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Relapse in childhood acute lymphoblastic leukemia is associated with a decrease of the Bax/Bcl-2 ratio and loss of spontaneous caspase-3 processing *in vivo*

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Dysfunction of the p53/Bax/caspase-3 apoptosis signaling pathway has been shown to play a role in tumorigenesis and tumor progression, ie the development of acquired drug resistance. Low expression of the apoptosis inducer Bax correlates with poor response to therapy and shorter overall survival in solid tumors. In the present study, we analyzed the p53/Bax/caspase-3 pathway in a paired and an unpaired sample series of children with acute lymphoblastic leukemia (ALL) at initial diagnosis and relapse. The data demonstrate that both Bax expression levels and the Bax/Bcl-2 ratio are significantly lower in samples at relapse as compared with samples at initial diagnosis ($P = 0.013$, Wilcoxon signed rank test (paired samples); $P = 0.0039$, Mann–Whitney *U* test (unpaired samples)). The loss of Bax protein expression was not a consequence of Bax frameshift mutations of the G_8 tract and could not be attributed to mutations of the p53 coding sequence (exons 5 to 8) which were detected to a similar extent in *de novo* ALL samples and at relapse. Analysis of the downstream effector caspase-3 showed loss of spontaneous caspase-3 processing at relapse. Whereas nine out of 14 (64%, paired samples) or 37 out of 77 (48%, unpaired samples) ALL patients at initial diagnosis displayed spontaneous *in vivo* processing of caspase-3, this was completely absent in patients at relapse (paired samples) or detected in only one out of 34 patients at relapse (2.9%, unpaired samples). We therefore conclude that in ALL relapse a severe disturbance of apoptotic pathways occurs, both at the level of Bax expression and caspase-3 activation. *Leukemia* (2000) **14,** 1606–1613.
Keywords: relapsed childhood acute lymphoblastic leukemia; Bax expression; Western blot; videodensitometry; caspase-3 processing; apoptosis

Introduction

Apoptosis, a morphologically and biochemically defined form of cell death,[1] plays a role in a wide variety of biological systems.[2–4] This process is a highly orchestrated cellular pathway leading to the activation of the death machinery. Although apoptosis can be p53-independent, apoptosis after DNA damage often occurs in a p53-dependent manner[5] which is regulated by members of the Bcl-2 protein family.[6] The central executioner of the death machinery is a proteolytic system involving a family of cysteinyl proteases called caspases (for review see Ref. 7). Due to the hierarchical activation of this proteolytic system it is possible to distinguish between initiator caspases, eg caspase-8 and caspase-9, being at the apex of the apoptotic cascade and executioner caspases, eg caspase-3 and caspase-6. Triggering of the apoptotic programme by different DNA-damaging death stimuli, such as ionizing radiation[8] and chemotherapeutic drugs,[9] culminates in specific cleavage of a set of regulatory proteins, degradation of cellular DNA and complete disassembly of the cell.

ALL is the most frequent malignant disease in childhood. After therapy, relapse occurs in about 25% of patients, and a high proportion of these patient develop incurable, chemoresistant disease. There is considerable evidence that the inactivation of apoptosis signaling pathways is a central event in the development of cytotoxic drug resistance. Antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xL, and death-promoting members, such as Bax, Bak and Bik/Nbk, regulate this form of cell death.[6] In this context, we demonstrated that expression of Bax in breast cancer cells[10] and Bik/Nbk in corticosteroid-resistant T cell lymphoma[11] increased the sensitivity of these cells to cytotoxic drugs. Analysis of the p53/Bax pathway in colorectal cancer revealed that loss of Bax expression is a frequent event in solid tumors[12,13] and a negative prognostic factor for therapy response.[14] This was especially observed in patients with wild-type p53. Clinical resistance to therapy is also associated with an increased expression of Mcl-1 in adult ALL[15] and Bcl-2 expression in acute myeloid leukemia.[16] In contrast, a recent report showed that increased expression of the proapoptotic Bax in childhood ALL at diagnosis is associated with an increased risk of relapse.[17]

The mechanisms by which p53 promotes apoptosis are not fully understood. However, it has been shown that Bax is a transcriptional target for wild-type p53 (for review see Ref. 5). To further investigate the role of members of the p53/Bax apoptosis pathway in the development of relapse in childhood ALL we analyzed changes of this pathway in samples of patients at initial diagnosis and in samples of patients at relapse by quantitative detection of Bax protein expression, frameshift mutation analysis of the bax gene and mutation analysis of p53 by single-stranded conformational polymorphism (SSCP) polymerase chain reaction (PCR). In addition, we extended our study to effector caspase-3 as central executioner of the downstream cell death machinery and determined its expression and processing in childhood ALL.

Materials and methods

Patients

Paired samples containing at least 1×10^7 cells of 14 children with ALL at initial diagnosis between 1986 and 1996 and at relapse between 1992 and 1997 were analyzed in this study. Out of 14 patients, three were female and 11 were male. All patients suffered relapses between 3 months and 6 years after first presentation. The median age at first presentation was 7 years (range 2 to 13 years), and the median age at relapse was 9 years (range 3 to 18 years). The median leukocyte count at initial diagnosis was 35.7×10^9/l (range 10.2 to 270×10^9/l) and the median leukocyte count at relapse 11.2×10^9/l (range

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3.2 to 440 × 10^9/l). The diagnosis was established by immunophenotyping of leukemia cells according to Béné *et al*.[18] Within the group of 14 patients the following immunological subgroups were found: one pro-B cell ALL, eight common-ALL, two pre-B cell ALL and three T cell ALL.

Additionally, unpaired samples of 77 children with ALL at initial diagnosis between 1986 and 1996 and 34 children at relapse between 1992 and 1997 were analyzed in this study. Out of 111 patients, 34 were female and 77 were male. The median age at first presentation was 6 years (range 1 to 16 years), and the median age at relapse was 8 years (range 2 to 18 years). The median leukocyte count at initial diagnosis was 28.6 × 10^9/l and the median leukocyte count at relapse 10.2 × 10^9/l. The diagnosis was established by immunophenotyping of leukemia cells according to Béné *et al*.[18] Within the group of 111 patients the following immunological subgroups were found: 14 pro-B cell ALL, 50 common ALL, 14 pre-B cell ALL, one pre-T cell ALL, 11 T cell ALL and 21 cortical T cell ALL.

Patients with *de novo* ALL in this study had been entered into one of four therapy protocols from the Gesellschaft für Pädiatrische Onkologie und Hämatologie (GPOH) ALL-BFM-83, ALL-BFM-86, ALL-BFM-90 and ALL-BFM-95.[19,20] Relapsed patients received therapy according to the protocols from GPOH ALLREZ-BFM-90 and ALLREZ-BFM-96.[21] According to these therapy protocols, patients were treated with multiagent chemotherapy.

Antibodies

Polyclonal rabbit anti-human caspase-3 antibody (developed against human recombinant protein and recognizing unprocessed procaspase-3 and the 17 kDa subunit of active caspase-3) from PharMingen (Hamburg, Germany) was used at a dilution of 1:1000. Monoclonal anti-human Bax antibody developed against recombinant human bax (clone 4F11) from Immunotech (Hamburg, Germany) was used at 1:100. This antibody specifically recognizes the 21 kDa isoform of human bax (bax-) which is generated by alternative splicing.[22] Control experiments with another monoclonal anti-bax antibody from Trevigen (Gaithersburg, MD, USA) (clone YTH-2D2) recognizing a N-terminal peptide (amino acids 3–16) were performed and both antibodies virtually gave the same results (data not shown). Monoclonal anti-human Bcl-2 antibody developed against the synthetic peptide sequence ^{41}GAAPAP-GIFSSQP^{54}GC-$_{COOH}$ of human bcl-2 (clone bcl-2/100/D5) from Novocastra (Newcastle upon Tyne, UK) was used at 1:100. Secondary anti-rabbit and anti-mouse horse radish peroxidase conjugated antibodies were from Promega (Mannheim, Germany).

Preparation of samples

All samples were bone marrow (BM) material and were processed within 24 h of BM aspiration. Within this time interval no significant changes of expression of the proteins in question were detected in control experiments where samples of the same patient were either processed immediately after aspiration or incubated for 24 h at room temperature followed by the standard processing procedure and cryopreservation of cells described below (data not shown). Lymphoblasts and mononuclear cells were separated by centrifugation over Ficoll. The percentage of leukemic lymphoblasts was above 90% which is in accordance with a former study investigating samples from patients with childhood ALL.[17] Furthermore, the high blast count should not have significant impact on the outcome of the present study since it has been recently shown that white cell count is not related to apoptotic and proliferative activity in childhood ALL.[23] Cells were cryopreserved in liquid nitrogen prior to Western blot analysis.

Preparation of cell free extracts

1 × 10^7 cryopreserved cells were thawed and immediately lysed for 1 h on ice in 100 l buffer L containing 10 mmol/l Tris/HCl, pH 7.5, 2 mmol/l EDTA, 1% Triton X-100, 0.1% sodium dodecylsulfate (SDS) and protease inhibitors pepstatine, leupeptine and phenylmethyl-sulfonylfluoride. The lysates were microcentrifuged at 14 000 *g* and 4°C for 20 min and protein concentration was determined using bicinchoninic acid (Pierce, Rockford, IL, USA).[24] The supernatants were stored at –80°C.

Immunoblotting

15 g protein were subjected to SDS-polyacrylamide gel electrophoresis on 16% polyacrylamide gels according to the method of Laemmli.[25] Paired samples at initial diagnosis and relapse of the same patient were loaded on adjacent lanes. For the unpaired sample series, six samples from patients at initial diagnosis and two samples from patients at relapse were loaded on the same gel. After electrophoresis, immunoblotting was performed as described.[26] Briefly, proteins were transferred to nitrocellulose membranes, the membranes were blocked and incubated with the respective primary antibody for 1 h. Secondary antibody was applied for 1 h and bands were detected using the enhanced chemiluminescence system from Amersham (Braunschweig, Germany). Films were exposed for exactly the same length of time which was optimized for all antibodies used in this study.

The integrated optical density of the resulting bands was determined by densitometric videoscanning[27] using a Gel Doc 2000 apparatus (BioRad, München, Germany). For standardization and interassay comparisons, protein measurements of all samples were performed using the bicinchoninic assay from Pierce and equal amounts of protein (15 g/lane) were loaded on the gel; transfer efficiency of the Western blots was routinely checked by staining the membranes with 0.5% Ponceau red in 1% acetic acid; 15 g of a protein standard from the human Burkitt-like lymphoma cell line BJAB were included on every gel and detection of Bax, Bcl-2 and caspase-3 by the antibodies described in the manuscript was performed one after another on the same membrane. Due to the high specificity of the first antibodies and the switch from monoclonal mouse to polyclonal rabbit antibodies, no overlapping bands were detected on the respective blots. Standard curves of all three proteins were linear in the range of 12 to 20 g total protein (Figure 1). Interblot reproducibility of identical samples was checked and the samples showed a coefficient of variation of 9.0%, 2.0% and 8.3% for Bax, Bcl-2 and caspase-3, respectively.

Mutation analysis for p53 and Bax

After DNA extraction using the InViSorb Genomic DNA Kit II (InViTek, Berlin, Germany), p53 mutations in the DNA bind-

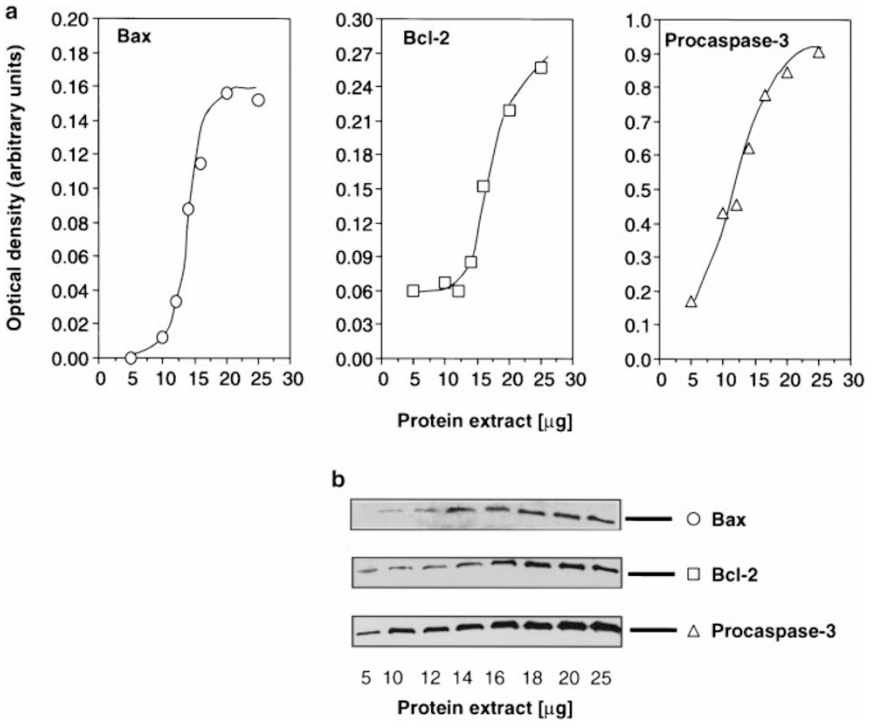

Figure 1 Standard curves for quantitative Western blot analysis of Bax, Bcl-2 and pro-caspase-3 protein expression. Different amounts of protein extract from the human Burkitt-like lymphoma cell line BJAB were loaded on a SDS polyacrylamide gel and subjected to Western blot analysis. Videodensitometric quantitation (a) of the specific bands as shown in the lower part of the Figure (b) was performed as described in Materials and methods. The mean values of optical density of two determinations are given. The experiment was repeated and similar results were obtained.

ing region (exon 5 to 8) and bax mutations in the G_8 tract in exon 3 were investigated by SSCP-PCR analysis (p53) and fragment length analysis (bax). Oligonucleotide primer sequences, PCR conditions and SSCP protocols for p53 and bax mutation analysis were as described elsewhere.[14] For bax mutation analysis, Lovo cells showing bax frameshift mutations on both alleles were used as positive controls in the paired sample series.

Statistical analysis

The levels of caspase-3, Bax and Bcl-2, and the Bax/Bcl-2 ratio of ALL at initial diagnosis and relapse of the paired sample series were analyzed by the Wilcoxon signed rank test. The levels of caspase-3, Bax and Bcl-2, and the Bax/Bcl-2 ratio of ALL at initial diagnosis and relapse of the unpaired sample series were analyzed by the Mann–Whitney *U* test. For the analysis of caspase-3 processing in *de novo* as compared with relapsed ALL, for the analysis of caspase-3 processing in samples with high as compared with low Bax levels and for the analysis of caspase-3 processing in samples with high as compared with low Bcl-2 levels, Fisher's exact test was used. Caspase-3 processing was also analyzed by the ^{2} test and in all cases the Fisher's exact test and the ^{2} test gave similar values of significance (data not shown).

Results

Using SSCP-PCR, we did not detect any p53 mutations in paired samples of 14 children with ALL at initial diagnosis and relapse (data not shown). In an additional series with unpaired samples, we found p53 mutations in seven of 77 patients with *de novo* ALL (9.1%) and two of 34 patients with ALL relapse (5.9%) thereby confirming that p53 mutations are relatively rare in childhood ALL, as has already been shown by others.[28] Furthermore, these data exclude p53 mutation as an indirect cause for impaired Bax expression since p53 mutations did not coincide with low bax expression ($P = 0.321$, Fisher's exact test).

We found no frameshift mutations in the G_8 tract of the bax gene of *de novo* patients either in the paired or in the unpaired sample series (data not shown) thereby confirming that bax frameshift mutations, in contrast to findings in hematopoietic cell lines, are infrequent in primary childhood ALL.[29] Furthermore, no such mutations were detected in either sample series at relapse.

Since low Bax expression is a negative prognostic factor especially in patients with p53 wild type,[14] we determined the Bax- expression in samples of 14 children with *de novo* and relapsed ALL. For this, we developed a method to measure the protein expression by videodensitometric analysis of the respective Western blots. Figure 1 illustrates the linearity of the standard curves for the quantitation of Bax, Bcl-2 and pro-caspase-3 protein expression. As shown in Figure 2a and c, all patient samples with the exception of R14, A10 and R10, contained significant amounts of Bax- (molecular weight: 21 kDa). In parallel, the protein expression of Bcl-2 (molecular weight: 23 kDa) was investigated and high expression levels of this anti-apoptotic factor were detected (Figure 2b and d). Videodensitometric and subsequent statistical analysis of the 14 paired samples revealed that Bax- expression was significantly lower in relapsed ALL as compared with *de novo*

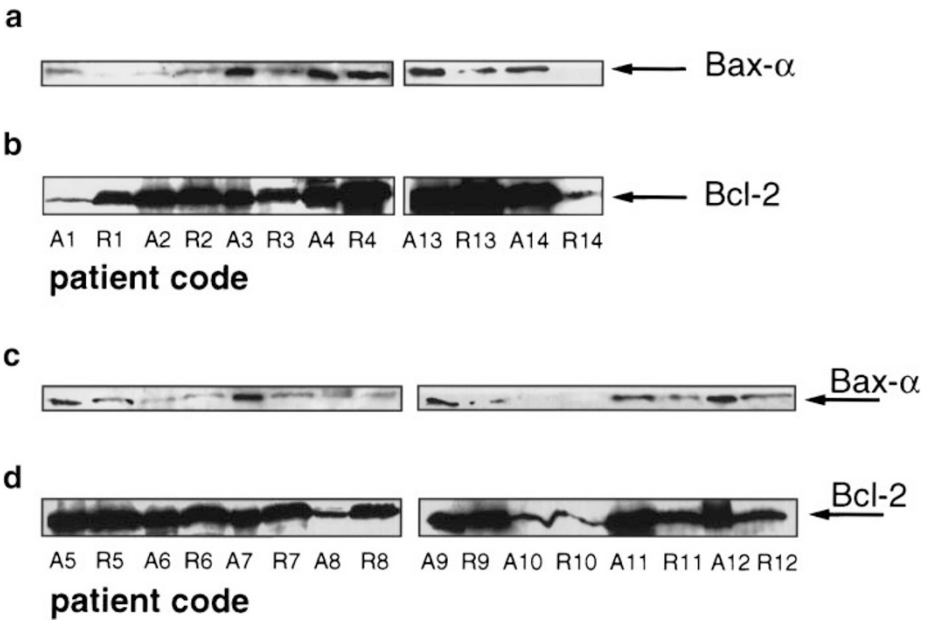

Figure 2 Expression of Bax- and Bcl-2 in paired samples of childhood ALL at initial diagnosis and relapse. 15 g of total protein from each of 14 patients with *de novo* ALL (patient code: A1–A14) and ALL relapse (R1–R14) were subjected to Western blot analysis. Immunoblots developed with anti-Bax-antibody (a and c) and anti-Bcl-2-antibody (b and d) are shown. The experiment was repeated and similar results were obtained.

ALL ($P = 0.023$, Figure 3a). Mean Bax- expression at relapse only reached 37% of its level at the initial presentation. This was in clear contrast to the quantification of Bcl-2. Although considerable variability was seen in Bcl-2 levels when comparing different patients (Figure 2b and d), mean Bcl-2 levels of the 14 paired samples did not change (Figure 3b). Consequently, the Bax- /Bcl-2 ratio was reduced at the time of relapse ($P = 0.013$, Figure 3c). To verify these observations in a larger unpaired sample series, Bax- and Bcl-2 expression were investigated by the same experimental procedure in samples of 77 children with *de novo* ALL and 34 children with relapsed ALL. The data from the unpaired sample series clearly confirm the data mentioned above showing a significant decrease of Bax- expression ($P = 0.0003$, Figure 4a) and reduction of the Bax- /Bcl-2 ratio ($P = 0.0039$, Figure 4c) at relapse. On the other hand, Bcl-2 levels in the samples varied over a broad range but no statistically significant differences between *de novo* and relapse situation were detected (Figure 4b). To further investigate the apoptotic cascade, we used an antibody against caspase-3 which detects both the proenzyme and the cleaved forms of the enzyme. In all paired samples of ALL at initial diagnosis and relapse high levels of procaspase-3 were detected. Complete loss of the enzyme was not observed (Figure 5). Videodensitometric and subsequent statistical analysis of the proenzyme forms (bands migrating at a molecular weight between 32 and 29 kDa) showed that the mean procaspase-3 level was slightly lower in relapsed ALL (Figure 6a) and reached 70% of procaspase-3 as compared with the *de novo* situation. However, this reduction of procaspase-3 was not significant ($P = 0.052$, Figure 6a). More interestingly, nine out of 14 samples from *de novo* patients contained spontaneously processed forms of caspase-3 with apparent molecular weights in the range 17–21 kDa. By comparison with the pattern of specific caspase-3 bands appearing after activation of cytosol from the Burkitt-like lymphoma cell line BJAB with 10 M cytochrome C and 1 mM dATP,[30] we were able to identify three different forms of processed caspase-3: the p21 and p20 intermediate forms and the p17 mature form (Figure 5). One patient (A3) showed all three processed caspase-3 forms, seven patients showed

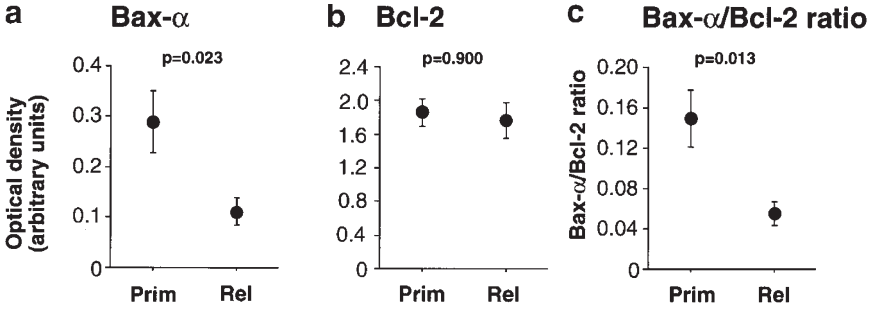

Figure 3 Comparison of expression of Bax- and Bcl-2 and Bax/Bcl-2 ratio in paired samples of childhood ALL at initial diagnosis and relapse. The expression of Bax- (a) and Bcl-2 (b) in paired samples from *de novo* ALL (Prim) and ALL relapse (Rel) was analyzed by Western blotting and subsequent videodensitometry. Mean values of optical density ± s.e.m. ($n = 14$) are given. Additionally, the Bax/Bcl-2 ratio (c) was determined and mean values ± s.e.m. ($n = 14$) are given. *P* values were calculated using the Wilcoxon signed rank test.

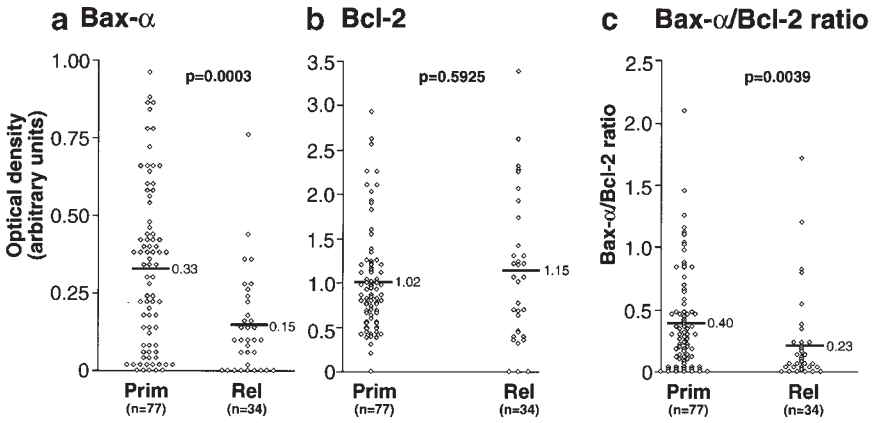

Figure 4 Comparison of expression of Bax- and Bcl-2 and Bax/Bcl-2 ratio in unpaired samples of childhood ALL at initial diagnosis and relapse. The expression of Bax- (a) and Bcl-2 (b) in unpaired samples of patients with *de novo* ALL and ALL relapse was analyzed by Western blotting and subsequent videodensitometry. Values of optical density of 77 *de novo* ALL samples (Prim) and 34 ALL relapse samples (Rel) are given. Additionally, the Bax- /Bcl-2 ratio (c) was determined and values of 77 *de novo* ALL samples (Prim) and 34 ALL relapse samples (Rel) are given. Mean values of Bax- , Bcl-2 and Bax- /Bcl-2 ratio are given as numbers and marked by a horizontal line. *P* values were calculated using the Mann–Whitney *U* test.

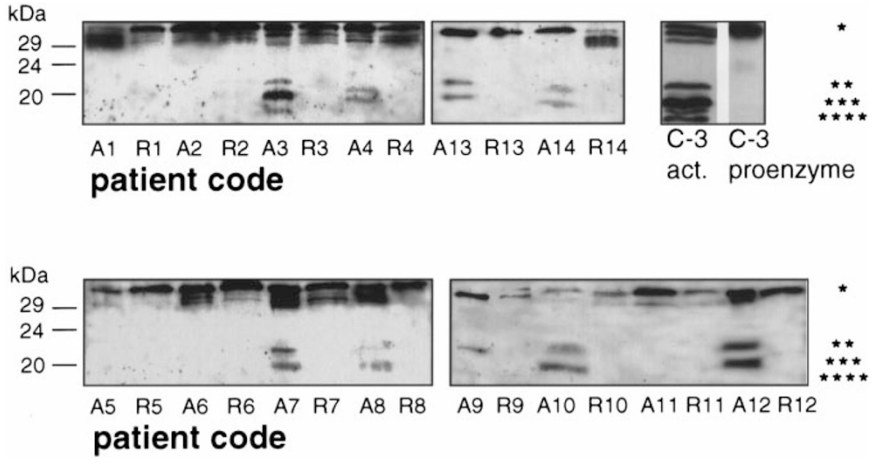

Figure 5 Expression of caspase-3 in paired samples of childhood ALL at initial diagnosis and relapse. 15 g of total protein from each of 14 patients with *de novo* ALL (patient code: A1–A14) and ALL relapse (R1–R14) were subjected to Western blot analysis. Immunoblots developed with anti-caspase-3-antibody are shown. Additionally, an *in vitro* activated BJAB cell lysate (C-3 act) and a control BJAB cell lysate (C-3 proenzyme) are shown. *, Procaspase-3 isoforms; **, p21 intermediate form; ***, p20 intermediate form; and ****, mature p17. The experiment was repeated and similar results were obtained.

the p21 and p20 intermediate forms (A4, A7, A8, A10, A12, A13 and A14) and one patient only showed the p21 band (A9). In clear contrast, processing of caspase-3 was absent in all patients with relapsed ALL. Statistical analysis of spontaneous caspase-3 processing proved this phenomenon to be highly significant ($P = 0.0003$, Figure 6b). In the larger unpaired sample series of 77 children with *de novo* ALL and 34 children with relapsed ALL, we were able to confirm that loss of spontaneous caspase-3 processing is a dominant feature in cells of patients with ALL relapse. While caspase-3 processing was observed in 37 of 77 samples at initial diagnosis this was detected in only one of 34 samples at relapse (P 0.0001, Figure 7b). On the other hand, no statistically significant differences of procaspase-3 levels between the *de novo* and relapse situation were found (Figure 7a), thereby excluding that the missing detection of processed caspase-3 forms is simply attributed to lower levels of the proenzyme. Although procaspase-3 levels of individual samples of the unpaired sample series were in the same range as compared with the paired sample series (see Figures 7 and 6, respectively) the mean values of pro-caspase-3 expression slightly differed between these two sample series. This might be attributed to the fact that the paired sample series only included patients who suffered from relapse while in the unpaired sample series the percentage of relapse patients was less than 31%.

Furthermore, we statistically evaluated whether there is a dependency between high Bax expression and spontaneous caspase-3 cleavage. To this end, the samples were split by the median value of Bax expression of the unpaired sample series (0.215 arbitrary units). Indeed, we could show that caspase-3 cleavage is preferentially seen in samples with high Bax levels (0.215 arbitrary units). 53% of the samples with high Bax contained processed caspase-3, whereas caspase-3 cleavage was detected in only 14% of the samples with low Bax (P 0.0001, Fisher's exact test). This observation was specific since caspase-3 cleavage was totally independent from Bcl-2 levels ($P = 0.843$, Fisher's exact test). This suggests that the

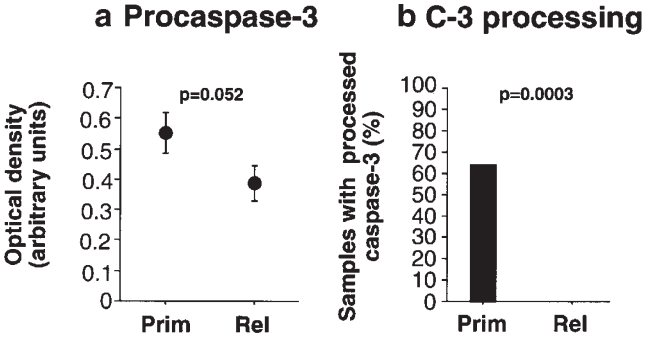

Figure 6 Comparison of expression of procaspase-3 and caspase-3 processing in paired samples of childhood ALL at initial diagnosis and relapse. The expression of procaspase-3 (a) in paired samples from *de novo* ALL (Prim) and ALL relapse (Rel) was analyzed by Western blotting and subsequent videodensitometry. Mean values of optical density ± s.e.m. ($n = 14$) are given. *P* value was calculated using the Wilcoxon signed rank test. Additionally, caspase-3 processing (b) was observed in nine of 14 samples at initial diagnosis and in 0 of 14 samples at relapse. The percentage of samples from *de novo* ALL (Prim) and ALL relapse (Rel) which contain processed caspase-3 is given. *P* value was calculated with Fisher's exact test.

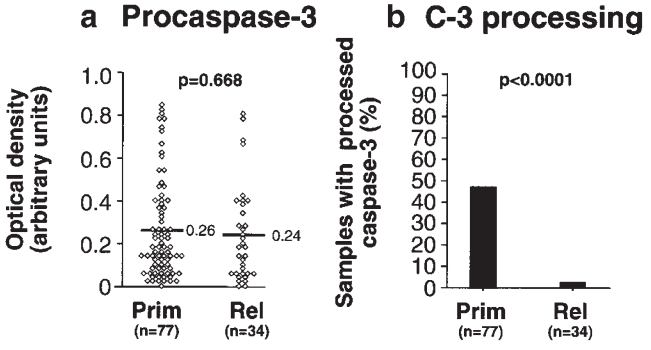

Figure 7 Comparison of expression of procaspase-3 and caspase-3 processing in unpaired samples of childhood ALL at initial diagnosis and relapse. The expression of procaspase-3 (a) in unpaired samples of patients with *de novo* ALL and ALL relapse was analyzed by Western blotting and subsequent videodensitometry. Values of optical density of 77 *de novo* ALL samples (Prim) and 34 ALL relapse samples (Rel) are given. The mean values of procaspase-3 expression are given as numbers and marked by a horizontal line. *P* value was calculated using the Mann–Whitney *U* test. Additionally, caspase-3 processing (b) was observed in 37 of 77 samples at initial diagnosis and in one of 34 samples at relapse. The percentage of samples from *de novo* ALL (Prim) and ALL relapse (Rel) which contain processed caspase-3 is given. *P* value was calculated with Fisher's exact test.

reduction of Bax protein might be responsible for the loss of *in vivo* caspase-3 processing.

Discussion

In the present study, we analyzed the p53/Bax/caspase-3 pathway in paired samples at initial diagnosis and relapse of 14 children with ALL and in an unpaired sample series with 111 patients (77 *de novo* ALL and 34 relapsed ALL). In the paired sample series, we did not find any p53 mutations in lymphoblasts from patients with *de novo* ALL and relapsed ALL. On the other hand, in the larger unpaired sample series 8.1% of the patients carried p53 mutations, but there was no difference between *de novo* (9.1%) and relapsed ALL (5.9%). This is in line with the analysis of a series of 330 cases which had shown that p53 mutations are very rare in childhood ALL (2%).[28] Similarly, mutations of the p53 gene occurred in only three of 57 patients (5%) with T-ALL at diagnosis and one of 14 patients (7%) at relapse, whereas p53 mutations were found in 67% of 18 T cell ALL cell lines.[31]

Bax frameshift mutations as a possible cause for the observed reduction of Bax protein expression in the relapsed ALL cells were absent at initial diagnosis and relapse as could be expected from a recent study.[32] However, using the same protocol we were able to detect bax frameshift mutations in Lovo cells which were therefore used routinely as a positive control (data not shown). As described above for the p53 gene, bax mutations are very uncommon in freshly isolated blasts but are likely to be selected during the establishment of cell lines (50% of 14 cell lines).[32] These results confirm that mutations in the p53 coding sequence or in the G_8 tract of bax are of minor importance for development and evolution of ALL *in vivo* but frequently occur in cell lines.

Many reports suggested, however, that dysregulation of apoptotic pathways on the protein expression level of members of the Bcl-2 family plays a role in tumorigenesis, prognosis of the disease and survival in solid tumors,[12–14,33] as well as leukemia.[15,34] Furthermore, it has been shown that relapsed ALL cells are more resistant to various drugs, such as glucocorticoids, L-asparaginase, anthracyclins, and thiopurines[35] and more frequently exhibit reduced apoptosis than newly diagnosed ALL.[36] We therefore investigated the Bax- and Bcl-2 levels in lymphoblasts at first presentation and relapse from patients with childhood ALL.

We found that Bax expression was significantly decreased in samples of relapse as compared with the samples obtained at the initial presentation. In contrast, we did not see a difference in Bcl-2 expression between lymphoblasts from *de novo* ALL and relapse. Consequently, we also observed a significantly lower Bax/Bcl-2 ratio at the time of relapse. This is in accordance with a former study in leukemia cell lines demonstrating that the Bax/Bcl-2 ratio, as determined by Western blotting, rather than Bcl-2 alone is important for survival after drug-induced apoptosis *in vitro* and is largely dependent on Bax- levels.[37] Although we did not find a significant difference in Bcl-2 expression between *de novo* disease and relapse, almost all of the marrow leukemic lymphoblasts expressed high levels of Bcl-2 protein. This fits well with data showing that high expression of Bcl-2 protein is a common feature in acute leukemia.[34,38]

It has been reported recently that increased Bax expression is associated paradoxically with an increased risk of relapse.[17] This report is not compatible with the concept that low Bax, as a key promoter of apoptosis, is a negative prognostic factor and is associated with poor response rates to chemotherapy, at least in patients with solid tumors.[14,33] However, in the light of the present investigation, a relatively high expression of Bax- in *de novo* ALL might be a feature of the lymphoblasts at initial presentation but appears to be irrelevant for the progression of the disease since Bax- levels drastically decline in relapsed ALL. In our hands, the analysis of paired, as well as unpaired samples from patients in relapse showed a consistent decrease of Bax protein as compared with Bax expression at primary diagnosis. This implicates an involvement of the loss of Bax expression in the development and progression of the resistant disease.

Caspase-3 is a central executioner of the p53/Bax apoptosis signaling pathway downstream of the activation of the apoptosome. This cytoplasmic structure is composed of apoptosis-

activating factor-1 (Apaf-1) and procaspase-9, and cytochrome c that has been released from the mitochondria.[39] In first clinical investigations, caspase-3 has been determined to be a significant predictor of complete remission in adults with ALL.[40] Interestingly, we detected spontaneous *in vivo* caspase-3 processing, a phenomenon which has also been described in acute myeloid leukemia,[41] in approximately 50% of the samples from patients at first presentation whereas, with the exception of one patient, caspase-3 processing was absent at relapse. Our data suggest that this is related to the loss of Bax since caspase-3 cleavage is preferentially seen in samples with high Bax levels (P 0.0001, Fisher's exact test).

Taken together, our data demonstrate for the first time that the p53/Bax/caspase-3 pathway of apoptosis is disturbed in relapsed ALL leading to different tumor biology and the development of therapy–refractory disease in ALL relapse in comparison with the disease at the time of initial diagnosis. The question of the prognostic significance of our observations is addressed in the ongoing ALLREZ-BFM trial.

Acknowledgements

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