Genes contributing to minimal residual disease in childhood acute lymphoblastic leukemia: prognostic significance of *CASP8AP2*

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In childhood acute lymphoblastic leukemia (ALL), early response to treatment is a powerful prognostic indicator. To identify genes associated with this response, we analyzed gene expression of diagnostic lymphoblasts from 189 children with ALL and compared the findings with minimal residual disease (MRD) levels on days 19 and 46 of remission induction treatment. After excluding genes associated with genetic subgroups, we identified 17 genes that were significantly associated with MRD. The caspase 8–associated protein 2 *(CASP8AP2)* gene was studied further because of its reported role in apoptosis and glucocorticoid signaling. In a separate cohort of 99 patients not included in the comparison of gene expression profiles and MRD, low levels of *CASP8AP2* expression predicted a lower event-free survival ($P = .02$) and a higher rate of leukemia relapse ($P = .01$) and were an independent predictor of outcome. High levels of *CASP8AP2* expression were associated with a greater propensity of leukemic lymphoblasts to undergo apoptosis. We conclude that measurement of *CASP8AP2* expression at diagnosis offers a means to identify patients whose leukemic cells are highly susceptible to chemotherapy. Therefore, this gene is a strong candidate for inclusion in gene expression arrays specifically designed for leukemia diagnosis. (Blood. 2006;108:1050-1057)

Introduction

Response to therapy in childhood acute lymphoblastic leukemia (ALL) is ultimately linked to the expression of genes that control cellular drug sensitivity and propensity to apoptosis. The discovery of such genes is important because it could provide a means to enhance classification systems based on relapse hazard and to identify signaling pathways that could be productively targeted with novel therapies. Genome-wide expression profiling technology promises to significantly facilitate these discoveries, as shown by studies determining gene expression changes in response to methotrexate and/or mercaptopurine,[1,2] and by correlative studies based on drug sensitivity findings in vitro.[3-5]

Minimal residual disease (MRD) assays provide a direct measure of treatment response in vivo that is likely to depend not only on the resistance of leukemic cells to individual drugs, but on other factors as well, including drug interactions and pharmacokinetic/pharmacogenetic variables.[6-8] Such assays have revealed considerable heterogeneity in the response of childhood ALL patients to remission induction therapy, which was not appreciated from conventional microscopic analyses.[9-14] While some patients can show profound reductions in their leukemia cell counts (to less than one leukemic cell among 10 000 normal bone marrow cells) after only 2 weeks of remission induction chemotherapy,[13,15] others require additional remission induction chemotherapy to achieve a similar level of leukemia cytoreduction, or retain detectable MRD beyond the completion of remission induction and consolidation treatment.[9-13] Thus, treatment response measured by MRD assays has consistently been the most powerful prognostic indicator in childhood ALL.[7,16]

In this study, we sought to identify genes whose expression is closely associated with the in vivo response to multiagent chemotherapy. We therefore compared the gene expression profiles of ALL cells obtained at diagnosis from 189 children with MRD findings on days 19 and 46 of remission induction chemotherapy. Seventeen genes whose expression was specifically associated with MRD at both time points were identified, including *CASP8AP2* (caspase 8–associated protein 2), which encodes a key mediator of apoptosis and glucocorticoid signaling,[17-20] and whose expression in this study was inversely related to persistent MRD during remission induction therapy. In a separate cohort of 99 children with ALL, *CASP8AP2* expression was a strong and independent predictor of treatment outcome.

Materials and methods

Patients and treatment

Bone marrow samples were collected at diagnosis from 288 children with ALL enrolled in St Jude Total Therapy Studies XIII, XIV, or XV. Samples were also collected during remission induction chemotherapy from 189 patients: MRD was studied on day 19 in 187 and on day 46 in 188 of these patients. At diagnosis, the immunophenotypic and karyotypic features of the leukemic cells were determined according to standard techniques.[21,22]

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The presence of *BCR-ABL, E2A-PBX1,* and *TEL-AML1* fusions or *MLL* gene rearrangements was detected by reverse transcriptase–polymerase chain reaction (RT-PCR).[23] Among the 288 ALL cases studied (189 to identify genes associated with MRD and 99 to test the clinical significance of the identified genes), 47 were classified as T-lineage ALL and 241 as B-lineage ALL. The latter included 16 cases with *BCR-ABL,* 22 with *E2A-PBX1,* 18 with *MLL* rearrangements, 57 with *TEL-AML1,* 52 with hyperdiploidy (> 50 chromosomes), and 76 with other features.

Initial treatment consisted of methotrexate alone followed 4 days later by 6 weeks of remission induction therapy with prednisone, vincristine, daunorubicin, asparaginase, and etoposide plus cytarabine.[24-26] Once they attained a complete clinical remission, all patients received 2 weeks of consolidation therapy with high-dose methotrexate and mercaptopurine, followed by risk-directed continuation therapy. The studies were approved by the St Jude institutional review board, with informed consent obtained from the parents or guardians of each child.

Gene expression profiling

Gene expression profiling studies were performed as previously described.[27,28] Briefly, bone marrow mononuclear cells obtained at diagnosis were enriched with a density gradient, washed twice, and cryopreserved. We isolated total RNA from bone marrow mononuclear cells using the Trizol reagent (Invitrogen, Carlsbad, CA). After generating cDNA, we prepared biotin-labeled cRNA hybridization solutions according to the protocols of Affymetrix (Santa Clara, CA). The solutions were hybridized to HG-U133A oligonucleotide microarrays (Affymetrix). After staining with phycoerythrin-conjugated streptavidin, the arrays were read with a laser confocal scanner (Agilent, Palo Alto, CA). Signal values were computed from the image files using Affymetrix GeneChip Operating Software. Signal intensities were normalized to a standard target value of 500. Detection calls (present, marginal, or absent) were determined by default parameters. Intensity values for a total of 22 283 probe sets on the U133A microarray were obtained.

Minimal residual disease studies

Studies of MRD were performed by flow cytometry as previously described.[9,12-14,29] Bone marrow mononuclear cells were labeled with various combinations of monoclonal antibodies conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, and allophycocyanin. For each case, optimal marker combinations specific for the leukemic clone were selected by labeling bone marrow mononuclear cells at diagnosis with antibody combinations previously shown to distinguish leukemic from normal cells; the combinations were then applied during clinical remission. Cell staining was analyzed using a dual laser FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson, San Jose, CA). The flow cytometry protocol used for MRD detection has been described in detail elsewhere.[12,13,30] In all samples, the data represent all mononuclear cells in each test tube ($> 1 \times 10^5$). These detection methods allow the identification of one leukemic cell among 10 000 or more normal bone marrow cells,[30] produce results that are highly concordant with those obtained by PCR analysis of antigen receptor genes,[31] and are currently applicable to more than 95% of patients.

CASP8AP2 expression studies

For detection of *CASP8AP2* expression by RT-PCR, we obtained cDNA by reverse transcription of RNA of established ALL cell lines and primary ALL samples with random hexamers and amplified it using the primers 5′-GAAGGTAATCATCCTGCATT-3′ (sense) and 5′-GAGCTTCATT-AGCTGCTGGA-3′ (antisense). PCR amplification was performed for 30 cycles (95°C for 45 seconds, 56°C for 45 seconds, and 72°C for 60 seconds). The PCR products were separated on a 2% agarose gel, and the DNA was visualized by ethidium bromide staining.

For flow cytometry, we used a specific rabbit polyclonal antibody anti-CASP8AP2 from ProSCI (Poway, CA). Cells were permeabilized with 8E, a reagent developed in our laboratory, and incubated with the antibody or with nonreactive rabbit immunoglobulin (as a control). After 2 washes in phosphate-buffered saline containing 0.2% serum albumin and 0.2% sodium azide, cells were incubated with a goat anti–rabbit Ig antibody conjugated to phycoerythrin (Jackson Laboratories, West Grove, PA). Tests in which the anti-CASP8AP2 antibody was preincubated with the immunizing peptide (ProSCI) were performed to ensure specificity of staining. Cells were analyzed with a FACSCalibur flow cytometer.

In vitro culture of leukemic lymphoblasts

In vitro cultures of leukemic lymphoblasts on bone marrow mesenchymal cells were performed as previously described.[32] Briefly, the leukemic cells were resuspended in AIM-V medium (Gibco, Grand Island, NY) at a final concentration of 1.5×10^6/mL. Of the suspension, 200 μL was then placed in a 96-well tissue culture plate or seeded onto confluent bone marrow mesenchymal cell layers. In all samples, cell viability exceeded 80% by trypan-blue dye exclusion. All cell cultures were performed at 37°C under 5% CO_2.

At the termination of cultures, cells were harvested by vigorous pipetting. B-lineage ALL samples were incubated with CD19 monoclonal antibody conjugated to FITC; T-ALL samples were incubated with FITC-conjugated CD7. All antibodies were from Becton Dickinson. Samples were analyzed with a FACScan flow cytometer with Cell Quest software, as previously described.[32,33] After 7 days of culture, the percentage of cell recovery was calculated as follows: (no. of $CD19^+$ or $CD7^+$ lymphoblasts after 7 days of culture) × 100/(no. of $CD19^+$ or $CD7^+$ lymphoblasts after 1 hour of culture). Results are reported as the means of at least duplicate experiments. Leukemic cells were counted without knowledge of the patient's level of *CASP8AP2* expression.

Statistical analysis

Individual genes associated with MRD adjusted for lineage and genetic subtypes were identified with an analysis-of-variance (ANOVA) model; *t* test analysis was used to identify individual genes associated with MRD without adjustment for other factors. Statistical significance and false discovery rate (FDR) estimates in this part of the analysis were determined using the profile information criterion and the FDR estimator, as described.[34]

Correlations between gene expression, clinicobiologic features of ALL, and MRD status were performed with the Kruskal-Wallis test for multiple samples or Wilcoxon–Mann-Whitney tests for 2 samples. Event-free survival and cumulative incidence of relapse (where death in remission and second malignancy were treated as competing risks) were analyzed by a proportional hazard regression model, as well as by log rank and Gray test, respectively. Cumulative incidence of relapse in relation to competing known prognostic factors of childhood was analyzed with a Fine and Gray model.[35] All analyses were performed with the R (The R Project, http://www.r-project.org/), SAS (SAS Institute, Cary, NC), and S-plus (Insightful, Seattle, WA) programs.

Results

Identification of individual genes associated with MRD during remission induction therapy

We compared gene expression data for the diagnostic bone marrow samples of 189 patients with results of MRD measurements obtained in 187 of these patients on day 19, and in 188 on day 46 of remission induction therapy. MRD positivity was defined as 0.01% or more cells expressing the leukemia-associated immunophenotype identified at diagnosis among bone marrow mononuclear cells. By this criterion, 109 (58.3%) of 187 patients were MRD positive on day 19, and 43 (22.9%) of 188 on day 46, in agreement with our previous findings in different patient cohorts.[12,13] After eliminating the possible confounding influence of genetic subtypes known to be associated with treatment response (*BCR-ABL, MLL* gene

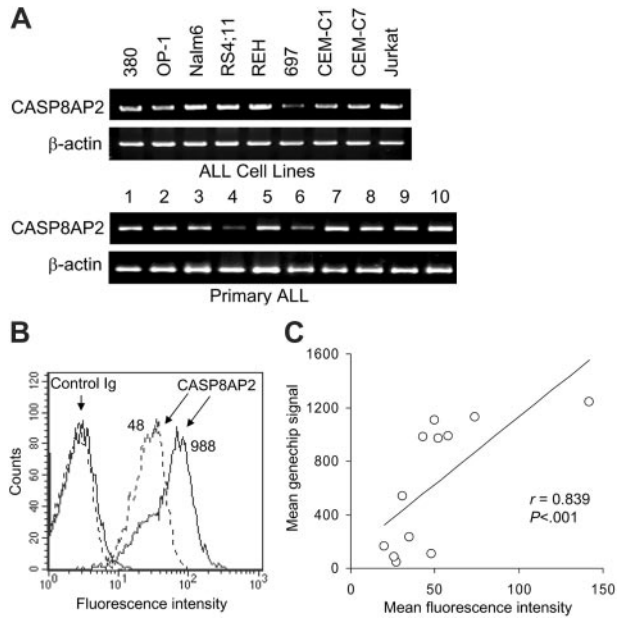

Figure 1. Expression of *CASP8AP2* in leukemic lymphoblasts. (A) Expression of *CASP8AP2* transcripts by RT-PCR in ALL cell lines and 10 primary ALL samples. (B) Expression of CASP8AP2 protein by flow cytometry in 2 cases of ALL. Overlay histograms indicate staining with anti-CASP8AP2 antibody and with nonreactive rabbit immunoglobulin (Control Ig) in each sample. The corresponding *CASP8AP2* signal by gene array in each sample is shown. (C) Relation between *CASP8AP2* transcript expression by gene array and protein expression by flow cytometry. Spearman correlation coefficient is shown.

rearrangements, *TEL-AML1,* and hyperdiploidy > 50 chromosomes), and applying a *P* value threshold of .001 by *t* test (estimated FDR, 17.1%),[34] we identified 105 probe sets whose expression was associated with MRD on day 46. The probe sets corresponded to 85 named genes; 53 were overexpressed in diagnostic samples from patients with MRD on day 46, and 32 were underexpressed (Table 1). Expression of 17 of the 85 genes was also significantly ($P < .02$) related to the presence or absence of MRD at day 19: 10 such genes were overexpressed in diagnostic samples of patients who had MRD at days 19 and 46, whereas 7 were underexpressed (Table 1).

A review of the reported functions of the 17 genes associated with MRD at both time points led us to select *CASP8AP2* (caspase 8–associated protein 2), also known as *FLASH* (FLICE-associated huge protein), for further study. This gene encodes a protein that interacts with caspase 8, a key mediator of apoptosis,[17,18] and has been shown to be a determinant of glucocorticoid signaling as well.[19,20] The reported function of CASP8AP2 together with its underexpression in leukemic cells from patients with a poor initial early response to remission induction therapy (as demonstrated by the presence of MRD on days 19 and 46) provided a compelling rationale for assessing its clinical significance in childhood ALL.

CASP8AP2 transcripts were detectable in ALL cells by RT-PCR. In all 9 ALL cell lines (B-lineage: 380, OP-1, NALM6, RS4;11, REH, and 697; T-lineage: CEM-C1, CEM-C7, and Jurkat) and in 10 primary ALL samples (not included in the gene expression array study), a transcript of approximately 700 kb corresponding to *CASP8AP2* was clearly detectable (Figure 1A). To demonstrate that the *CASP8AP2* transcript was translated into the encoded protein in ALL cells, we used a rabbit polyclonal antibody anti-CASP8AP2. Labeling of the ALL cell line REH with this antibody after cell membrane permeabilization stained virtually all cells; staining was prevented by preincubating the antibody with the immunizing peptide corresponding to amino acids 1966 to 1981 of the human CASP8AP2 protein (not shown). In general, levels of protein expression correlated with those of the CASP8AP2 transcripts, as shown by staining of 12 primary ALL samples selected among those studied by microarray ($r = 0.839$, $P < .001$ by Spearman correlation test; Figure 1B-C). We noted, however, that differences in levels of transcript expression were generally higher than those measured by flow cytometry. It is unclear whether this was due to the low affinity of the polyclonal antibody used or to posttranslational regulatory mechanisms. Such a discrepancy has been reported for other molecules expressed in ALL cells.[36]

Relation of *CASP8AP2* expression to selected clinicobiologic features of ALL and treatment outcome

To assess the relationship of *CASP8AP2* expression at diagnosis to the clinical and biologic features of ALL in 288 children with ALL, we divided the patients into 3 groups of 96 patients each according to level of *CASP8AP2* expression measured by gene array. Among clinical features, low levels of expression were significantly more prevalent among patients younger than 1 year of age, a known adverse prognostic feature ($P = .016$),[6] whereas patients with hyperdiploidy (> 50 chromosomes), a feature associated with favorable outcome,[6] generally had higher levels of *CASP8AP2* expression ($P < .001$) (Table 2). Using *CASP8AP2* expression as a continuous variable, we found that low levels of expression were again significantly associated with age younger than 1 year ($P = .012$) and less favorable genetic subtypes ($P < .001$). No significant associations between *CASP8AP2* and other presenting clinicobiologic features were apparent (Table 2). Levels of *CASP8AP2* were not different among patients enrolled in the 3 sequential treatment protocols (Total XIII, XIV, and XV; $P = .16$ by Kruskal-Wallis test).

As expected, low *CASP8AP2* expression was significantly associated with slow early treatment response as defined by the presence of MRD at day 19 ($P = .006$) and at day 46 ($P < .001$;

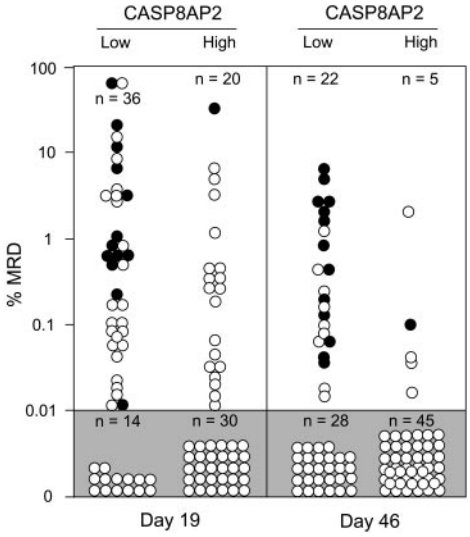

Figure 2. Prevalence of MRD (by level) on days 19 and 46 in 2 groups of patients with the lowest and highest expression of *CASP8AP2* among 189 patients studied. Solid circles denote persistent MRD and/or hematologic relapse after day 46, while open circles denote continuous complete remission without MRD

Table 1. Genes significantly associated with MRD on day 46

Probe set	Representative public ID	Gene title
Genes underexpressed in MRD+		
206502_s_at	NM_002196	Insulinoma-associated 1
217281_x_at	AJ239383	IgM rheumatoid factor RF-TT9, variable heavy chain
217098_s_at	Z98745	Zinc finger protein 96
218736_s_at	NM_017734	Palmdelphin
207894_s_at	NM_020552	T-cell leukemia/lymphoma 6
220657_at	NM_018143	Kelch-like 11 (Drosophila)
206142_at	NM_003436	Zinc finger protein 135 (clone pHZ-17)
203702_s_at	AL043927	Tubulin tyrosine ligase-like family, member 4*
207940_x_at	NM_001840	Cannabinoid receptor 1 (brain)
207979_s_at	NM_004931	CD8 beta polypeptide 1*
218986_s_at	NM_017631	Hypothetical protein FLJ20035
215717_s_at	X62009	Fibrillin 2 (congenital contractural arachnodactyly)
212419_at	AA131324	Chromosome 10 open reading frame 56
219364_at	NM_024119	Likely ortholog of mouse D11lgp2
209760_at	AL136932	KIAA0922 protein*
218033_s_at	NM_003498	Stannin
216444_at	AK024138	SMAD-specific E3 ubiquitin protein ligase 2
203276_at	NM_005573	Lamin B1*
209502_s_at	BC002495	BAI1-associated protein 2
205888_s_at	AI962693	Jak and microtubule interacting protein 2
203422_at	NM_002691	Polymerase (DNA directed), delta 1, catalytic subunit 125 kDa
211717_at	BC005853	Ankyrin repeat domain 40
203963_at	NM_001218	Carbonic anhydrase XII
218115_at	NM_018154	ASF1 antisilencing function 1 homolog B *(S cerevisiae)**
219165_at	NM_021630	PDZ and LIM domain 2 (mystique)
209499_x_at	BF448647	Tumor necrosis factor (ligand) superfamily, member 13
202326_at	NM_006709	Euchromatic histone-lysine *N*-methyltransferase 2*
222201_s_at	AB037736	CASP8-associated protein 2*
38158_at	D79987	Extra spindle poles-like 1 *(S cerevisiae)*
218586_at	NM_018270	Chromosome 20 open reading frame 20
204804_at	NM_003141	Tripartite motif-containing 21
204599_s_at	NM_006428	Mitochondrial ribosomal protein L28
Genes overexpressed in MRD+		
201429_s_at	NM_000998	Ribosomal protein L37a
200025_s_at	NM_000988	Ribosomal protein L27
200034_s_at	NM_000970	Ribosomal protein L6
200038_s_at	NM_000985	Ribosomal protein L17
200949_x_at	NM_001023	Ribosomal protein S20
212042_x_at	BG389744	Ribosomal protein L7
200716_x_at	NM_012423	Ribosomal protein L13a
208904_s_at	BC000354	Ribosomal protein S28
208856_x_at	BC003655	Ribosomal protein, large, P0
216520_s_at	AF072098	Tumor protein, translationally controlled 1
200019_s_at	NM_001997	Ribosomal protein S30
200927_s_at	AA919115	RAB14, member RAS oncogene family
202649_x_at	NM_001022	Ribosomal protein S19
200937_s_at	NM_000969	Ribosomal protein L5
211927_x_at	BE963164	Eukaryotic translation elongation factor 1 gamma
212773_s_at	BG165094	Translocase of outer mitochondrial membrane 20 homolog (yeast)
213890_x_at	AI200589	Ribosomal protein S16
208724_s_at	BC000905	RAB1A, member RAS oncogene family
200081_s_at	BE741754	Ribosomal protein S6
200005_at	NM_003753	Eukaryotic translation initiation factor 3, subunit 7 zeta, 66/67 kDa
217915_s_at	NM_016304	Chromosome 15 open reading frame 15
218268_at	NM_022771	TBC1 domain family, member 15
217747_s_at	NM_001013	Ribosomal protein S9
201259_s_at	AI768845	Synaptophysin-like 1
217719_at	NM_016091	Eukaryotic translation initiation factor 3, subunit 6 interacting protein
211937_at	NM_001417	Eukaryotic translation initiation factor 4B
218041_x_at	NM_018573	Solute carrier family 38, member 2
214351_x_at	AA789278	Ribosomal protein L13

Table 1. Continued

Probe set	Representative public ID	Gene title
221726_at	BE250348	Ribosomal protein L22
204102_s_at	NM_001961	Eukaryotic translation elongation factor 2
209510_at	AF064801	Ring finger protein 139
200024_at	NM_001009	Ribosomal protein S5
201337_s_at	NM_004781	Vesicle-associated membrane protein 3 (cellubrevin)*
200632_s_at	NM_006096	N-myc downstream regulated gene 1
218084_x_at	NM_014164	FXYD domain containing ion transport regulator 5*
212202_s_at	BG493972	DKFZP564G2022 protein
221646_s_at	AF267859	Zinc finger, DHHC-type containing 11
203544_s_at	NM_003473	Signal transducing adaptor molecule 1*
218562_s_at	NM_018202	Transmembrane protein 57
206890_at	NM_005535	Interleukin 12 receptor, beta 1
204426_at	NM_006815	Coated vesicle membrane protein
208330_at	NM_021926	Aristaless-like homeobox 4
221718_s_at	M90360	A kinase (PRKA) anchor protein 13
209288_s_at	AL136842	CDC42 effector protein (Rho GTPase binding) 3*
202393_s_at	NM_005655	Kruppel-like factor 10
209732_at	BC005254	C-type lectin domain family 2, member B*
209795_at	L07555	CD69*
212509_s_at	BF968134	Matrix-remodeling associated 7*
209543_s_at	M81104	CD34
213075_at	AL050002	Olfactomedin-like 2A*
201904_s_at	BF031714	CTD small phosphatase-like
215177_s_at	AV733308	Integrin, alpha 6*
201325_s_at	NM_001423	Epithelial membrane protein 1*

*Genes also associated with MRD on day 19.

Wilcoxon–Mann-Whitney test; data not shown). Figure 2 illustrates the prevalence of MRD on days 19 and 46 in 2 groups of patients with the lowest and highest levels of *CASP8AP2* expression, respectively. On day 19, MRD was detected in 36 of the 50 patients with low expression of this gene versus 20 of the 50 with high expression; on day 46, these prevalence rates were 22 of 50 versus 5 of 50, respectively. Among the positive samples, higher levels of MRD were found primarily in cases with low *CASP8AP2* expression. For example, 7 patients with low *CASP8AP2* expression, compared with 1 with high expression, had an MRD level higher than 1% at day 46, a feature typically associated with a dismal outcome and an indication for hematopoietic stem cell transplantation.[12] Indeed, all but 1 of the 7 patients with this finding and low *CASP8AP2* expression have relapsed or shown persistent MRD after day 46 of remission induction therapy.

To test the suggestive relationship of *CASP8AP2* level with clinical outcome in Figure 2, we focused our analysis on a separate group of 99 patients enrolled in St Jude Total Therapy Study XIII.[24] As a continuous variable, *CASP8AP2* expression measured by gene array at diagnosis was significantly associated with event-free survival ($P = .023$) and with cumulative incidence of ALL relapse ($P = .013$) in a proportional hazard regression model. When the patients were divided into 3 groups of 33 each according to level of *CASP8AP2* expression, those with high levels of expression had a significantly better event-free survival rate than those with intermediate or low levels ($P = .011$ by log rank test; Figure 3A), and a lower cumulative incidence of relapse ($P = .043$ by Gray test; Figure 3B). In a cumulative incidence regression model including all major presenting features associated with prognosis in childhood ALL, expression of *CASP8AP2* remained a significant predictor of outcome (Table 3). Note that age older than 10 years was the only factor that increased the relapse hazard more than low *CASP8AP2* expression did in this analysis. The results indicate that *CASP8AP2* levels measured in diagnostic samples of leukemic blasts are a powerful predictor of treatment response in childhood ALL.

We performed similar analyses with 2 other genes included in the group of 17 genes associated with MRD: integrin α6 and tissue matrix remodeling-like gene (MXRA7; Table 1). Both genes were overexpressed in patients with a positive MRD assay at days 19 and 46. However, we found that expression of neither gene was significantly associated with ALL relapse in the independent cohort of 99 patients (not shown).

CASP8AP2 expression is associated with the capacity of ALL cells to grow in vitro

To begin to define the role of *CASP8AP2* in ALL cell biology, we determined the association between *CASP8AP2* expression and the capacity of leukemic cells to survive and grow in vitro. For this purpose, we used a 7-day culture assay in which the survival of leukemic cells is supported by bone marrow mesenchymal cells.[37] This assay is well suited to test the growth potential of leukemic cells, a feature that correlates with treatment outcome.[38] We previously found that cells from approximately 50% of ALL cases expand in vitro when grown on mesenchymal cell layers, whereas in the remaining cases, the leukemic cells undergo apoptosis.[32]

After dividing 24 cases of ALL studied by expression array into 2 equal groups based on the expression of *CASP8AP2,* we compared recovery of leukemic cells after 7 days of culture. As shown in Figure 4, the recovery of lymphoblasts was significantly lower in cases with higher *CASP8AP2* expression ($P = .018$), in agreement with morphologic and flow cytometric evidence of apoptosis (not shown). This result together with the reported function of CASP8AP2 suggests that the lower prevalence of MRD and better outcome in patients with high *CASP8AP2* expression could be related to a higher propensity of the cells to undergo apoptosis and a lower capacity for expansion.

Table 2. Correlation between *CASP8AP2* gene expression and presenting clinicobiologic features

Presenting feature	Patients studied, no.	*CASP8AP2* expression, no.			*P**
		Lower	Middle	Higher	
Age					
Younger than 1 y	9	7	1	1	.016
1 to 9 y	203	58	71	74	
10 y or older	76	31	24	21	
Race					
White	214	74	67	73	.401
Black	55	19	21	15	
Other	19	3	8	8	
Sex					
Male	169	58	56	55	.905
Female	119	38	40	41	
WBC, × 10^9/L					
Less than 10	90	27	30	33	.328
10 to 50	85	27	26	32	
50 to 100	52	16	17	19	
More than 100	61	26	23	12	
BCR-ABL					
Present	16	6	5	5	.936
Absent	272	90	91	91	
TEL-AML1					
Present	57	15	19	23	.352
Absent	231	81	77	73	
MLL-AF4					
Present	18	10	5	3	.099
Absent	270	86	91	93	
Ploidy					
Hyperdiploid†	52	8	15	29	< .001
Others	236	88	81	67	
DNA index					
1.16 or higher	47	8	11	28	< .001
Less than 1.16	241	88	85	68	

WBC indicates white blood cell count.
*Calculated by general association test.
†More than 50 chromosomes.

Discussion

The wealth of information generated by microarray studies provides unprecedented opportunities for identifying molecules that influence the propensity of leukemic cells to undergo apoptosis and hence their susceptibility to multiagent chemotherapy in vivo. We postulated that genes whose expression is associated with the presence of MRD during remission induction should have significant prognostic impact. After adjusting for associations with known ALL subtypes and using *P* values below .001 as a cutoff, we found 85 genes whose expression level was associated with MRD on day 46. Comparison of the reported functions of these genes led us to select *CASP8AP2 (FLASH),* a member of the apoptosis signaling complex that activates caspase 8 and facilitates Fas-induced apoptosis,[17] as a candidate for further study. CASP8AP2 participates in apoptosis,[17,18] and its enforced expression in brain cells increases glucocorticoid receptor–mediated transactivation.[20] The pro-apoptotic function of CASP8AP2, together with the down-regulation of *CASP8AP2* in the leukemic lymphoblasts of patients with persistent MRD during remission induction therapy, strongly suggested that this gene may be an important prognostic factor in ALL.

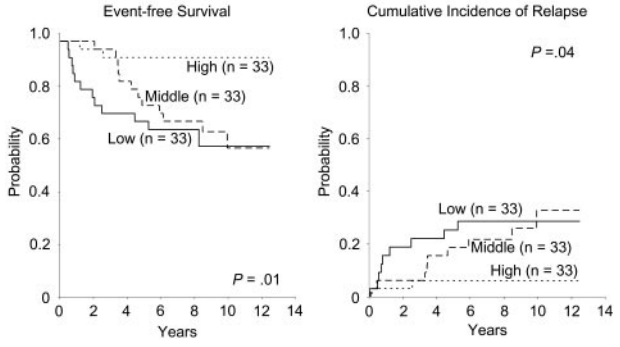

Figure 3. Prognostic impact of *CASP8AP2* expression in a group of 99 patients enrolled in St Jude Total Therapy Study XIII. Patients were divided into 3 equal groups according to level of *CASP8AP2* expression measured by gene array at diagnosis. Event-free survival and cumulative incidence of relapse are shown.

In an independent cohort of children with ALL, we found a striking association between low levels of *CASP8AP2* expression and a high rate of leukemia relapse. In this cohort, low expression of *CASP8AP2* was a very strong predictor of ALL relapse, second only to age older than 10 years. Low *CASP8AP2* expression was more prevalent among patients younger than 1 year of age, a subgroup with generally poor response to therapy,[39] whereas high *CASP8AP2* expression was more prevalent among patients with hyperdiploidy, a favorable genetic abnormality.[40-42] However, the

Table 3. Cumulative risk of ALL relapse according to *CASP8AP2* expression in relation to that of other selected clinical and biologic variables

Variable	Hazard ratio	95 % CI of hazard ratio		*P*
		Lower	Upper	
***CASP8AP2* expression**				
Low vs high*	7.98‡	1.45	43.83	.017
Log-transformed signal†	6.32‡	1.83	21.79	.004
Age, y				
Younger than 1 vs 1 to 10	5.63	0.71	44.61	.100
Older than 10 vs 1 to 10	10.33‡	3.00	35.61	< .001
WBC, × 10^9/L				
More than 10 to 49 vs 10 or less	0.86	0.22	3.38	.830
50 to 99 vs 10 or less	2.72	0.50	14.72	.240
100 or more vs 10 or less	4.15	0.95	18.22	.059
Genotype (B-lineage only)				
BCR-ABL vs other	6.45‡	1.69	24.65	.001
E2A-PBX vs other	0.96	0.26	3.47	.940
MLL-AF4 vs other	1.70	0.54	5.38	.370
TEL-AML1 vs other	5.18	0.96	27.92	.056
Hyperdiploidy vs other	0.37	0.03	4.62	.440
Lineage				
T vs B	6.12‡	1.58	23.78	.009

Analyzed using Fine and Gray's estimator.[35]
*Comparisons of relapse hazards for 33 patients with the lowest levels of *CASP8AP2* expression versus the 33 patients with the highest levels.
†Increase of risk per unit decrease in log *CASP8AP2* expression.

relation between *CASP8AP2* expression with age and chromosome number was not absolute, and, in a multivariate analysis, it remained a significant predictor of outcome.

Recently, Holleman et al[4] reported correlations between expression of apoptosis-related genes and drug sensitivity in vitro in samples of childhood ALL cells. Their study focused on the prognostic significance of another apoptosis regulator, BCL2L13, but we noted that in their analysis *CASP8AP2* expression was, on average, twice as high in prednisone-sensitive cases and was associated with higher sensitivity to asparaginase and daunorubicin. In our study, higher *CASP8AP2* levels were related to a reduced capacity of leukemic lymphoblasts to grow in vitro, suggesting that this feature confers a general propensity to undergo apoptosis. *CASP8AP2* was not detected in 2 other screenings of genes associated with early treatment response in ALL. The study by Chiaretti et al[43] analyzed genes associated with early treatment response in 33 adult patients with T-ALL and was performed with the HG-U95A Affymetrix GeneChip, which does not include probes for *CASP8AP2*. The study of Cario et al[44] analyzed gene expression in 51 patients classified as either "MRD standard risk" or "MRD high risk" according to the BFM2000 criteria but was performed on a different microarray platform.[45] Both groups of investigators noted that low expression of *TTK* (a gene encoding a kinase involved in cell-cycle regulation)[46] was associated with poorer treatment response. Remarkably, low expression of *TTK* was also associated with the presence of MRD on both days 19 and 46 in our series, although its association with MRD was weaker than that of *CASP8AP2* and of the other genes listed in Table 1. Nevertheless, the consistent association of this gene with treatment response clearly merits further investigation.

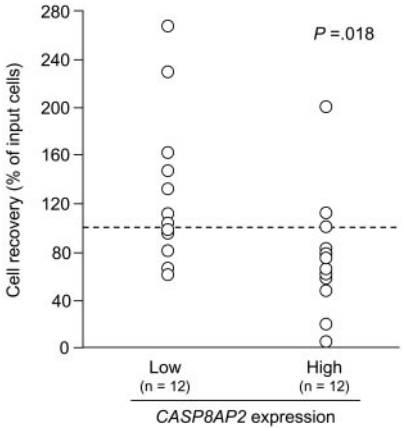

Figure 4. Cell recovery of primary ALL cells after culture on mesenchymal cell layers according to *CASP8AP2* expression. Primary ALL cells, selected from cases with known *CASP8AP2* levels by GeneChip, were cultured on confluent mesenchymal cells for 7 days in serum-free medium. The number of viable ALL cells recovered at the end of the cultures was compared with the number of cells originally seeded. The dashed line indicates median cell recovery for the 24 cases. *P* value was calculated by Wilcoxon 2-sample test.

Further progress in the treatment of childhood ALL will require optimization of risk assignment to avoid overtreatment and undertreatment of patients as well as the development of new antileukemic agents capable of overcoming drug resistance.[6,47] We suggest that measurements of *CASP8AP2* expression could help to identify patients whose leukemic cells are highly susceptible or highly resistant to chemotherapy. Indeed, this gene is a strong candidate for inclusion in gene arrays specifically designed for leukemia diagnosis. Since patients with detectable MRD during remission induction therapy appeared to have a much higher risk of relapse if their leukemic cells had low levels of *CASP8AP2,* measurements of this gene's levels could also be used to augment the informative power of MRD studies.

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