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Bcl-2 family members in childhood acute lymphoblastic leukemia: relationships with features at presentation, *in vitro* and *in vivo* drug response and long-term clinical outcome

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We have found that, in addition to Bcl-2 and Bax, the expression levels of apoptosis inducers (Bad, Bak) and inhibitors (Bcl-x_L, Mcl-1) were highly variable in blasts from 78 children with newly diagnosed acute lymphoblastic leukemia (ALL). The patients were enrolled in the national study ALL-7 of the Dutch Childhood Leukemia Study Group. In contrast to Bcl-2 that inversely correlated with %S-phase cells and WBC, and was lower in T than in B-lineage ALL, the Bcl-2 family members were not found to be associated with features at presentation. These expression levels were also compared with drug resistance in *in vitro* MTT (methyl-thiazol-tetrazolium) assays for prednisolone, vincristine and asparaginase in 46 children. Protein expression levels of the Bcl-2 family were not found to correlate with *in vitro* resistance to the individual drugs or the combined drug resistance profile. In addition, neither peripheral blast reduction after 1 week of prednisone monotherapy nor long-term disease-free interval or survival showed a correlation with protein expression. Our results indicate that the anti-proliferative function of Bcl-2 dominates its anti-apoptotic function in ALL, but neither Bcl-2 nor the Bcl-2 family members gained prognostic information in the risk-adapted protocol ALL-7.

Keywords: acute lymphoblastic leukemia; Bcl-2 family; apoptosis; drug resistance

Introduction

The (proto)oncogene Bcl-2 was initially identified because of its involvement in the chromosomal translocation t(14;18), which is frequently found in follicular B cell lymphomas.[1,2] Bcl-2 contributes to the expansion of (pre) neoplastic cells, by promoting cell survival rather than by stimulating proliferation.[3,4] In fact, recent studies indicate that Bcl-2 can inhibit proliferation by retardation of the cell cycle entry,[5–9] which is in line with clinical observations that high Bcl-2 associates with slowly progressing disease and a low percentage of S-phase cells in some malignancies, notably lymphoma.[10] Bcl-2 promotes survival by antagonizing spontaneous programmed cell death (apoptosis). In addition, Bcl-2 protects against apoptosis induced by several cytostatic drugs and elevated levels of Bcl-2 are therefore considered to confer a special form of multidrug resistance.[4] Bcl-2 functions in the context of the Bcl-2 family consisting of members with apoptosis inhibiting and inducing activities that can form hetero- and homo-dimers.[3] Bax, Bad, Bak, and Bcl-x_s (a rarely detected protein product of the shorter splice variant of *bcl-x*$_L$) are proteins with apoptosis-inducing functions, whereas Bcl-2, Bcl-x_L and Mcl-1 show apoptosis-inhibiting actions. The role of the Bcl-2 family in cancer is not precisely known, but clinical data indicate that at least the Bcl-2 level is a marker of poor prognosis for cancers arising from different cell types, including acute myeloid leukemia.[4,11]

Bcl-2 is highly variable among cases with newly diagnosed childhood acute lymphoblastic leukemia (ALL).[12–14] Despite the fact that Bcl-2 expression levels in cell lines modulate the sensitivity to most clinically relevant drugs, the pretreatment Bcl-2 levels do not predict 2.5-year survival rates in ALL or the *in vitro* response to cytostatic treatment.[12,13] It has been speculated that the balance of apoptosis-inducers *vs* inhibitors decides whether or not a cell survives upon apoptotic stimuli.[15,16] We therefore studied the prognostic value of protein expression levels of Bcl-2 family members in newly diagnosed ALL patients enrolled in the Dutch study ALL-7. To this end, we have tested whether correlations exist between expression levels of Bcl-2 family members and clinical or cell biological features at presentation. In addition, we have investigated whether there was a correlation between the pretreatment expression levels of the Bcl-2 family and drug response at three levels; (1) *in vitro* drug resistance for prednisolone (PRD), vincristine (VCR) and l-asparaginase (ASP) and its combination; (2) *in vivo* response at day 8 after 1 week monotherapy with prednisone; and (3) long-term clinical outcome.

Materials and methods

Patients

The study panel involved newly diagnosed children with ALL, who were treated according to protocol ALL-7 of the Dutch Childhood Leukemia Study Group (DCLSG), accrual period from July 1988 until December 1991,[17] after informed consent was obtained from the parents. The treatment protocol was identical to the Berlin–Frankfurt–Münster (BFM) protocol ALL-BFM86 (but without cranial irradiation in CNS prophylaxis), which included 7-day monotherapy with prednisone (60 mg/m^2/day) and one intrathecal injection with methotrexate (MTX) at day 1 of treatment.[17,18] Subsequently the patients were stratified into one of the three risk-groups: standard risk (SR) if the BFM risk factor (RF or leukemic mass, based on WBC, liver and spleen size) was <0.8, excluding patients with mediastinal mass, T-ALL, extramedullary disease and/or features defined for the experimental group (EG); EG in case of immunophenotypically acute undifferentiated leukemia (AUL), poor response to prednisone monotherapy ($\geqslant$1000 blasts/μl in peripheral blood (PB)) at day 8, and/or no complete remission (CR) after 33 days of induction therapy, and risk group (RG), ie all cases excluding the ones that were included in the SR or EG groups. Patient characteristics and

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cell biological variables are detailed in Table 1. Mononuclear cells were isolated as described previously[14] at the DCLSG. Analysis of immunophenotype was centrally determined by the DCLSG.[17,19] The remaining blasts were cryopreserved at the DCLSG. Determination of hyperdiploidy (ie DNA index of $\geqslant$1.16 and $<$1.35) and of the fraction of S-phase cells (%S) were performed at the Netherlands Cancer Institute as described previously.[20] Karyotyping was performed in the laboratories of the university hospitals as described.[21] From a total of 218 study patients, 78 cryopreserved bone marrow (BM) (n = 70) or PB (n = 8) samples contained above 80% of blasts and were available to study the expression levels of Bcl-2 family proteins.

Detection of Bcl-2 family members

Bcl-2 family members were assessed by Western blotting in duplicate experiments. A third experiment was performed if the variation between duplicates was greater than 20%. Not all patient samples could be tested for Bad, Bak, Bcl-x_L and Mcl-1 because of insufficient material. The protein levels were determined in samples of 2×10^5 cells boiled in Laemmli sample buffer. Protein lysates were separated on a 12–14% SDS-polyacrylamide gel (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) by electrophoresis for 3 h at 1000 V, 40 mA, 10 W at 15°C using the Multiphorll flatbed electrophoresis system (Amersham Pharmacia Biotech, Uppsala, Sweden) and transferred to Immobilon PVDF membranes (Millipore, Bedford, MA, USA) in 1 h using a semi-dry transfer apparatus (Pharmacia). The membranes were dried at room temperature and cut into two sections. The section containing proteins $<$35 kDa was either used to detect Bcl-2/Bax (DAKO: clone 124, Santa Cruz Biotechnology: sc-493) as described previously[14] or used to detect either Bcl-x_L (sc-634), Bak (sc-832) or Bad (Transduction Laboratories: 36420), while the section containing proteins of $>$35 kDa was used to detect actin (Boehringer Mannheim Biochemica, Mannheim, Germany: clone C4) for normalization or Mcl-1 (sc-819). Because actin and Mcl-1 migrate at the same molecular weight (42 kDa), equal loading was determined separately in an identical gel. Primary antibody incubations were performed as previously described but without a preceding blocking step.[14] Secondary antibodies were ^{125}I sheep-anti-whole-mouse Ig and ^{125}I donkey-anti-whole-rabbit Ig (Amersham International, Buckingham, UK). Protein levels were quantitated with a phosphor imager. In each blot the levels of Bcl-2 family proteins were compared to those in the reference DoHH2 cells, a follicular non-Hodgkin's B cell lymphoma that harbours the t(14;18). The protein levels and the Bax to Bcl-2 ratio in DoHH2 cells were all arbitrarily set at 1.0. The values for Bcl-2, Bax, Bad, Bak, Mcl-1 and Bcl-x_L in normal peripheral blood lymphocytes (PBL) were determined from a minimum of nine healthy adults.

Table 1 Characteristics of study patients (n = 86)

Variable	*Mean, s.d. (median; min-max)*
Age in years	5.7, 3.7 (4.5; 0–15)
Sex	boys, 43 girls, 35
WBC $\times 10^9$/l	106, 165 (41; 2.5–729)
Immunophenotype	T lineage, 18 B lineage, 60: common, 40, including 6 pro B pre B, 20
Risk factor (leukemic mass)	1.14, 0.36 (1.12; 0.47–2.07); <0.8 (14), >0.8 (64)
Risk group classification	SR, 13 RG, 56 EG, 9
Splenomegaly	no, 25 yes, 53
Hepatomegaly	no, 19 yes, 59
Lymphnodes	negative, 64 positive, 14
Ploidy (n = 80)	non-hyperdiploid, 63 hyperdiploid, 12
%S-phase BM (n = 75)	10.2, 4.8 (10.5; 2–23.5)
%S-phase PB (n = 60)	5.9,3.9 (5; 0–16)

In vitro drug resistance

The *in vitro* drug resistance profiles for PRD, VCR and ASP using the cell culture MTT (methyl-thiazol-tetrazolium) assay, were performed at the University Hospital Vrije Universiteit.[19] Drug resistance was expressed by the drug concentration lethal to 50% of the cells (LC_{50}).

Statistical analysis and treatment outcome in 78 patients

A full account of the outcome of study ALL-7 will be published by the Dutch Childhood Leukemia Study Group.[17] Follow-up for the subset of patients in the present study was closed at the end of 1996. Median follow-up was 62 months. CR was defined as less than 5% leukemic blasts in BM aspirates at day 33. For the disease-free interval (DFI), patients without CR (DFI = 0) or with a relapse were counted as failures. A stepwise procedure using proportional hazard (PH) regression analysis[22] was used to identify prognostic factors (Table 1) with respect to DFI and survival. Time was measured from the date of diagnosis. Life-table calculations were performed using the product-limit method of Kaplan and Meier.[23] SEs were calculated according to the method of Peto *et al*.[24]

The correlations between Bcl-2 family members and immunologic subtype, ploidy, risk group classification, spleen or liver involvement and *in vitro* drug resistance were analyzed using one-way analysis of variance, after ln-transformation of the marker values. The drug response was classified as sensitive, intermediate-sensitive and resistant as was defined in the previous study.[19]

All statistical calculations were performed using SAS6.12 for windows.

Results

Bcl-2 family members are biological variables in ALL

The values of the Bcl-2 family members were successfully determined by quantitative Western blotting in blasts of 78 ALLs. Of these, one sample was discarded because of degradation of actin and Bcl-x_L and two other samples because of extensive amounts of necrotic/apoptotic cells. Figure 1 illustrates the high variation of Bcl-2, Bax, Bcl-x_L, Bak, Mcl-1 and Bad among ALL cases as summarized in Table 2. Actin and

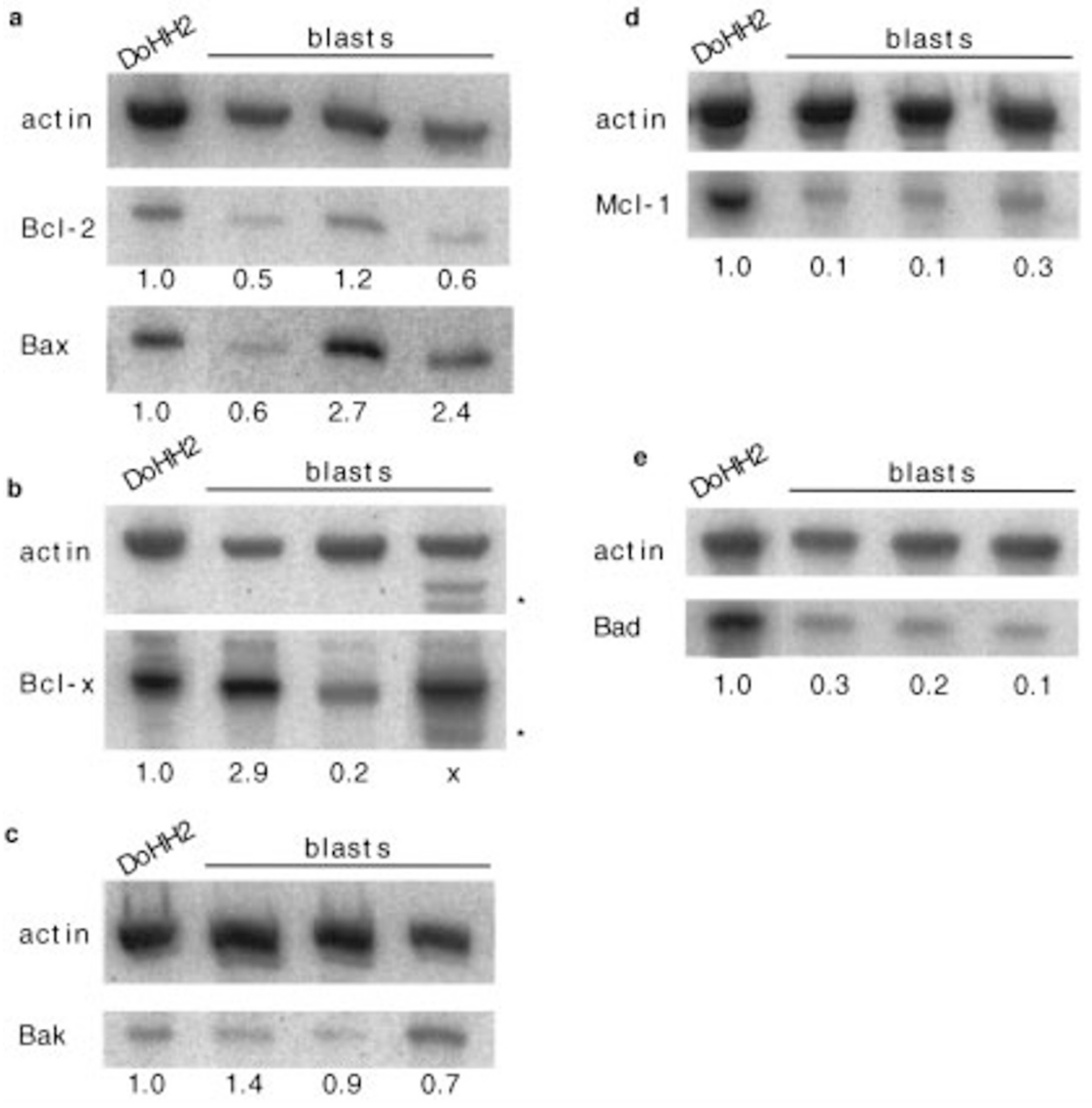

Figure 1 Western blots illustrating the highly variable expression levels of the Bcl-2 family members Bcl-2 and Bax (a), Bcl-x_L (b), Bak (c), and the lower, but also variable expression levels of Mcl-1 (d) and Bad (e) in blasts of newly diagnosed ALL. In each panel blasts from different patients are used to demonstrate the variable expression levels for each protein. The protein values were determined by normalization for actin and were expressed relative to DoHH2 reference cells that were arbitrarily set at 1.0 for each protein as depicted in the Figures. One patient was excluded from the study because of degradation of both actin and Bcl-x_L as indicated by the asterisks (*) in panel b.

Table 2 High variation in the expression levels of Bcl-2 family members among cases with newly diagnosed ALL

Protein	*Geometric mean (CoV)*	*range*	*n*	*Normal PBL*
Bcl-2	0.81 (62%)	0.11–2.46	75	0.2
Bax	0.76 (65%)	0.06–2.67	75	0.26
Bak	1.19 (78%)	0.16–19.9	66	0.39
Bad	0.28 (61%)	0.07–1.36	72	0.09
Bcl-x_L	0.48 (69%)	0.13–6.83	66	0.11
Mcl-1	0.19 (117%)	0.01–1.05	60	0.18
Bax:Bcl-2	0.94 (84%)	0.09–7.68	75	1.3

Bcl-x_L degradation is illustrated (Figure 1b) in the one sample that was excluded from this study as mentioned above. Bcl-x_L, Bak, Bcl-2 and Bax showed a similarly large variation in expression levels (Figure 2b and c). Bcl-x_L varied from 0.13–6.83 times that of the follicular lymphoma reference cells (see below: Table 2 and Figure 1) with a geometric mean of 0.48 and a CoV of 69%. Bcl-x_L in normal PBL was 0.11 (CoV = 36%). Bak varied from 0.16–19.9 with a geometric mean 1.19 and a CoV of 78% and was 0.39 (16%) in normal PBL. In the present study, Bcl-2 and Bax varied 0.11 to 2.46 and 0.05–2.67 with a geometric mean of 0.81 (CoV = 62%) and 0.71 (CoV = 65%), respectively. In normal lymphocytes these Bcl-2 and Bax levels were 0.20 (CoV = 60%) and 0.26 (CoV = 49%), respectively. Mcl-1 and Bad were also variable (0.01–1.10 and 0.07–1.36, respectively) but the levels were usually low (Figure 2d and e) and the geometric means (Mcl-1: 0.19,

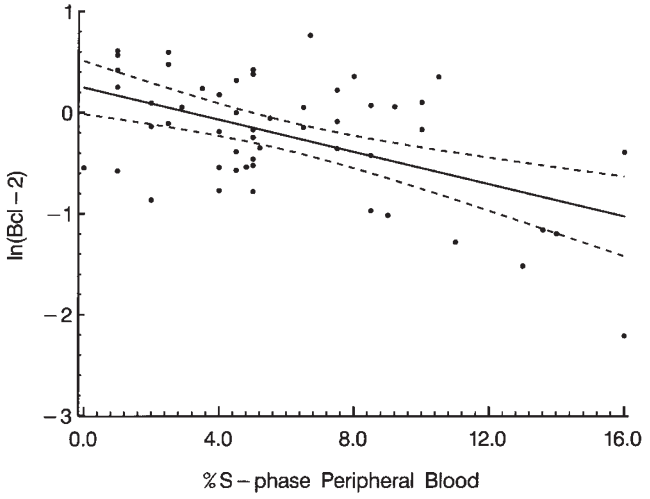

Figure 2 Bcl-2 level inversely correlates with the %S-phase cells in ALL. The Bcl-2 levels were correlated with the %S phase cells in peripheral blood by linear regression analysis after logarithmic transformation of Bcl-2. The correlation coefficient (*r*) was –0.52, with $P < 0.0001$. In the Figure both the regression line (solid line) and its 95% confidence interval (broken lines) are drawn.

CoV 117%, Bad: 0.28, CoV 61%) were more in the range of those found in normal lymphocytes: Mcl-1 0.18 (72%) and Bad 0.09 (79%).

Bcl-2 family members do not associate with clinical features in contrast to Bcl-2

We also tested whether these protein levels associated with the cell biological and clinical features as described Table 1. No correlations were found between these variables and Bcl-xL, Bad, Mcl-1, Bak, Bax and the Bax to Bcl-2 ratio. By contrast, our studies (Table 3) confirmed reported correlations between Bcl-2 and immunophenotype and WBC.[12,14] However, this study is the first to reveal in ALL a significant inverse correlation between Bcl-2 levels and the percentage S-phase cells in both PB ($P < 0.0001$, $r = –0.52$; Figure 2, Table 3) and BM blasts ($P = 0.0044$, $r = –0.34$). In PB the determination of %S may be complicated by contaminating normal cells. However, this does not apply to BM samples, containing at least 80% blasts. In addition a weak correlation was found for Bcl-2 and high risk factor ($P = 0.016$) and hyperdiploidy ($P = 0.048$).

Table 3 Correlations of Bcl-2 with presenting features and cell biological characteristics in newly diagnosed childhood ALL

Feature	*Bcl-2 level*	*P value*
High WBC[a]	low	0.0017
Immunophenotype:[b]		
T-lineage	low	<0.0001
B-lineage	high	
High risk factor (leukemic mass)	low	0.016
Risk group classification (SR, RG, EG)[b]	no difference	0.18
Spleen involved (yes/no)[b]	no difference	0.48
Liver involvement (yes/no)[b]	no difference	0.43
Lymphnodes (positive/negative)[b]	no difference	0.17
High %S-phase cells in:[a]		
BM	low	0.0044
PB	low	<0.0001
Hyperdiploidy[b]	high	0.048

The *P* values were considered significant at the $P \leqslant 0.01$ level, and weakly significant if larger but $P \leqslant 0.05$ because of the large amount of performed statistical tests.
[a]Correlations were tested by comparing absolute values using linear regression after ln-transformation of Bcl-2 and WBC, but not of %S-phase cells or leukemic mass.
[b]Correlations between Bcl-2 and variables were tested with the analysis of variance after ln-transformation of Bcl-2.

Relationship between the expression levels of Bcl-2 family members and in vitro and in vivo drug response and clinical outcome

In a previous study of 152 newly diagnosed ALLs we showed that the combined *in vitro* data of PRD, VCR and ASP provided a drug resistance profile with independent prognostic significance.[19] We also reported a significant relation between *in vitro* PRD resistance and the clinical response to the 1 week prednisone monotherapy, and a significant *in vitro* cross resistance between all 13 drugs tested in 166 children with newly diagnosed ALL.[25] In the present study no relationship could be found either between Bcl-2 expression levels and the *in vitro* drug resistance for PRD ($n = 46$), VCR ($n = 45$) and ASP ($n = 39$) alone, or with the combined data ($n = 39$). These relationships were tested with the one-way analysis of variance, yielding $P = 0.88$, 0.92, 0.11 and 0.13, respectively. In addition no relationship between the other members of the Bcl-2 family or the Bax:Bcl-2 and the *in vitro* drug resistance profile was found. In view of the small size of the study panel this conclusion should be interpreted with caution. However, it is of note that the subgroup of 39 patients can be considered as a proper representation of the entire study population of 152 cases[19] because the prognostic value of the combined resistance data in the total population was confirmed for this subgroup by the proportional hazard regression analysis (data not shown).

Seventy-six out of 78 patients received 1 week prednisone monotherapy according to the ALL-7 study protocol. No differences between poor responders ($n = 7$) and responders ($n = 69$) were observed with respect to the expression levels of the individual Bcl-2 family members or of Bax:Bcl-2.

For the subpopulation of 78 out of 218 ALL-7 study patients, the proportional hazard regression analysis was used to study whether or not one of the Bcl-2 family members, clinical or cell biological characteristics, as described in Table 1, could be considered as a prognostic factor with respect to DFI and survival. Although in study ALL-7 statistical analysis showed no prognostic value of WBC,[17] in this subset proportional hazard regression analysis revealed that WBC was prognostic for survival ($P = 0.0045$) but not for DFI. No evidence was found for a prognostic value of Bcl-2 (DFI: $P = 0.57$; survival: $P = 0.11$ adjusted for WBC), Bax:Bcl-2 ratio (DFI: $P = 0.61$; survival: $P = 0.17$ adjusted for WBC) or of the other Bcl-2 family members. Figure 3 gives the DFI and survival curves according to the Bcl-2 levels (Figure 3a and c, respectively) and the Bax:Bcl-2 ratio (Figure 3b and d, respectively). In addition, when adjusted for WBC, no combination of the biological markers was found that could predict survival.

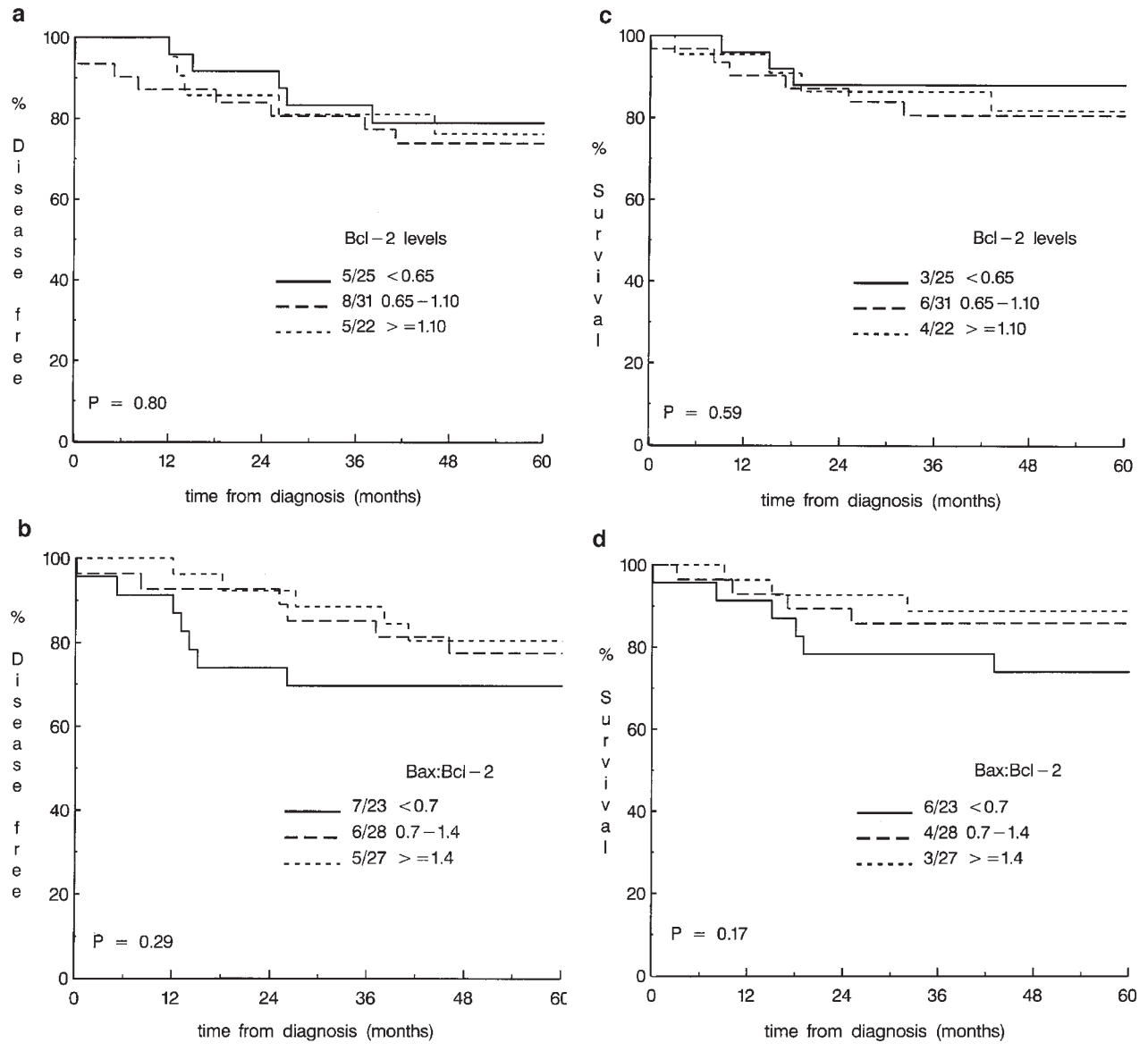

Figure 3 Disease-free interval and overall survival according to Bcl-2 expression levels (a, c) and the Bax:Bcl-2 (b, d) in blasts from newly diagnosed ALL. Patients were divided in three approximately equal-sized groups as indicated in the Figure. Bcl-2 level and the Bax:Bcl-2 ratio did not show evidence for an effect on DFI or survival rates.

Discussion

It has been reported that in newly diagnosed childhood ALL, Bcl-2 levels do not correlate with clinical outcome[12] or *in vitro* drug resistance.[13] We therefore addressed the question whether in addition to Bcl-2, the expression levels of apoptosis-inducers (Bax, Bad, Bak) and inhibitors (Bcl-2, Bcl-x_L, Mcl-1) are variable between patients and, if so, whether these markers correlated with features at presentation, *in vitro*, and *in vivo* drug response and long-term clinical outcome.

We found a marked variation in the expression level of Bcl-2, Bax, Bak, Bcl-x_L, Mcl-1 and Bad, although the latter two showed usually low expression and were more in the range of normal PBL. Bax, Bad, Bak, Bcl-x_L and Mcl-1 were not found to correlate strongly with the features at presentation. We confirmed an inverse correlation between Bcl-2 and WBC, as well as a relation with immunophenotype.[12,14] In addition a weak correlation was found between Bcl-2 and high RF and hyperdiploidy. More importantly, this is the first study that reveals an inverse relationship in ALL between Bcl-2 and %S phase cells, suggesting that Bcl-2 has an adverse effect on the rate of proliferation of leukemic blasts. Recently, a similar correlation between Bcl-2 and %S phase cells was observed in lymphoma.[10] These anti-proliferative effects may explain the observation of Uckun *et al*[12] that the growth of ALL blasts in SCID mice was inhibited by elevated levels of Bcl-2 and are also in agreement with the proliferation-suppressing effect of Bcl-2 in cell lines[7,26,27] and thymocytes from transgenic mice.[5,6,8] Thus ALL can be grouped among malignant disorders in which high levels of Bcl-2 are associated with slowly progressing, indolent disease.[28,29] In the case of ALL, a low percentage of S phase cells has been associated with superior survival in earlier studies.[20]

The combined *in vitro* response to PRD, VCR and ASP, as measured with the MTT assay, provides a drug-resistance profile with prognostic independent significance.[19] No relation-

ship between each of the Bcl-2 family members or for Bax:Bcl-2, and the response to any of these drugs or their combination was identified in 41 unselected ALLs. Our data confirm a former study that failed to establish a relation between Bcl-2 levels and resistance to seven drugs, including dexamethasone and VCR.[13] It has been argued that it is not the pretreatment levels of the Bcl-2 family but the capacity of a cell to regulate these proteins upon apoptotic stimuli that is crucial to sensitivity, eg after ionising radiation.[30] Although this has not been addressed in the present study, it is of note that in acute myeloid leukemia elevated pretreatment levels of Bcl-2 do correlate with a poor prognosis[11] and that in leukemia and lymphoma cell lines, as well as in primary thymocytes, clinically relevant pretreatment settings of the Bax:Bcl-2 modulate sensitivity to various p53-independent apoptotic stimuli, including dexamethasone.[14,31]

The individual Bcl-2 family members nor the Bax:Bcl-2 could predict the *in vivo* response in 76 patients to 1 week of PRD monotherapy. Although in agreement with the *in vitro* findings, the absence of a correlation with Bcl-2 remains puzzling in view of the frequently reported straightforward correlation between Bcl-2 (and the Bax:Bcl-2) and sensitivity to GC and because, contrary to genotoxic drugs, apoptosis is the only known mode of GC-mediated cell death. As yet, the small number of poor responders does not allow definitive conclusions. However, it can not be excluded that these few patients were resistant due to other, Bcl-2-independent mechanisms, eg by deficiencies in hormone binding and receptor activation.[32–34]

We and others did not find an independent relationship between Bcl-2 and treatment outcome.[12,13] In addition, the present study indicates no gain in prognostic information by including Bax, Bak, Bcl-x_L and Bad. Larger studies may elucidate significant correlations but such studies will most likely not result in information relevant for the individual patient. Although a moderate increase of Mcl-1 in relapsed leukemia has been observed,[35] no correlation between pretreatment levels of this protein and DFI or survival was found in the present study.

In conclusion, apart from Bcl-2, the Bcl-2 family does not gain insights in ALL. Contrary to findings in cell lines no evidence was found for an important role of the Bcl-2 family in drug resistance. Elevated levels of Bcl-2 correlate with low WBC, low %S-phase cells, B-lineage ALL, and possibly with hyperdiploidy and leukemic mass, but did not predict treatment outcome. These factors were previously associated with a favorable response.[20] However, recent protocols have greatly diminished these associations, including in the risk-adapted protocol ALL-7.[17] The latter is in agreement with the tendency that the impact of prognostic markers decreases as treatment improves.[36] The present study indicates instead that Bcl-2 is anti-proliferative in ALL, a propensity which may dominate over its anti-apoptotic capacity in determining the response to cytostatic treatment.

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