Expression and prognostic significance of the apoptotic genes *BCL2L13*, *Livin*, and *CASP8AP2* in childhood acute lymphoblastic leukemia

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ABSTRACT

Improved treatment of childhood acute lymphoblastic leukemia (ALL) depends on the identification of new molecular markers that are able to predict treatment response and clinical outcome. The development of impaired apoptosis in leukemic cells is one factor that may influence their response to treatment. We investigated the expression of three apoptosis related genes, *BCL2L13*, *CASP8AP2*, and *Livin*, as well as their prognostic significance, in a retrospective study of 90 pediatric ALL patients diagnosed between 1996 and 2007 in Taiwan. Univariate analysis revealed that high expression of *BCL2L13* was associated with inferior event-free survival and overall survival ($p < 0.001$ and 0.005 , respectively). Multivariate analysis for EFS and OS demonstrated that high expression of *BCL2L13* was an independent prognostic factor for childhood ALL in this ethnic group.

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1. Introduction

Recent progress in the use of DNA microarrays has identified genes that regulate cell cycle control, DNA repair, and apoptosis which may also participate in disordered cell proliferation and cancer progression in leukemia [1,2]. Alterations in the basal level of expression of these genes may also affect the drug response and clinical outcome of leukemic patients. The cure rate of childhood acute lymphoblastic leukemia (ALL) has increased from 10 to 80% in the developed countries, a fact which may largely be a consequence

of the stringent application of prognostic factors for risk-factor-directed therapy [3,4]. The identification of new such gene markers is therefore important not only to gain a basic understanding of the signaling pathways that operate in leukemogenesis, but also to implement enhancements to disease classification systems and to productively target disease with novel therapies.

It has been shown that decreased apoptosis may be an important step in the acquisition of cellular drug resistance in pediatric acute leukemia. Holleman et al. used microarrays to investigate the expression of 70 apoptosis genes, and revealed that *BCL2L13* expression was an independent prognostic factor [5]. Flotho et al. analyzed gene expression in diagnostic lymphoblasts, and compared the findings to minimal residual disease (MRD) levels on days 19 and 46 of remission induction therapy. They identified 17 genes that were significantly associated with MRD level. Among these, the gene coding for caspase 8-associated protein 2 (*CASP8AP2*) was studied further and showed a strong correlation with prog-

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nosis [6]. Chio et al. studied the expression of *Livin*, a member of the IAP family of childhood ALL [7]. Unexpectedly, *Livin* expression was an independent prognostic factor in their cohort. These studies demonstrated an association between dysregulated apoptosis pathways and treatment prognosis, and suggested that the identified genes might serve as functionally defined risk factors for treatment stratification, in addition to those factors currently used.

If specific patterns of gene expression can be correlated with clinical features in childhood ALL, a refinement of current prognosis-based stratification systems might be possible. Thus, we analyzed *BCL2L13*, *CASP8AP2*, and *Livin* gene expression in the childhood ALL of a specific ethnic group, and investigated their correlation with treatment outcomes.

2. Design and methods

2.1. Patients

Viable diagnostic bone marrow (BM) or peripheral blood (PB) was obtained from 90 children who were diagnosed with ALL between July 1996 and August 2007 at National Taiwan University Hospital (NTUH) and National Cheng Kung University Hospital (NCKUH). Of these, 78 patients had been newly diagnosed with B-precursor ALL, and 12 patients with T-cell ALL. Forty-seven patients were treated with TPOG-93-ALL protocols and 43 patients were treated with TPOG-2002-ALL protocols, which are described in detail elsewhere [8,9]. The diagnosis of ALL was made based on the morphologic findings of BM aspirates, as well as on immunophenotype analyses of leukemic cells by flow cytometry. Conventional cytogenetics analyses were performed as part of the routine workup.

Patients were prospectively assigned to one of three risk groups (standard, high, and very high) based on their presenting clinical features and the biological features of their leukemic cells. Patients were considered to have standard-risk (SR) ALL if they were between 1 and 9 years old with a presenting leukocyte count less than 10×10^9 (cells/L) or were between 2 and 7 years old with a presenting leukocyte count between 10×10^9 and 50×10^9 . Patients were considered to have high-risk (HR) ALL if they were between 1 and 9 years old with a presenting leukocyte count between 50×10^9 and 100×10^9 , or between 1 and 2 or 7 and 10 years old with a presenting leukocyte count between 10×10^9 and 50×10^9 . Patients with at least one of the following were assigned to the very-high-risk (VHR) group: age younger than 1 year, initial leukocyte count greater than 100×10^9 , T-cell ALL, or presence of *BCR-ABL*, *MLL-AF4* or other *MLL* rearrangements in pre-B ALL.

The risk-directed Taiwan Pediatric Oncology Group (TPOG) protocols consisted of multiple chemotherapeutic agents of different intensities. The treatment protocol was upgraded if complete remission was not achieved after initial induction therapy. Events were defined as any relapse, death, or secondary malignancy. This study was approved by the Medical Ethics Committee of National Taiwan University Hospital. Informed consent was obtained from the patients or their parents before sample collection.

2.2. Methods

2.2.1. RNA isolation

Mononuclear cells from bone marrow (BM) or peripheral blood (PB) were Ficoll-purified and immediately stored in liquid nitrogen. Cryopreserved samples were thawed and washed in FBS-supplemented RPMI 1640 medium prior to RNA extraction. Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen, Paisley, United Kingdom).

2.2.2. Determination of *BCL2L13*, *Livin* and *CASP8AP2* expression by comparative real-time quantitative-reverse transcriptase polymerase chain reaction (Q-RT-PCR)

The mRNA expression levels of *BCL2L13*, *Livin*, *CASP8AP2* and *glyceraldehydes-3-phosphate dehydrogenase (GAPDH)* were measured by Q-RT-PCR using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Mature mRNAs were reverse-transcribed into cDNA using oligo-dT and random hexamers as primers by standard methods as described previously [10]. Quantitative PCR amplification of individual cDNAs was performed using the pre-developed Taqman gene expression assay for *BCL2L13* (Hs00209789.m1), *Livin* (Hs00223384.m1) and *CASP8AP2* (Hs01594281.m1) (Applied Biosystems, Foster City, CA). TaqMan endogenous control assay for the *GAPDH* was combined with the assay for target gene in the same reaction (Applied Biosystems). All reactions were performed in duplicate at a minimum. The 20 μ L PCR reaction mixture contained 12.5 ng cDNA, 900 nM primers, 250 nM probe, and 1 $\times$ Taqman universal master mix, combining AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTPs with dUTP, and optimized buffer components. After an initial incubation at 50 °C for 2 min, and a denaturing step at 95 °C for 10 min, a 2-step PCR (95 °C for 15 s followed by 60 °C for 1 min) was performed for 45 cycles to amplify and detect the target sequence.

The expression levels of the target genes in unknown samples were normalized standardized for expression of *GAPDH* and analyzed by the $\Delta\Delta C_t$ method

$[\Delta\Delta C_t = (C_{t_{\text{target gene}}} - C_{t_{\text{GAPDH}}})_{\text{sample}} - (C_{t_{\text{target gene}}} - C_{t_{\text{GAPDH}}})_{\text{calibrator}}]$. The average of ΔC_t ($C_{t_{\text{target gene}}} - C_{t_{\text{GAPDH}}}$) from all samples combined was defined as 0, as the calibrator. The amplification efficiencies for target genes and *GAPDH* were calculated and showed the same slopes. A negative control without the templates was also included in each experiment.

2.2.3. Statistical methods

Comparison of baseline clinical variables across groups was made using the Fisher's exact test for categorical data. The nonparametric Mann–Whitney *U*-test was applied for continuous variables. A *p*-value <0.05 (two-sided) was considered significant.

Patients analyzed for *BCL2L13* expression were initially divided into two groups according to their median level of *BCL2L13* (median = 5.91 as cut-off point). We divided the patients into three groups of 30 patients each according to the *CASP8AP2* expression levels (5.5 and 10.5 as cut-off point). The patients were categorized into two groups according to the presence or absence of the *Livin* expression.

OS was calculated using the Kaplan–Meier method, and the log-rank test was used to compare differences between survival curves. OS was measured from the protocol commencement date until the date of death regardless of cause, excluding patients who were alive at last follow-up. EFS was defined only for patients who achieved complete remission, and was measured from the date of attaining CR until the date of relapse. Patients with no report of relapse by the end of the follow-up observation were censored on the date of last follow-up.

Cox proportional hazard models were constructed for EFS and OS. The following covariates were included in the full model of OS and EFS: *BCL2L13* expression (low vs. high), *CASP8AP2* expression (low, median and high), *Livin* expression (presence or absence), sex, WBC (<100,000/ μ L vs. 100,000/ μ L), age (<10 years vs. >10 years), immunophenotypes (B or T), hospital, and genetic subtypes. Stepwise backward selection was performed. All calculations were performed using the SAS software package, version 9 (SAS Inc.).

3. Results

3.1. Patient characteristics (Table 1)

The clinical characteristics of patients at the time of diagnosis are presented in Table 1. The median age among 90 patients (46 boys and 44 girls) was 4.3 years (range: 0–17 years), and their median leukocyte count was 20.0 (range: 0.2–1826). The number of SR, HR, and VHR patients was 24, 31, and 35 respectively.

3.2. The association of *BCL2L13*, *CASP8AP2* and *Livin* expression level with clinical outcomes

Patients were defined as low and high *BCL2L13* as described in Section 2. The *BCL2L13* expression level did not differ among the different risk groups (Table 1). Patients with lower *BCL2L13* expression had better EFS ($p < 0.001$), and better OS ($p = 0.005$) (Fig. 1).

Patients were defined as low, median and high *CASP8AP2* as described above. The *CASP8AP2* expression level did not differ among the different risk groups (Table 1). The prognosis did not differ between different *CASP8AP2* expression groups (Fig. 2).

As expected, approximately 21.1% of patients expressed *Livin*, as was reported by Choi et al. In our series, 20 patients expressed *Livin* and 70 patients did not. We compared EFS and OS between patients who did and did not express *Livin*. There was no statistically significant difference between these two groups of patients (Fig. 3).

3.3. Multiple variable (Cox regression) analysis reveals that *BCL2L13* was an independent prognostic factor

We performed multivariable Cox regression analyses examining the correlation between EFS or OS and *BCL2L13*, *CASP8AP2* and *Livin* status, other known prognostic factors, and age and white count listed in Table 1. When other significant predictors of EFS were controlled for in the final model, *MLL* gene rearrangement, initial white counts more than 100,000/ μ L, risk groups and the expression level of *BCL2L13* were correlated with EFS (hazard ratio for *BCL2L13* = 4.11, $p = 0.0025$) (Table 2). When other significant predictors of OS were controlled for in the final model, *MLL* gene rearrangement, initial white counts more than 100,000/ μ L, and the expression level of

Table 1

The clinical features of patients at diagnosis and their association with three genes studied.

	<i>BCL2L13</i>			<i>CASP8AP2</i>				<i>Livin</i>		
	Low number	High number	<i>p</i> -value*	Low number	Median number	High number	<i>p</i> -value*	Positive number	Negative number	<i>p</i> -value*
WBC										
<100,000/ μ L	36	12	0.462	24	21	23	0.749	16	52	0.771
>100,000/ μ L	9	13		6	9	7		4	18	
Immunophenotype										
B	36	42	0.118	23	24	27	0.578	18	16	1.000
T										
Age										
<10 years	33	32	1.000	21	22	22	1.000	16	49	0.572
>10 years	12	13		9	8	8		4	21	
Risk groups										
Standard risk	14	10	0.335	9	7	8	0.929	7	17	0.592
High risk	17	14		11	11	9		7	24	
Very high risk	14	21		10	12	13		6	29	
Hyperdiploid										
Negative	36	41	0.230	23	28	26	0.217	58	19	0.283
Positive	9	4		7	2	4		12	1	
<i>t</i> (9;22)										
Negative	45	43	0.494	30	28	30	0.326	20	69	1.000
Positive	0	2		2	0	2		0	4	
<i>MLL</i> gene										
Negative	45	41	0.117	28	30	28	0.54	20	66	0.572
Positive	0	4		2	0	2		0	4	
Hospital										
NTUH	29	38	0.052	24	20	23	0.565	52	15	1.000
MCKUH	16	7		6	10	7		18	5	

* Fisher exact test.

BCL2L13 were correlated with OS (hazard ratio for *BCL2L13* = 3.41, $p = 0.0244$) (Table 3).

3.4. Three gene signature as a predictor as outcome

We attempted to develop a risk signature using the combination of three markers studied. The signature was $CASP8AP2 \times 0.03869 + BCL2L13 \times 0.02747 + Livin$ code (0 or 1) – 0.59135. The patients with high-risk signature had inferior EFS than patients with low-risk signature ($p = 0.0084$, median = 0.463 as cut-off point) (Fig. 4). We performed multivariable Cox regression analyses examining the correlation between EFS and other known prognostic factors, genetic types, age and white count listed in Table 1. The results were shown in Table 4. When other significant predictors of EFS were controlled for in the final model, *MLL* gene rearrangement,

Table 3

Multivariate Cox regression analysis for OS.

Variables	Hazard ratio (HR)	95% HR CI	<i>p</i> -value
<i>Livin</i>	0.49	0.11–2.29	0.365
<i>CASP8AP2</i>	1.12	0.58–2.17	0.738
<i>BCL2L13</i>	2.45	0.67–9.04	0.178
WBC > 100,000/ μ L	3.30	0.81–13.43	0.095
B vs. T	0.43	0.07–2.89	0.388
Age > 10	1.39	0.36–5.35	0.630
Risk groups	1.85	0.61–5.57	0.277
Hyperdiploid	0	0	0.992
<i>t</i> (9;22)	1.38	0.25–7.67	0.716
<i>MLL</i> gene rearrangements	6.28	1.38–28.56	0.017
<i>Step selection</i>			
<i>BCL2L13</i>	3.41	1.17–9.93	0.0244
WBC > 100,000/ μ L	5.25	2.05–13.44	0.0005
<i>MLL</i> gene rearrangements	8.05	1.99–32.54	0.0034

Table 2

Multivariate Cox regression analysis for EFS.

Variables	Hazard ratio (HR)	95% HR CI	<i>p</i> -value
<i>Livin</i>	0.73	0.24–2.25	0.587
<i>CASP8AP2</i>	1.12	0.53–2.00	0.695
<i>BCL2L13</i>	3.25	1.09–9.72	0.035
WBC > 100,000/ μ L	2.77	0.89–8.60	0.079
B vs. T	0.74	0.18–3.03	0.679
Age > 10	1.39	0.49–3.95	0.537
Risk groups	2.02	0.82–4.98	0.127
Hyperdiploid	0.44	0.06–3.52	0.44
<i>t</i> (9;22)	2.65	0.48–14.52	0.261
<i>MLL</i> gene rearrangements	5.75	1.42–23.23	0.014
<i>Step selection</i>			
<i>BCL2L13</i>	4.11	1.64–10.30	0.0025
WBC > 100,000/ μ L	2.72	1.02–7.26	0.0452
Risk groups	2.28	1.04–4.97	0.0386
<i>MLL</i> gene rearrangements	5.75	1.45–17.55	0.0110

Table 4

Multivariate Cox regression analysis for EFS using three gene signature as a parameter.

Variables	Hazard ratio (HR)	95% HR CI	<i>p</i> -value
Three gene signature	2.68	1.07–6.70	0.035
WBC > 100,000/ μ L		0.78–6.83	0.129
B vs. T	0.54	0.15–1.99	0.356
Age > 10	1.47	0.53–4.07	0.461
Risk groups	2.42	1.00–5.88	0.051
Hyperdiploid	0.47	0.06–3.77	0.478
<i>t</i> (9;22)	2.89	0.53–15.72	0.220
<i>MLL</i> gene rearrangements	6.98	1.78–27.30	0.005
<i>Step selection</i>			
Three gene signature	2.85	1.21–6.70	0.0163
WBC > 100,000/ μ L	6.36	1.83–22.08	0.0036
<i>MLL</i> gene rearrangements	3.51	1.82–6.76	0.0002

Fig. 1. Patients with lower *BCL2L13* expression had better EFS ($p < 0.001$), and better OS ($p = 0.005$).

risk groups and the three gene signature were correlated with EFS (hazard ratio for three gene signature = 2.85, $p = 0.0163$).

4. Discussion

The principal goal of this study was to measure the expression, in pediatric ALL patients, of three genes involved in apoptotic pathways, and to determine whether they showed any correlation with clinical outcomes. In our study, higher expression of *BCL2L13* was associated with inferior EFS and OS. The expressions of *CASP8AP2* and *Livin* were not associated with clinical outcomes in our cohort. We also attempted to develop a three gene signature to predict the clinical outcome in this study cohort.

BCL2L13 is a member of the *BCL-2* family, and has pro-apoptotic activity [11]. Our results were consistent with those of Holleman et al., who reported that high expression of *BCL2L13* was associated with inferior outcome, a finding that was validated by another cohort [5]. In theory, impaired apoptosis of leukemia cells might allow them to resist chemotherapy, and thus low expression of *BCL2L13* would be expected to indicate a better prognosis following cancer treatment. These apparently conflicting results suggest that *BCL2L13* may have a different apoptotic role in childhood ALL in comparison to its behavior in the cell lines that were used to characterize its apoptotic functions. One possible mechanism to explain this difference is alternative splicing, which is known to generate both anti- and pro-apoptotic variants of apoptosis genes

such as *Apaf-1* and *Livin* [12–14]. The explanation for the finding that high expression of *BCL2L13* was associated with inferior treatment response will thus require further study.

We attempted to develop a risk signature by the combination of three genes studied. We can use this signature to predict the clinical outcome in this cohort. After multivariate regression including this signature and other known risk factor, this signature can predict the EFS but not OS. However, the p -value and hazard ratio were less powerful than the expression of *BCL2L13* alone (HR: 2.81 and $p = 0.0163$ vs. HR: 4.11 and $p = 0.0025$ for EFS). This may be due to the clinical significance of the expression *BCL2L13* but not *Livin* and *CASP8AP2* in this ethical group. A larger prospective study cohort may be needed to validate this finding.

Genome-wide, gene-expression profiling offers a powerful new approach to the study of leukemia cell biology and potentially provides a new molecular classification of leukemia [15–19]. Although our sample size was small, the results differed in some respects from those previously reported. First, we found *BCL2L13* expression to predict survival in the same way as previously reported. We did not find any clinical significance of *CASP8AP2* and *Livin* individually. Several recent studies attempted to identify prognostically relevant genes in ALL by correlating in vitro drug sensitivities or treatment outcomes to chemotherapy drugs with gene expression profiles on microarrays [2,20,21]. Although several genes were identified, and were subsequently confirmed independently in patient populations, there was little overlap between gene expression signatures and established subgroups of patients, such as *t*(12;21), hyperdiploid BP-ALL, or T-cell ALL, or with established drug resistance genes. Catchpole and colleagues [22] examined the set of 14 genes that Floth et al. [23] recently identified. The results were not

Fig. 2. The EFS and OS did not differ between different *CASP8AP2* expression groups.

Fig. 3. There was no statistically significant difference for EFS and OS between these two groups of patients with *Livin* expression or not.

in agreement with the original report, suggesting that treatment outcome may be influenced by the other factors, such as pharmacogenetics, the intensity of chemotherapy, and the gene expression of primary leukemia cells. The value of gene expression profiles as

prognostic indicators depends on the treatment regimen. In fact, this caveat applies to any prognostic factor.

Over the past 10–20 years, chromosomal translocation has also emerged as an extremely important prognostic factor and means to assign patients to protocols. However, the importance of even the *t*(12;21) event remains under debate. For example, two large prospective studies focused on the prognostic impact of *t*(12;21) but reached different conclusions [24,25]. If more molecular prognostic factors can be found, it might be possible to provide accurately tailored individualized chemotherapy regimens. Despite this, the number of genes identified as being able to predict clinical outcome in ALL remains small. For examples, Cario et al. reported that expression of *MADL2* was associated with MRD on week 12 in the BFM2000 study [26]. *MADL2* was one of 14 genes identified by Flotho et al. [2]. Low expression of *TTK* (a gene encoding a kinase involved in cell-cycle regulation) was identified by two groups [26,27]. Searching for genes related to treatment response across different regimens might provide clues about general mechanisms that regulate drug sensitivity in leukemic cells.

In summary, in our retrospective studies, *BCL2L13* expression was an independent prognostic factor in childhood ALL. Upon confirmation of their prognostic significance in a larger ALL population, *BCL2L13* may be incorporated into a new classification system. Furthermore, these genes, or other genes related to treatment failure, might themselves represent molecular targets allowing ALL cells to be sensitized to chemotherapy drugs.

Conflict of interest

The authors declare no competing financial interests.

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Contributions. Yung-Li Yang and Shu-Rung Lin contributed equally to this article.

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Fig. 4. The patients with high-risk signature had inferior EFS than patients with low-risk signature.

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