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Research Article

Enhanced levels of the apoptotic BAX/BCL-2 ratio in children with acute lymphoblastic leukemia and high-risk features

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Abstract

It has been suggested that leukemia is characterized by an impaired balance between the proliferation of blood cells and their capacity to undergo apoptosis. The aim of this study was to examine the expression of key molecules related to apoptosis (BCL-2, BAX, FAS, FAS-L) in children with acute lymphoblastic leukemia (ALL). Measurement of *BCL-2* and *BAX* mRNA was performed by quantitative real-time PCR, and membrane expression of *FAS* and *FAS-L* was assessed by flow cytometry in bone marrow mononuclear cells, both at diagnosis and at remission following induction chemotherapy. At diagnosis, increased levels of the apoptotic *BAX/BCL-2* ratio were observed in children older than 10 years and with higher white blood cell counts. A DNA index < 1.16 was associated with increased *BAX/BCL-2*, both at diagnosis and at remission, and the del(9p) chromosome abnormality with increased *BAX/BCL-2* at remission. The expression of the apoptotic receptor FAS was significantly higher at remission compared to diagnosis, which might reflect enhanced sensitivity of the leukemic clone to apoptosis and response to treatment. Altogether, our results highlight the association of apoptosis-related genes with clinical and cytogenetic prognostic parameters in pediatric ALL. A better understanding of the mechanisms and regulation of apoptosis should enable the design of novel targeted therapies for these patients.

Keywords: leukemia, real-time PCR analysis, protein.

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Introduction

Acute lymphoblastic leukemia (ALL) accounts for nearly 1/3 of all pediatric malignancies and 75% of all childhood leukemias. The annual incidence of ALL has been estimated at 30 cases per million, with a peak incidence in children aged two to five years (Pui and Evans, 2006). Progress in diagnosis provided by novel molecular techniques, risk classification, and treatment strategy in ALL has led to cure rates that now exceed 80% (Pui and Evans, 2006). However, a significant proportion (20%) of patients fails to respond to therapy, and treatment failure can occur even in patients with favorable prognostic features (Winick *et al*., 2004; Carroll *et al*., 2006).

It has been suggested that leukemia results from an imbalance between the proliferation of blood cells and their capacity to undergo apoptotic death (Peters *et al*., 1998). At the cellular level, apoptosis is regulated by two major signaling pathways: a) the receptor-mediated (extrinsic), and b) the mitochondria-mediated (intrinsic) pathway (Hacker, 2000). The susceptibility of cells to apoptosis depends on the relative expression of intracellular molecules that enhance apoptosis (pro-apoptotic), with BAX being the major representative (Farrow and Brown, 1996), and of molecules that inhibit apoptosis (anti-apoptotic), such as BCL-2 (Hockenbery *et al*., 1993; Yang and Korsmeyer, 1996), BCL-X_L, and MCL-1 (Hacker, 2000). Activation of the above-mentioned pathways may be mediated by membrane receptors, such as FAS (APO-1/CD95), that is significantly expressed in neoplastic cells and interacts with FAS ligand (FAS-L) expressed in activated T-lymphocytes (Wood *et al*., 2003).

From a clinical standpoint, expression of apoptosis-related genes has been associated with outcome in various hematological malignancies. To this end, high *BAX* levels correlate with favorable prognosis in acute myeloblastic leukemia (AML) (Ong *et al*., 2000), whereas enhanced expression of *BCL-2* is a poor prognostic factor in lymphomas and in chronic lymphocytic leukemia (Hogarth and Hall, 1999). In the case of ALL, studies existing so far have yielded conflicting results. Thus, Aref *et al.* (2004) reported that *BCL-2* expression at the time of diagnosis is correlated with responsiveness to induction chemotherapy, but not patient outcome. In contrast, another study (Coustan-Smith *et al*., 1996) found no association between *BCL-2* levels and disease aggressiveness or resistance to therapy. Similarly, although high expression of *BAX* has been associated with increased risk for relapse in one study (Hogarth

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and Hall, 1999), other researchers have suggested that the *BAX/BCL-2* ratio - rather than individual *BAX* or *BCL-2* levels - may be a more reliable prognostic indicator in ALL (Prokop *et al*., 2000).

The aim of this study was to assess the expression of the apoptosis-related genes *BCL-2* and *BAX* in childhood ALL, both at the time of diagnosis and at remission achieved post induction treatment. In addition, we measured the levels of the apoptotic receptors FAS, FAS ligand, and their co-expression in patients' leukemic cells. To explore the prognostic significance of apoptosis-related genes in childhood ALL, we examined associations between expression levels and established clinical and cytogenetic disease parameters.

Materials and Methods

Patients

The study included 26 children (18 boys, eight girls) with newly diagnosed ALL (23 B-ALL, three T-ALL). All patients were diagnosed, treated and followed-up at the Department of Pediatric Hematology-Oncology, University of Crete, and received chemotherapy according to the ALL BFM 2000 protocol. Bone marrow specimens were obtained from all children, under informed consent signed by the parents/legal guardians. Cytogenetic abnormalities were screened by conventional karyotype analysis and FISH. Disease remission following induction therapy was assessed by bone marrow microscopic evaluation and flow cytometry. The study was approved by the Institute's Ethics Committee.

Bone marrow mononuclear cell isolation

Bone marrow mononuclear cells (MNCs) were isolated by Ficoll Hypaque separation (Lymphoprep-Nycomed d = 1077 g/mL) at the time of ALL diagnosis, upon remission on day 33, and at the end of therapy. The MNCs isolated consisted almost exclusively of leukemic blasts (> 90% bone marrow infiltration at the time of diagnosis). Cells were stored at -80 ºC immediately after collection.

RNA isolation and cDNA synthesis

Total RNA was isolated from frozen cells using the SV Total RNA Isolation kit (Promega, Madison, WI, USA). RNA was quantified by spectrophotometry at 260 nm, and the quality was evaluated by 1% agarose electrophoresis (Figure 1). An amount of 500 ng of RNA was used for cDNA synthesis with the IM-PROM Reverse Transcription System (Promega).

Quantitative real-time PCR

BAX and *BCL-2* mRNA levels were assessed by quantitative real-time PCR, using *GAPDH* as housekeeping gene for data normalization. A plot of C*t vs.* log was used for quantification, using a standard curve obtained from serial

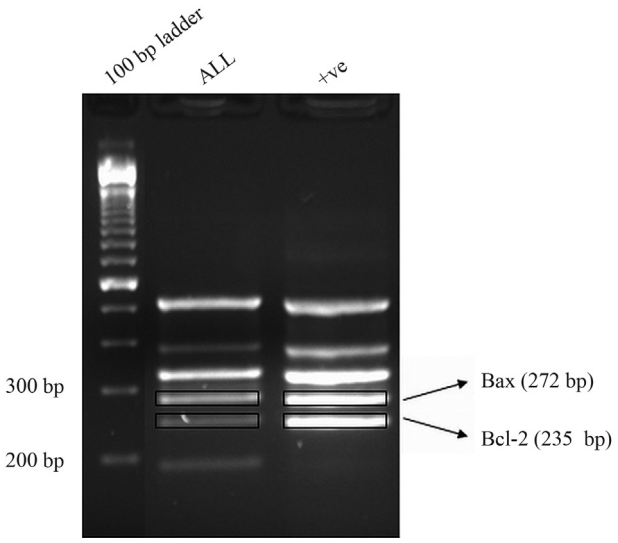

Figure 1 - Agarose electrophoresis results in one of the study samples.

dilutions of a reference cell line cDNA (HL-60). A total reaction volume of 20 µL was set up, containing 1x Quantitect SYBR Green mix (QIAGEN, Hilden, Germany), 1x Quantitect Primer mix (*Bax* QT00997381, *bcl-2* QT00025011, *Gapdh* QT01192646 (all from QIAGEN), and 1.2 µL cDNA. All reactions were performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions included a hot start step at 95 ºC for 15 min followed by 40 cycles at 95 ºC for 15 s, 55 ºC for 30 s, and 72 ºC for 30 s.

Flow cytometry

Anticoagulated whole bone marrow samples were transferred into polystyrene round-bottom tubes and incubated with 10 µL of PC5-anti-CD45 (A07785, Beckman Coulter, Brea, CA, USA), 20 µL FITC-anti-Fas (sc-52524, Santa Cruz Biotechnology Inc,, Santa Cruz, CA, USA), and 20 µL PE-anti-Fas-L (sc-19681, Santa Cruz Biotechnology). Control samples were stained with anti-CD45, PE-mouse IgG1 (A07796, Beckman Coulter) and FITC-mouse IgG1 (A07795, Beckman Coulter) isotype controls. The samples were incubated for 15 min at room temperature in the dark, washed with PBS supplemented with 2% FBSSV (Hyclone, Thermo Scientific, Logan, UT, USA) and 5% sodium azide (106688, Merck, Darmstadt, Germany) for 5 min at 290 x g. Supernatants were discarded and red blood cells were lysed by Immunoprep Reagent System (Beckman Coulter). Cells were analyzed on an Epics Elite Coulter cell sorter. The $CD45^+$ population was gated and lymphocytes were identified based on forward scatter and side scatter properties. The expression of surface FAS, FAS-L and their co-expression were determined.

Statistical analysis

Statistical analysis was performed using the SPSS 16.0 (Statistical Package for the Social Sciences) program.

The differences in gene expression among groups were evaluated with the nonparametric Mann-Whitney *U* test. Correlations were calculated with the Spearman test. A p-value < 0.05 was considered as statistically significant. Data are presented as mean ± standard error of the mean (SE), unless stated otherwise.

Results

Expression of the apoptosis-related genes *BAX* and *BCL-2* in children with ALL and association with clinical characteristics

We measured the levels of the *BAX*, *BCL-2*, and *BAX/BCL-2* ratio in bone marrow MNCs from 26 children (18 boys, eight girls) with ALL. The patients' demographic and clinical characteristics are presented in Table 1. At diagnosis, *BAX* levels were correlated with *BCL-2* ($\rho = 0.46$, $p = 0.001$) and *BAX/BCL-2* ($\rho = 0.47$, $p < 0.001$). A significant negative correlation between white blood cell count and *BCL-2* mRNA ($\rho = -0.57$, $p = 0.007$) was observed, whereas patients' age and hemoglobin showed no association with *BAX* or *BCL-2*. According to the BFM protocol criteria, 12 ALL patients were classified in the high-risk group and 14 in the median-risk group. We found no difference in *BAX*, *BCL-2*, or *BAX/BCL-2* levels between high-risk and median-risk patients. Older children (> 10 years) in the high-risk group presented higher values of *BAX/BCL-2* ratio compared to younger counterparts (0.71 ± 0.20 *vs.* 0.15 ± 0.24, $p = 0.031$).

Correlation of *BAX* and *BCL-2* levels at diagnosis with cytogenetic abnormalities in children with ALL

Hyperdiploidy (defined as DNA index ≥ 1.16) was correlated with higher levels of the anti-apoptotic *BCL-2* at diagnosis ($\rho = 0.71$, $p = 0.05$). In line with this finding, the DNA index showed negative correlation with the *BAX/BCL-2* ratio ($\rho = -0.88$, $p = 0.004$) (Figure 2). Conversely, the presence of a t(12;21)(p13;q22) translocation, observed in four children, was associated with significantly higher *BAX/BCL-2* ratios (1.45 ± 0.23 *vs.* 0.46 ± 0.30, $p = 0.030$). A deletion in chromosome 9p was also observed in four children, but did not correlate with a differential *BAX*, *BCL-2*, or *BAX/BCL-2* ratio.

Table 1 - Demographic and clinical characteristics of the ALL patients.

No. of patients	26[1]
Age (years)	7.1 ± 1.2
Gender (female/male)	8/18
ALL type (B/T)	23/3
White blood cell count (K/μL)	27.5 ± 10.6
Hemoglobin (g/dL)	9.1 ± 0.6
Cytogenetics	
Hyperdiploidy (no. of patients)	7
t(12;21) (no. of patients)	4
del(9p21) (no. of patients)	4
Other abnormalities	5
ALL risk (median/high)	17/9

[1]Data presented as *N* or mean ± SE.

Expression of *BAX* and *BCL-2* in ALL children at remission post induction therapy

Measurement of *BAX* and *BCL-2* in bone marrow MNCs was repeated in the ALL children at disease remission on day 33 after treatment initiation. The apoptotic *BAX/BCL-2* ratio was significantly lower at remission than at the time of diagnosis in the high-risk (0.05 ± 0.02 *vs.* 0.32 ± 0.12, $p = 0.019$), but not in the median-risk group. In line with this finding, patients in the high-risk group had lower *BAX/BCL-2* at remission compared to their median-risk counterparts (0.05 ± 0.02 *vs.* 0.58 ± 0.13, $p = 0.009$).

Expression of the apoptosis-related genes once remission has been achieved is correlated with certain baseline

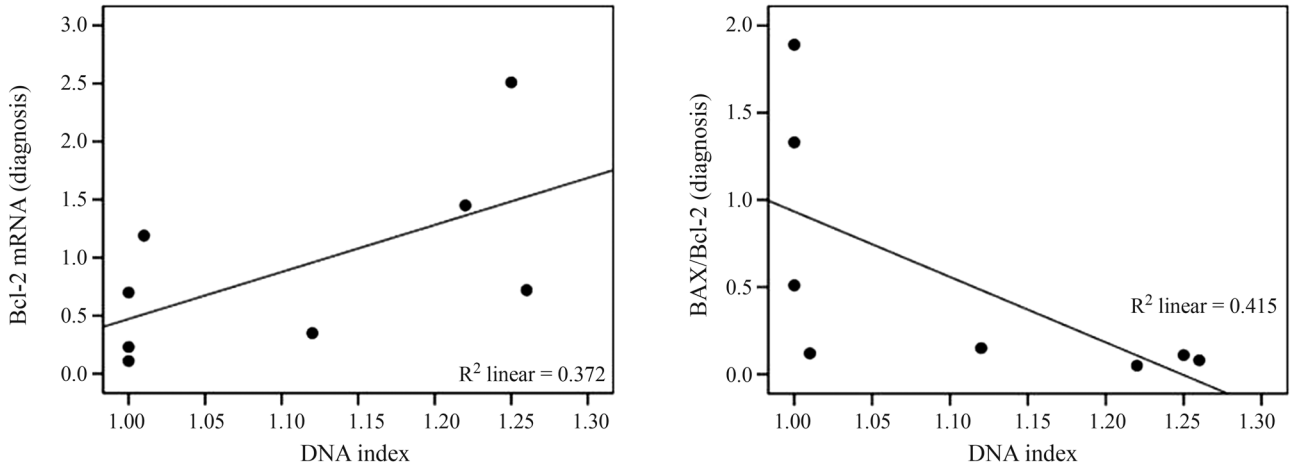

Figure 2 - Correlation between BCL-2 mRNA and BAX/BCL-2 ratio and the DNA index at ALL diagnosis.

cytogenetic findings. First, a negative correlation was observed between the pro-apoptotic *BAX* and DNA index (ρ = -0.85, p = 0.015). Accordingly, significantly higher levels of *BAX/BCL-2* were observed in patients with DNA index < 1.16 compared to those with DNA index ≥ 1.16 (hyperdiploidy) (0.80 ± 0.78 *vs.* 0.08 ± 0.03, p = 0.025). Moreover, patients with deletion in chromosome 9p had significantly higher *BAX/BCL-2* levels than those without the deletion (0.51 *vs.* 20, p = 0.007).

Expression of FAS and FAS-L in children with ALL

The membrane expression of the pro-apoptotic receptors FAS and FAS-L in bone marrow $CD45^+$ MNCs was determined in 13 children with B-ALL both at the time of diagnosis and at disease remission (day 33 after treatment initiation). The proportion of FAS^+ MNCs was significantly increased at remission compared to the time of diagnosis (24.0 ± 6.1% *vs.* 8.0 ± 1.9%, p = 0.035) (Figure 3). In contrast, a non-significant reduction in $FAS\text{-}L^+$ cells was observed at remission (2.4 ± 0.8% *vs.* 4.6 ± 1.4% at diagnosis). We found no association between FAS/FAS-L expression and clinical or cytogenetic parameters.

Discussion

Deregulation of apoptosis disrupts the balance between cell survival and death and may be a key pathogenetic aspect in hematologic malignancies. Herein, we report increased levels of the apoptotic *BAX/BCL-2* ratio in ALL children with high-risk features, such as higher white blood cell count, DNA index < 1.16, and the del(9p) chromosomal abnormality. Previous studies have failed to demonstrate a significant association between clinical prognostic parameters, such as age at diagnosis, gender, or white blood cell count, and *BAX* or *BCL-2* in pediatric ALL (Gala *et al*., 1994; Hogarth and Hall, 1999; Narayan *et al*., 2007). In our study, although the expression of the apoptosis-related genes did not vary significantly according to the risk group classification, a higher white blood cell count was correlated with reduced expression of the anti-apoptotic *BCL-2*. Another high-risk feature, namely age above 10 years, showed inverse association with the *BAX/BCL-2* ratio, albeit only in the high-risk group of patients. Altogether, in children with ALL, clinical parameters of adverse outcome are associated with increased levels of pro-apoptotic *vs.* anti-apoptotic genes in bone marrow MNCs.

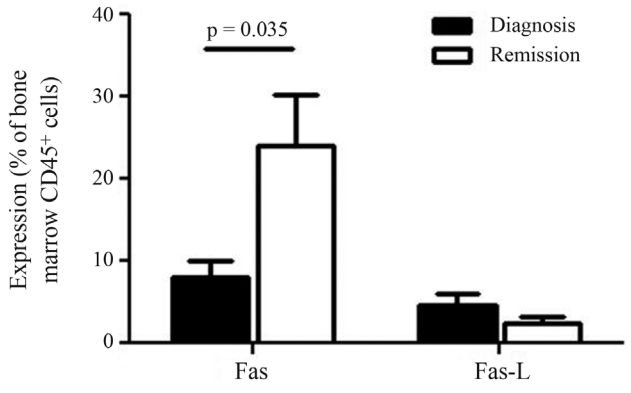

Figure 3 - Expression of the apoptotic receptors FAS and FAS-L in bone marrow MNCs of ALL patients at the time of diagnosis and at remission following induction therapy.

Ploidy has been recently recognized as a prognostic factor in ALL, with hyperdiploidy (DNA index ≥ 1.16) being associated with a favorable prognosis (Aricò *et al*., 2008). In our analysis, *BCL-2* mRNA showed a positive correlation with the DNA index, both at diagnosis and at the time of remission following induction therapy, whereas *BAX* expression was inversely correlated with the DNA index at remission. The *BAX/BCL-2* ratio was inversely correlated with the DNA index at diagnosis and remission, suggesting that the persistence of a high *BAX/BCL-2* ratio in bone marrow MNCs may be related to a less favorable prognosis.

Cytogenetic abnormalities have significant prognostic value in ALL. Abnormalities in chromosome 9 are common in children with ALL (11% according to large series) (Heerema *et al.*, 1999). Although in adult B-precursor ALL with abnormal chromosome 9p the prognosis has been reported to be poor (Nahi *et al*., 2008), data in children are scarce and controversial (Heerema *et al*., 1999). Since del(9p) was found in 15% of our patients, we investigated its association with apoptosis-related genes. Children with del(9p) had higher levels of *BAX* at diagnosis than children without this chromosome abnormality. Moreover, the *BAX/BCL-2* ratio at the time of remission was significantly higher in the del(9p) group than in patients without this abnormality. Although the small number of patients in this study precludes definitive conclusions, these data may suggest that the presence of del(9p) in children with ALL indicates a poor prognosis.

In our study, the ALL patients in the high-risk group had a higher *BAX/BCL-2* ratio at diagnosis than at remission, achieved after induction chemotherapy, whereas no significant change was observed in the median-risk group. This finding might be a further indication that a high *BAX/BCL-2* ratio at diagnosis points to a poor prognosis in children with ALL. Alternatively, longitudinal changes in the expression of apoptosis-related genes could reflect alterations in the apoptosis burden of the disease as response to chemotherapeutic regimens. Thus, changes in apoptosis during induction therapy may be indicative of the effectiveness of treatment and of the disease outcome (Schuler and Szende, 2004). Higher BAX levels were associated with increased risk of relapse in one study (Hogarth and Hall, 1999), whereas another one (Narayan *et al*., 2007) showed that low *BAX/BCL-2* ratio correlates with favorable prognosis.

It has been suggested that *FAS* expression could control the response of leukemic cells to chemotherapy and

have an impact on prognosis (Aref *et al*., 2004). In our study, the ALL patients had low levels of *FAS* at diagnosis, followed by a significant increase at disease remission. In some pediatric ALL studies, increased levels of *FAS* have been associated with longer survival in complete remission (Baryshnikov *et al*., 1999), whereas other studies found no association of *FAS* levels with outcome (Wuchter *et al*., 2000; Aref *et al*., 2004; Fulda, 2009). At this time, we suggest that increased *FAS* levels at remission post induction treatment could discriminate patients who are not resistant to chemotherapy, but additional studies with long-term follow-up are needed to further address this issue.

In conclusion, our study highlights the association between the apoptotic *BAX/BCL-2* ratio with high-risk features, such as older age, higher white blood cell count, the del(9p) abnormality, and DNA index < 1.16, in children with ALL. The increase in *FAS* expression, once remission has been achieved after induction treatment, could represent a prognostic factor of favorable response to chemotherapy and deserves further investigation. A better understanding of the role of apoptosis in pathogenesis and prognosis of pediatric ALL should enable the design of novel targeted therapies.

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