The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia


Childhood acute lymphoblastic leukemia (ALL) consists of various subtypes that respond differently to cytotoxic drugs and therefore have a markedly different clinical outcome. We used microarrays to investigate, in 190 children with ALL at initial diagnosis, whether 70 key apoptosis genes were differentially expressed between leukemic subgroups defined by lineage, genetic subtype, in vitro drug resistance, and clinical outcome. The expression of 44 of 70 genes was significantly different in T- versus B-lineage ALL, 22 genes differed in hyperdiploid versus nonhyperdiploid, 16 in TEL-AML1–positive versus –negative, and 13 in E2A-rearranged versus germ-line B-lineage ALL. Expression of MCL1 and DAPK1 was significantly associated with prednisolone sensitivity, whereas BCL2L13, HRK, and TNF were related to L-asparaginase resistance. BCL2L13 overexpression was also associated with unfavorable clinical outcome (P < .001). Multivariate analysis including known risk factors revealed that BCL2L13 expression was an independent prognostic factor (P = .011).

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Introduction

The treatment of pediatric acute lymphoblastic leukemia (ALL) has greatly improved over the past 3 decades, resulting in long-term disease-free survival (DFS) of approximately 80%.1 Despite this progress, therapy resistance still forms a major obstacle to success. In a heterogeneous disease consisting of various genetic subtypes such as t(9;22)/BCR-ABL, t(12;21)/TEL-AML1, hyperdiploid (> 50 chromosomes), 11q23/MLL rearranged, t(1;19)/E2A-PBX1, and T-lineage ALL, which differ markedly in their treatment response.2 The in vitro response to chemotherapy can be studied by exposure of primary patient samples to cytostatic drugs in a cell-kill assay such as the methyl-thiazol-tetrazolium (MTT) assay. We and others have previously demonstrated that children with ALL whose leukemia cells exhibit in vitro resistance to single drugs or a combination of drugs (ie, prednisolone, vincristine, and L-asparaginase [PVA]) have a significantly worse prognosis than patients with sensitive leukemic cells.3,5 In addition, leukemia subtypes with a relatively unfavorable prognosis have been associated with in vitro drug resistance8,10 and subtypes with a favorable prognosis with in vitro drug sensitivity.11,12

Apoptosis is the predominant form of cell death triggered in vivo and in vitro by drugs in hematologic malignancies.13 There are 2 major routes by which apoptosis can be induced: (1) the mitochondrial or intrinsic apoptosis pathway; and (2) the death receptor–mediated or extrinsic apoptosis pathway. Both apoptotic pathways have been extensively reviewed elsewhere.14-17 Briefly, the intrinsic route is initiated by mitochondrial damage that leads to release of apoptogenic factors, such as cytochrome c, Smac/Diablo, and apoptosis-inducing factor (AIF), from the mitochondrial intermembrane space.17 The release of these factors is mediated by Bcl-2 family proteins, a group of key regulators of the intrinsic apoptosis pathway that consists of proapoptotic and antiapoptotic members. Upon its release into the cytoplasm, cytochrome c forms a complex known as the apoptosome consisting of apoptotic protease-activating factor-1 (Apaf-1), ATP/dATP, and procaspase-9.18 Following its activation within the apoptosome, caspase-9 activates the downstream effector caspase cascade.19 Initiation of the extrinsic apoptosis pathway involves ligand-induced aggregation of death receptors and activation of procaspase-8 or procaspase-10 within the death-inducing signaling complex (DISC).20,21

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Activated procaspase-8 or -10 is released into the cytoplasm, where it induces activation of downstream effector caspases. The intrinsic and extrinsic apoptotic pathways converge at the level of caspase-3 activation.

Leukemia subtypes with a relatively unfavorable prognosis have been associated with in vitro drug resistance. Moreover, cellular drug resistance is associated with decreased ability to induce apoptosis in pediatric ALL. Therefore, one of the factors that may contribute to the different treatment response of genetic leukemia subtypes may be a differential propensity to undergo apoptosis. Apoptosis is controlled by various positive and negative regulators, responding to stimuli from inside and outside the cell. Most papers to date addressing causes of cellular drug resistance, however, only focus on a limited number of apoptosis molecules. In the present study we analyzed the expression patterns of 70 key apoptosis genes in leukemic cells of 190 children at initial diagnosis of ALL. The expression of these genes was tested for association with (1) lineage and genetic subtype, (2) in vitro drug resistance to 4 widely used drugs in treatment of ALL (ie, prednisolone, vincristine, L-asparaginase, and daunorubicin), and (3) clinical outcome. Last, we analyzed the relation between the expression of active Apaf-1 isoforms and cellular drug resistance.

Patients, materials, and methods

Patient samples

Bone marrow (BM) and peripheral blood (PB) were obtained after informed consent from 190 children with newly diagnosed ALL who were enrolled on treatment protocols 92 and 97 at the hospitals participating in the German Cooperative Study Group for Acute Lymphoblastic Leukemia (COALL) study or the ALL-9 Dutch Childhood Oncology Group (DCOG) protocol at the Erasmus MC-Sophia Children’s Hospital in Rotterdam (study cohort); and of 92 children enrolled as part of the Total Therapy protocols 13A25 and 13B26 of St Jude Children’s Research Hospital (SJCRH) in Memphis, TN (validation cohort). Approval was obtained from the Erasmus MC-Sophia Children’s Hospital and SJCRH institutional review board for these studies. Clinical characteristics of these patients are provided in Table 1.

Isolation of leukemia cells

Mononuclear cells were isolated by sucrose density centrifugation (Lymphoprep, density 1.077 g/mL; Nycomed Pharma, Oslo, Norway) within 24 hours after sampling. Cells were resuspended in culture medium consisting of RPMI 1640 (Dutch modification without L-glutamine; Gibco BRL, Life Technologies, Breda, the Netherlands) supplemented with 20% FCS (Integro, Zaandam, the Netherlands), 2 mM L-BRL, Life Technologies, Breda, the Netherlands) supplemented with 20% consisting of RPMI 1640 (Dutch modification without L-glutamine; Gibco BRL, Life Technologies, Breda, the Netherlands) within 24 hours after sampling. Cells were resuspended in culture medium (Lymphoprep, density 1.077 g/mL; Nycomed Pharma, Oslo, Norway) after sucrose density gradient centrifugation. Mononuclear cells were isolated by sucrose density gradient centrifugation.

In vitro drug-resistance assay

Responsiveness of leukemia cells to prednisolone (PRED; Bufa Pharmaceutical Products, Uitgeest, the Netherlands), vincristine (VCR; TEVA Pharma, Mijdrecht, the Netherlands), L-asparaginase (ASP; Paronal, Christiaens, Breda, the Netherlands), and daunorubicin (DN; Cerubidine, Rhône-Poulenc Rorer, Amstelveen, the Netherlands) was determined by the 4-day in vitro MTT drug resistance assay. The concentration ranges tested for these drugs were PRED, 0.008-250 μg/mL; VCR, 0.05-50 μg/mL; ASP, 0.003-10 IU/mL; and DNR, 0.002-2.0 μg/mL. The drug concentration lethal to 50% of the ALL cells (LC50 value) was used as the measure of cellular drug resistance. The cut-off LC50 values, used to assign cases as sensitive or resistant to each agent, were those previously shown to be associated with a good or poor treatment outcome in children with ALL.

<table>
<thead>
<tr>
<th>Gene expression profiling: purification, labeling, and hybridization of RNA</th>
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Total cellular RNA was extracted from leukemic cells of 190 patients with acute lymphoblastic leukemia and hybridized to the U133A GeneChip oligonucleotide microarray containing 22 283 probe sets (~12 700 genes) according to manufacturer’s protocols (Affymetrix, Santa Clara, CA). Gene expression values were scaled to the target intensity of 2500 using Affymetrix Microarray Analysis Suite (MAS) 5.0 software.29,30 Probe sets expressed in fewer than 5 patients were omitted, leaving 14 550 probe sets in the filtered dataset for subsequent analyses. Gene expression analysis of 173 out of these 190 patients was published previously; in this paper, we focus solely on genes involved in apoptosis. All analyses were carried out on log2-transformed gene-expression values.

<table>
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<th>REAL-TIME QUANTITATIVE PCR</th>
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Total cellular mRNA was extracted using Trizol reagent (Gibco BRL) and cDNA was synthesized using random hexamers and oligo dT. mRNA expression levels of total Apaf-1, “active” Apaf-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference were quantified using real-time quantitative (RTQ) polymerase chain reaction (PCR) analysis on a ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) as previously described.31,32 The comparative cycle time (Ct) value of the target PCR was normalized by subtracting the Ct value of GAPDH (ΔCt). The ΔCt value was used to calculate the relative expression level to GAPDH for each target PCR using the following formula: relative mRNA expression = 2△Ct × 100%.33 Primer sequences used were as follows: upper, 5'-ACCGAC CGCATACCTTT-3; lower, 5'-AGGGCCTCA-CAAGTTCGTG-3' (total Apaf-1); upper, 5'-GAGCCCTAGAGAGGATA TG-3'; lower, 5'-GTGGGGAGAAAGT CACAGTAC-3; upper, 5'-GAGCCCTAGAGAGGATA TG-3'; lower, 5'-AGGTCCTCGGTCGCTCTACTAG-3' (GAPDH). Probe sequences were 5'-CAATGCGCGCTGGCAAGAT-3' (total Apaf-1).
Statistical analysis

A selection of genes with known involvement in apoptosis was made by a search using GenMaPP version 2.0 and in literature (Gladstone Institutes, University of California, San Francisco, CA). Corresponding probe sets were retrieved using Affymetrix NetAffx (http://www.affymetrix.com/analysis/index.affx). From the total of 179 selected probe sets, 118 were present in the filtered dataset corresponding to 70 apoptosis genes (intrinsic pathway, 40; extrinsic pathway, 30) for subsequent analysis.

We applied the global test\(^\text{33}\) to identify those probe sets that are simultaneously differentially expressed between different subgroups defined by lineage, genetic subtype, in vitro drug resistance, and clinical outcome. Briefly, the global test compares 2 or more groups taking into account the association between probe sets as well as their individual effects.\(^\text{34}\) The advantage of the global test is that it is applied to the entire set of probe sets under study at the same time, yielding a single overall \(P\) value, rather than on individual probe sets consecutively. Thus, there are no multiple testing issues associated with the global test. In addition, the global test can be applied to multiple probe sets encoding one gene, since this test investigates the influence of each single probe set on the discrimination between the 2 studied groups. One of the outputs of this test is a so-called gene plot, which displays the individual influences of the probe sets on the test result. The gene plot was used to select those probe sets that were most strongly explaining the difference between 2 subgroups.

In addition, we applied the Wilcoxon rank-sum test to each probe set to identify those probe sets that were individually associated with the subgroups. \(P\) values were corrected for multiple testing using the false discovery rate (FDR) step-up procedure proposed by Benjamini and Hochberg.\(^\text{35}\) The global test has more power to detect differential expression when dealing with multiple probe sets with small effects, compared with tests applied probe set-wise, such as the Wilcoxon rank-sum test. The output of the global test and the FDR-corrected Wilcoxon rank-sum test is summarized in Figures 1 and 2 and Table 2.

The duration of disease-free survival (DFS) was defined as the time from diagnosis until the date of leukemia relapse (event) or the last follow-up (censored). Univariate analysis using Cox proportional hazard regression models estimated the relative risk of an event. Significant probe sets from the univariate analysis were entered in a multivariate analysis using Cox proportional hazards regression model, which included known risk factors white blood cell count (WBC), age, lineage, and genetic subtype. DFS curves were calculated by reversing the cumulative incidence curve.\(^\text{37}\) Presence of competing events were accounted for in comparisons of DFS curves,\(^\text{36,38}\) and in multivariate analysis.\(^\text{37}\)

The Wilcoxon rank-sum test was applied to compare Apaf-1 isoform mRNA expression in sensitive and resistant patients for each individual drug.

### Results

Apoptosis-related genes and immunophenotypic and genetic subtypes of pediatric ALL

The expression of 118 probe sets corresponding to 70 apoptosis-associated genes was compared between various leukemic subgroups (ie, T-lineage and B-lineage ALL [lineage]); hyperdiploid (ie, more than 50 chromosomes present at cytogenetic analysis) and non-hyperdiploid B-lineage ALL patients (ploidy); TEL-AML1–positive and –negative B-lineage ALL patients (TA); and E2A-rearranged and E2A germ-line B-lineage ALL patients (E2A). The global test applied to all 118 probe sets generated \(P\) values less than .001 for lineage, ploidy, TA, and E2A. Gene plots that visualize influences for individual probe sets on the
The global test and Wilcoxon rank-sum test were performed to identify which of the indicated 118 apoptosis probe sets were differentially expressed in B-lineage ALL cells sensitive and resistant to prednisolone (PRED), vincristine (VCR), L-asparaginase (ASP), and daunorubicin (DNR). For each drug, probe sets selected only by the global test ($P < .001$) are light gray, probe sets selected by the global test ($P < .001$) and Wilcoxon rank-sum test with FDR controlled at 5% are black, and probe sets not selected by none of the tests are white. The numbers indicated in colored boxes are the ratio per significant gene between PRED-resistant and PRED-sensitive ALL samples (PRED), VCR-resistant and VCR-sensitive ALL samples (VCR), ASP-resistant and ASP-sensitive ALL samples (ASP), and DNR-resistant and DNR-sensitive ALL samples (DNR).

The global test and Wilcoxon rank-sum test with FDR controlled at 5% were performed to identify which of the 118 apoptosis probe sets under study were differentially expressed in leukemic cells taken at initial diagnosis of ALL from patients who achieved and remained in continuous complete remission (CCR) and patients who achieved a complete remission but relapsed during or after completion of chemotherapy. Probe sets selected only by the global test ($P < .001$).

*B Probe sets selected by the global test and Wilcoxon rank-sum test with FDR less than 5%.

**Table 2. Apoptosis genes associated with disease-free survival in pediatric ALL**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Probe set ID</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAG1</td>
<td>211475_s_at</td>
<td>0.91</td>
</tr>
<tr>
<td>BAG1+</td>
<td>203939_at</td>
<td>0.81</td>
</tr>
<tr>
<td>BAG5</td>
<td>202984_s_at</td>
<td>1.26</td>
</tr>
<tr>
<td>BAG5</td>
<td>202985_s_at</td>
<td>1.26</td>
</tr>
<tr>
<td>BCL2L13</td>
<td>217955_at</td>
<td>0.72</td>
</tr>
<tr>
<td>BID</td>
<td>204493_at</td>
<td>0.67</td>
</tr>
<tr>
<td>BIK</td>
<td>205780_at</td>
<td>0.25</td>
</tr>
<tr>
<td>XIAP</td>
<td>206537_at</td>
<td>1.30</td>
</tr>
<tr>
<td>CASP9</td>
<td>203984_s_at</td>
<td>0.79</td>
</tr>
<tr>
<td>CYCS</td>
<td>208905_at</td>
<td>0.83</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>208982_at</td>
<td>1.33</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>208981_at</td>
<td>1.33</td>
</tr>
<tr>
<td>CASP1</td>
<td>211367_s_at</td>
<td>0.77</td>
</tr>
<tr>
<td>FAS</td>
<td>216252_x_at</td>
<td>0.99</td>
</tr>
<tr>
<td>TRAIL-R2</td>
<td>210405_x_at</td>
<td>1.73</td>
</tr>
<tr>
<td>TRAIL-R2</td>
<td>209295_at</td>
<td>1.36</td>
</tr>
<tr>
<td>TRAIL-R4</td>
<td>210654_at</td>
<td>1.53</td>
</tr>
</tbody>
</table>

**Figure 2. Apoptosis genes associated with resistance to four individual chemotherapeutic agents in B-lineage ALL**

The global test and Wilcoxon rank-sum test were performed to identify which of the indicated 118 apoptosis probe sets were differentially expressed in B-lineage ALL cells sensitive and resistant to prednisolone (PRED), vincristine (VCR), L-asparaginase (ASP), and daunorubicin (DNR). For each drug, probe sets selected only by the global test ($P < .001$) are light gray, probe sets selected by the global test ($P < .001$) and Wilcoxon rank-sum test with FDR controlled at 5% are black, and probe sets not selected by none of the tests are white. The numbers indicated in colored boxes are the ratio per significant gene between PRED-resistant and PRED-sensitive ALL samples (PRED), VCR-resistant and VCR-sensitive ALL samples (VCR), ASP-resistant and ASP-sensitive ALL samples (ASP), and DNR-resistant and DNR-sensitive ALL samples (DNR).
resistance in both tests toward prednisolone or L-asparaginase, respectively. Gene plots for each drug are shown in Figure S2 and probe-set identification, gene names, and median expression are shown for each drug in Table S2.

The expression of apoptosis-related genes and clinical outcome in pediatric ALL

From the 190 patients included in this study (median follow-up at risk of event, 4.8 years; range, 0.3-10.5 years), 45 had disease-related events and 2 had a competing event, which was censored at the time of occurrence. Apoptosis gene expression profiles measured at initial diagnosis were compared between patients who entered and remained in continuous complete remission (CCR) and those who relapsed during follow-up. Gene plots for each drug are shown in Figure S3 and probe-set identification, gene names and median expression are shown for each drug in Table S3. Seventeen probe sets (13 genes) influenced the global test P value by more than 2 standard deviations. Out of these 17 probe sets, 4 probe sets (3 genes) were also selected by the univariate Cox regression analysis with FDR controlled at 5% (marked with an asterisk in Table 2). Subsequently, each of these 4 significant probe sets was analyzed in a multivariate Cox regression analysis with inclusion of conventional risk criteria (ie, age, white blood cell count, lineage, and genetic subtype [Tables 3-4]). BCL2L13 was the only gene that was independently and significantly associated with treatment outcome (P = .011; Table 3). BCL2L13 expression was significantly associated with treatment outcome when used as continuous variable (P < .001) and when divided into 2 equally sized groups (P = .002; Figure 3). The 5-year probability of disease-free survival (pDFS) plus or minus SE was 85% ± 5.2% for patients with low (below median) and 66% ± 7.3% for patients with high (above median) expression of BCL2L13.

For 92 patients enrolled at St Jude the median follow-up was 6.2 years. Of these patients, 15 had disease-related events and 8 had a competing event, which was censored at the time of occurrence. In this independent cohort treated with the same chemotherapeutic agents but on a different protocol at the St Jude Children’s Research Hospital the association between BCL2L13 expression and outcome was significant in a univariate analysis when treated as a continuous variable (P = .025), but not significant when patients below and above the median were compared (P = .28). In a multivariate Cox analysis including the above-mentioned known risk factors BCL2L13 expression showed the same trend for an association with outcome in patients treated according to St Jude protocols (P = .051; Table 4).

Expression of Apaf-1 isoforms and cellular drug resistance in pediatric ALL

The expression of Apaf-1 splice variants has been linked to functional apoptosis in tumor cell lines.39-41 The presence of an

![Figure 3. Disease-free survival according to BCL2L13 expression in pediatric ALL.](image)

Table 3. Multivariate proportional-hazards analysis of the risk of relapse: COALL/DCOG cohort (N = 190)

<table>
<thead>
<tr>
<th>Age at diagnosis</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10 y</td>
<td>1.00*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Older than 10 y</td>
<td>1.07</td>
<td>0.77-1.53</td>
<td>.242</td>
</tr>
<tr>
<td>WBC count at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 10 x 10^9 cells/L</td>
<td>1.00*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-49 x 10^9 cells/L</td>
<td>1.05</td>
<td>0.81-1.38</td>
<td>.241</td>
</tr>
<tr>
<td>50-100 x 10^9 cells/L</td>
<td>1.12</td>
<td>0.90-1.37</td>
<td>.827</td>
</tr>
<tr>
<td>More than 100 x 10^9 cells/L</td>
<td>1.49</td>
<td>0.45-4.06</td>
<td>.441</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genetic subtype</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-lineage</td>
<td>4.80</td>
<td>1.00*</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>1.33</td>
<td>0.34-5.13</td>
<td>.681</td>
</tr>
<tr>
<td>E2A rearranged</td>
<td>1.10</td>
<td>0.31-3.86</td>
<td>.886</td>
</tr>
<tr>
<td>MLL rearranged</td>
<td>9.45</td>
<td>2.42-36.88</td>
<td>.001</td>
</tr>
<tr>
<td>TEL-AML1</td>
<td>4.44</td>
<td>0.23</td>
<td>0.07-0.80</td>
</tr>
<tr>
<td>Hyperdiploid†</td>
<td>9.28</td>
<td>0.08-9.79</td>
<td>.045</td>
</tr>
<tr>
<td>T-lineage</td>
<td>8.66</td>
<td>0.28-1.60</td>
<td>.359</td>
</tr>
<tr>
<td>BCL2L13‡ expression</td>
<td>1.94</td>
<td>1.47</td>
<td>0.77-1.53</td>
</tr>
</tbody>
</table>

Multivariate Cox regression analysis among 190 patients of the original COALL/DCOG cohort was performed to quantify the independent contribution of BCL2L13 to disease-free survival. Age, white blood cell (WBC) count and genetic subtype were considered as discrete and BCL2L13 expression as a continuous variable in the analysis. A hazard ratio greater than 1 indicates increased probability of relapse. CI denotes confidence interval.

*Reference group.
†Cytogenetic analysis revealed more than 50 chromosomes.
‡Continuous variable.
additional C-terminal WD-40 repeat encoded by exon 18 appears to be required for in vitro activation of pro-caspase-9 and -3. The Affymetrix probe sets are unable to distinguish between the individual isoforms of Apaf-1 (Figure 4). To investigate whether the relative expression of proapoptotic (active) Apaf-1 isoforms (ie, the isoforms containing exon 18) is linked to sensitivity to antileukemic agents, real-time quantitative PCR was carried out in 36 children with ALL at initial diagnosis. Two primer pairs were used: one pair recognizes both pro- and antiapoptotic Apaf-1 isoforms, and one pair hybridizes to exon 18 and is thus specific for the proapoptotic isoform of Apaf-1 (Figure 4). The relative expression of proapoptotic Apaf-1 isoform ranged between 2% and 69% of total Apaf-1 and did not differ significantly in patients sensitive and resistant to prednisolone (P = .74), vincristine (P = .33), L-asparaginase (P = .79), or daunorubicin (P = .95). In addition, the absolute expression of the proapoptotic isoform did not differ significantly in patients sensitive and resistant to prednisolone (P = .96), vincristine (P = .20), L-asparaginase (P = .25), or daunorubicin (P = .67).

**Discussion**

Leukemic subtypes with an unfavorable prognosis may have a decreased tendency to undergo apoptosis compared with subtypes with a favorable prognosis. Gene expression signatures discriminative for lineage,27,42 genetic subtype,27,42 in vitro,29 and in vivo20,42 drug response were previously reported. Among the discriminative genes identified in these studies were virtually no apoptosis genes. This does not rule out a role for apoptosis genes in these leukemic subtypes per se, because these genes may be significant at a lower level than the cut-off P values used for the construction of these signature models. Therefore, we analyzed the expression patterns of 70 selected key apoptotic genes in leukemic cells of 190 children at initial diagnosis of ALL and correlated the expression of these genes to lineage, genetic subtype, in vitro drug resistance, and clinical outcome.

Children with T-lineage ALL have an increased risk of treatment failures compared with children with B-lineage ALL,43 which can be attributed to the presence of numerous adverse presenting features, such as older age, high white blood cell count, and in vitro resistance to a variety of drugs.8,44 However, intensification of treatment regimens has resulted in remarkably improved outcomes for children with T-ALL.45 Although T-ALL has been associated with aberrant expression of some apoptosis genes,46,47 the underlying causes of in vitro drug resistance have not yet been fully determined. Global test analysis indicated that the expression of apoptosis genes differs between T-lineage and B-lineage ALL (P < .001). A large number of apoptosis genes (44 of 70 examined genes) were most discriminative between T-lineage and B-lineage ALL (as defined by $\geq 2$ SD influence on the global test P value; “Patients, materials, and methods”).

The death-receptor Fas has been linked to apoptosis and nuclear factor–κB (NF-κB)–related inflammatory response pathways.48 Activation of NF-κB inhibits drug-induced apoptosis in various cell-line studies and was shown to be linked to drug resistance in childhood ALL.49,51 Interestingly, we observed simultaneous up-regulation of Fas and its downstream effectors (ie, Fas-associated death domain [FADD], caspase-8, and caspase-10) in T- compared with B-lineage ALL and up-regulation of several NF-κB target genes, such as cIAP1, cIAP2, survivin, and FLIP. The relative high expression of NF-κB–associated genes may point to enhanced NF-κB activity in T-lineage compared with B-lineage ALL.

Children with hyperdiploid and TEL-AML1–positive ALL have a favorable prognosis, which is associated with a relatively high in vitro sensitivity to various drugs, including L-asparaginase.11,12 Interestingly, the TNF receptor ligand (TNF) is expressed higher in hyperdiploid and TEL-AML1–positive B-lineage ALL patients (Figure 1). Moreover, TNF is 0.6-fold less expressed in L-asparaginase–sensitive cases (Figure 2). Since both hyperdiploid and TEL-AML1–positive B-lineage ALL are in vitro sensitive to L-asparaginase,11,12 these data point to novel insights in the apoptotic changes underlying L-asparaginase cytotoxicity. Another notable feature of hyperdiploid B-lineage ALL cells is the simultaneous overexpression of TNF-R1, TRAIL-R2, and TRAIL-R4. The overexpression of these cytokine receptors was not previously observed in hyperdiploid B-lineage ALL but may contribute to their marked apoptotic propensity in allogeneic bone marrow–derived stromal layers that contain the microenvironment to trigger these receptors.32

The relation between the expression of apoptosis genes and in vitro53-55 or in vivo response56-59 has been extensively studied in ALL. However, these studies each focused on the expression of only a few genes out of the large family of apoptosis-related genes. In this study, analysis of 70 key apoptotic genes revealed that only 2 and 3 genes were significantly associated with resistance toward prednisolone and L-asparaginase, respectively. Bcl-2 family members are thought of as the central regulators of apoptosis by regulating cytochrome c release upstream of the mitochondria.60 We observed increased expression of the antiapoptotic Bcl-2 family member MCL1 in prednisolone-resistant B-lineage ALL cells and decreased expression of the proapoptotic Bcl-2 family member HRK in L-asparaginase–resistant B-lineage ALL cells. The differential expression of these Bcl-2 family members may contribute to the apoptotic blockage we previously observed upstream of the mitochondria in prednisolone- and L-asparaginase–resistant ALL cells.22 The fact that we observed decreased apoptosis in vincristine- and daunorubicin-resistant ALL cells in the former study,22 and that no apoptosis gene was associated with vincristine and daunorubicin resistance in the present study, suggests that resistance to these drugs is caused by mechanisms that do not appear transcriptionally. Alternatively, VCR and DNR resistance in childhood ALL may be caused by a defect further upstream of the mitochondria. Aberrant expression and function of cytoskeleton-associated genes29,61 and lack of ceramide generation62 are examples of upstream defects that were previously observed in leukemic samples resistant to VCR and DNR, respectively.

**Figure 4.** Quantification of the expression of Apaf-1 isoforms in pediatric ALL. The structure of the Apaf-1 gene and 2 Apaf-1 transcript variants. Indicated is the location of the Affymetrix probe sets (211553_s_at and 204859_s_at) and the Taqman primer pairs (1 and 2).
BCL2L13 (Bcl-rambo) is a recently discovered member of the Bcl-2 family with proapoptotic activity.63,64 Remarkably, however, in this study we observed that a high mRNA expression of BCL2L13 was associated with in vitro L-asparaginase resistance and an unfavorable long-term clinical outcome in children with ALL. This finding suggests BCL2L13 may have a different apoptotic role in primary leukemic cells of children compared with the cell lines used to describe its apoptotic role. Alternative splicing is known to generate both anti- and proapoptotic variants of a single apoptosis gene (eg, Apaf-1; Figure 4).65,66 Therefore, an alternative explanation for our finding may be the existence of a previously unrecognized antiapoptotic splice variant. Probe sets designed by Affymetrix are (in general) not suitable to recognize previously unrecognized antiapoptotic splice variant. Probe sets designed by Affymetrix are (in general) not suitable to recognize differentially expressed splice variants of a single gene. Most importantly, high expression of the BCL2.13 probe set was associated with resistance toward L-asparaginase and independently linked to an unfavorable prognosis compared with other known risk factors. Since BCL2L13 expression was also associated with an inferior outcome in a second (differently treated) validation cohort, this gene may represent a new risk factor in childhood ALL. The fact that only one of the 70 apoptosis genes was independently associated with treatment outcome in this study suggests that treatment outcome in childhood ALL is largely dependent on genes involved in other pathways than the apoptosis pathway. This notion is supported by the absence of apoptosis genes among the genes previously associated with treatment response in several studies in diagnostic childhood ALL samples.62,67,68

In conclusion, this study is the first to describe an association between the differential expression of key apoptosis genes and lineage, genetic subtype, and in vitro drug resistance in children with ALL. In addition, we identified a single gene, BCL2L13, which is related to both L-asparaginase resistance and treatment outcome independent from known prognostic factors in 2 independent cohorts of children with B-lineage ALL. To establish BCL2L13 expression as a true prognostic factor in childhood ALL, prospective validation is required. Also, the currently identified genes warrant further studies on expression and function at the protein level to further increase our insight in the causes of drug resistance and therapy failure in pediatric ALL. It was recently demonstrated that inhibition of McI-1 by the cyclin-dependent kinase (CDK) inhibitor seliciclib induced significant cytotoxicity in multiple myeloma cells sensitive and resistant to conventional therapy.69 In addition, depletion of McI-1 levels by antisense McI-1 oligonucleotides sensitized lung cancer cells to apoptosis induced by cytotoxic agents as well as by ionizing radiation.70 Likewise, it can be hypothesized that down-regulation of BclL13 by antisense oligonucleotides or specific inhibitors may sensitize ALL cells to L-asparaginase and eventually other drugs.

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References
The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia