protein level by Western blotting,[9,35,36] immunohistochemistry,[6,8,13,15,37] or flow cytometry.[36] We show that mRNA expression of several genes has a distinct prognostic value at the time of initial diagnosis (eg Bad, Bax) as well as after completing ICT (eg Bax, Bad, Bcl-2/Bax ratio, Mdm-2).

The clearest correlation exists between high expression of the proapoptotic genes Bad and Bax and poor prognosis. This is surprising since one might expect an inverse correlation, at least at the protein level.[25,28,37] Remarkably, the combination of high Bad and Bax values appear to be as strong predictive parameters as the conventional risk factors, relapse and treatment protocol, but with the considerable advantage of being prospectively available. Regarding Bax, similar observations were made in non-Hodgkin's lymphoma,[15] acute lymphoblastic leukemia (ALL),[38] breast,[13] pancreatic[4] and ovarian carcinomas.[8] Our findings are also consistent with the observation that patients with small-cell lung cancer and Bax-positive/Bcl-2-negative tumors have a poor prognosis,[7] and that higher ratios of Bcl-2/Bax correlate with failure to achieve CR in AML[39–41] or chronic lymphocytic leukemia (CLL).[24] In contrast, increased Bax protein expression was found to be

Table 5 Transcript amounts and ratios correlated with survival of patients divided into non-responders (NR) and complete responders (CR)

Transcript or ratio	*NR*				*CR*			
	At diagnosis		*After ICT*		*At diagnosis*		*After ICT*	
	P	*RR*	*P*	*RR*	*P*	*RR*	*P*	*RR*
Treatment protocol	0.08[a]	3.14[a]	0.09[a]	11.36[a]	0.10[a]	1.78[a]	0.22[a]	2.04[a]
Relapsed *vs de novo*	0.18[a]	3.41[a]	0.04[a]	22.50[a]	0.14[a]	1.90[a]	0.11[a]	2.88[a]
Secondary *vs de novo*	0.43[a]	1.71[a]	0.07[a]	5.35[a]	0.30[a]	1.46[a]	0.23[a]	1.76[a]
Bax	0.001	4.97	0.001	9.62	0.41	1.26	0.39	1.41
Bad	0.01	3.42	0.045	3.46	0.21	1.45	0.84	1.08
High Bax + Bad	0.002	6.14	0.002	10.82	0.15	1.55	0.36	1.59
Bcl-2/Bax	0.67	1.25	0.38	1.80	0.73	1.10	0.05	2.26
Bcl-x_L/Bad	0.56	1.32	0.70	1.23	0.91	1.03	0.04	2.52

P and RR values obtained from multivariate Cox regression analysis correspond to either high expression levels or high transcript ratios compared with low expression levels or ratios, respectively. High levels were always associated with poor prognosis. Bcl-2, Bcl-x_L and Mdm-2 data were always not significant.
[a]Mean values obtained from the Cox analysis of each gene expression parameter assessed separately for its impact on prognosis.

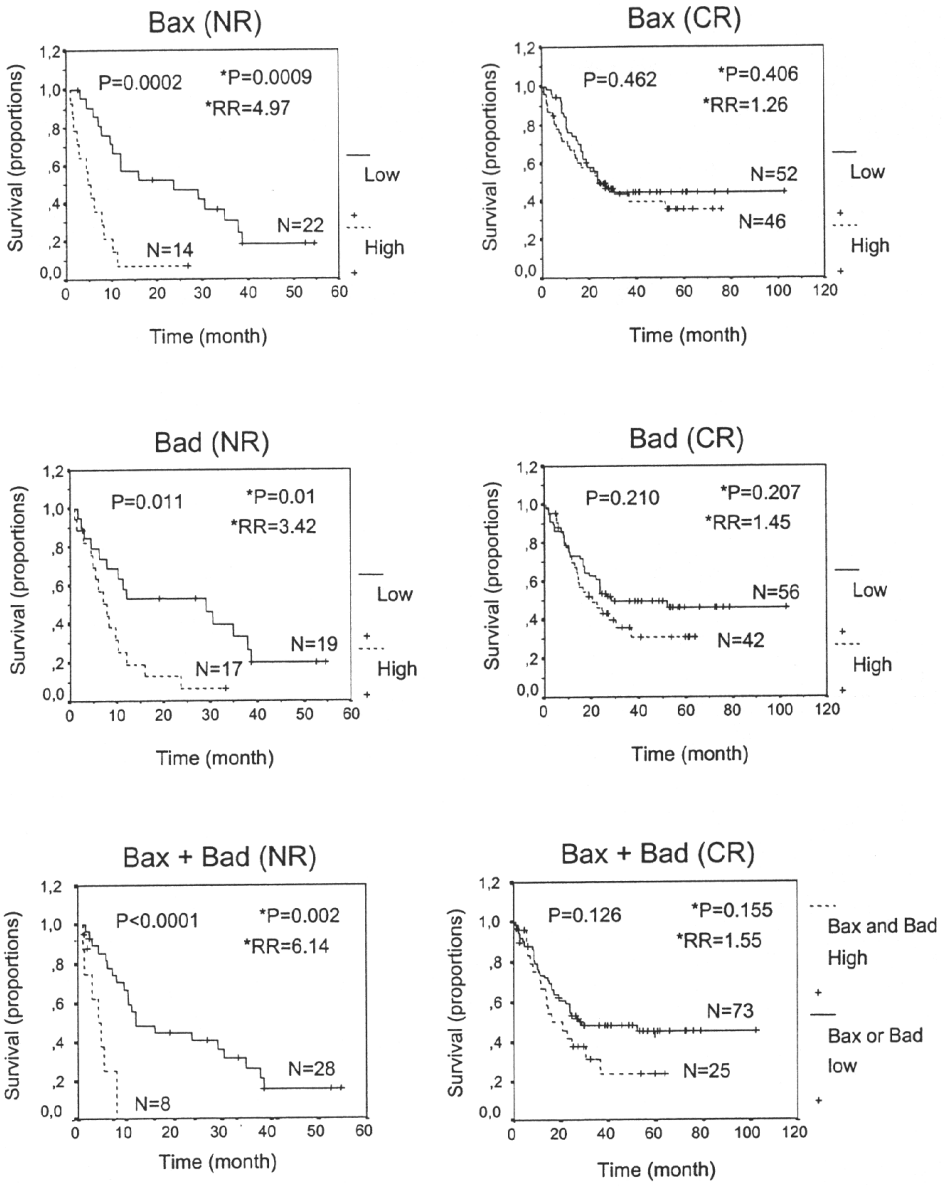

Figure 3 Bax, Bad and interacting Bax and Bad expression at diagnosis and response to treatment. Kaplan–Meier survival analysis and log-rank test separately performed for NR and CR AML patients expressing high or low mRNA amounts of Bax, Bad, or high Bax and Bad compared with low Bax or Bad levels, respectively. *Data confirmed by multivariate Cox regression analysis.

correlated with improved *in vitro* survival of CLL cells,[24] cervical,[12] pancreatic,[4] low-grade urinary bladder cancer,[11] esophageal squamous cell carcinoma[25] and hepatic metastases of colorectal cancer.[6] It should be stated here that in a significant number of studies only the Bax-alpha isoform was characterized,[3,25,42] whereas the assay used in this study detects the majority of human alternative spliced Bax mRNA isoforms (ie alpha, beta, delta, sigma, zeta) except gamma and epsilon. A recent study found no correlation to prognosis in AML at diagnosis.[41]

Furthermore, an important observation is that the uniform NR group may be subdivided into a high and low risk subgroup based on Bax or Bad expression at diagnosis as well as after ICT. Surprisingly, this correlation was restricted to refractory patients. It is not clear why high Bax mRNA levels at diagnosis are not predictive of poor outcome if patients achieve a CR. One possible explanation is that if ICT fails to eliminate the clone that expresses high levels of Bax and Bad, all subsequent attempts will also fail to achieve this. Alternatively, one could speculate that the physiological function of Bax in an AML cell prior to chemotherapy is different from its function after the cell has evolved under the selective pressure of cytotoxic agents. An explanation for the unexpected correlation between high Bax and Bad mRNA levels and poor prognosis may be that transcripts may not necessarily reflect protein levels. This is, however, somewhat unlikely because a significant number of studies that investigated protein levels yielded results very similar to ours.[7–9,13,15] On the other hand, overexpression of Bax may be the result of mutations in the DNA-binding motif of p53; such mutations have recently been demonstrated in non-small-cell lung cancer.[43] p53 mutations have also been reported in a significant proportion of AML[44] and we are currently investigating whether such mutations are correlated with high levels of Bax. Another possible expla-

nation is loss-of-function mutations of Bax, which have been found in 21% of human hematopoetic cell lines, most commonly in ALL subset[45] and some gastrointestinal cancers.[46] These mutations may affect the capability of Bax to dimerize,[45] and may result in a compensatory increase of Bax expression. To our knowledge, no data with regard to Bax mutations in AML are available. Since Bax plays a role in the control of proliferation as well as apoptosis,[18,47] a third explanation for the relationship between Bax overexpression and poor prognosis could be that its major function has shifted towards enhancement of cell proliferation.[9,18] Because Bad is able to displace Bax from Bcl-x_L[48] its simultaneously high-level expression may further enhance the putative proliferative effect of Bax.

Little is known from the literature that could explain the potential physiological role of increased Bad mRNA levels and their strong correlation with poor survival in AML. Interestingly, in CLL, higher levels of the anti-apoptotic proteins Mcl-1 and Bag-1, which are also death ligands, were strongly correlated with failure to achieve CR after chemotherapy.[14] The Bad protein has been shown to be rapidly inactivated by serine-phosphorylation on residues 112 and 136 followed by its sequestration in the cytosol by 14–3-3 proteins.[18] This inactivation can be induced by survival-promoting cytokines such as IL-3 which lead to the activation of kinases like Akt and Raf-1 that phosphorylate Bad.[18,49] Interestingly, up to 90% of AML tumor cells appear to secrete IL-1β, which can induce cells in the microenvironment to produce IL-3.[50] Therefore, up-regulation of proapoptotic Bad may be an attempt to compensate for increased post-translational inactivation of the protein. In line with this concept, a recent publication demonstrated that in 40 out of 41 AML samples the Bad protein was phosphorylated on both possible residues.[51] Whether this is the case in our samples is currently under investigation.

In summary, our data provide a rationale to use Bad and Bax transcript levels as independent predictive factors to assess prognosis in AML patients at diagnosis as well as after ICT. If confirmed in a prospective study, measurement of certain apoptosis-related genes could guide risk-adapted therapy to aid treatment stratification in the future.

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