Comparison of Prognostic Value of *In Vitro* Drug Resistance and Bone Marrow Residual Disease on Day 15 of Therapy in Childhood Acute Lymphoblastic Leukemia

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Abstract. *Aim: The analysis of the prognostic impact of residual disease at day 15 of induction therapy, individual tumor response testing (ITRT) at diagnosis, initial factors and initial therapy response to the risk of relapse in children with precursor B-cell acute lymphoblastic leukemia (ALL). Patients and Methods: A total of 87 children were tested at diagnosis for ITRT and for persistence of blasts in bone marrow at day 15 (BML15>0.5%) and were followed-up in long-term analysis. Results: The probability of disease-free survival (pDFS) was significantly better for patients with an ITRT profile showing sensitivity to prednisolone, vincristine, daunorubicin, and L-asparaginase. Patients with BML15>0.5% had higher ITRT for prednisolone, daunorubicin, L-asparaginase, and etoposide. Three factors had predictive impact for relapse: BML15>0.5%, ITRT for prednisolone and high combined ITRT profile for prednisolone, vincristine and L-asparaginase (PVA score). Conclusion: Persistence of blasts in bone marrow at day 15, ITRT showing resistance to prednisolone and high PVA score were the strongest and comparable prognostic factors predicting relapse in childhood ALL.*

Acute lymphoblastic leukemia (ALL) is the most frequent malignant disease of childhood. Pediatric ALL is a disease stratified according to prognostic factors. Over the decades, some factors lost their value, while new ones were discovered. Currently, the following factors are regarded as the most important in predicting disease-free survival: response to one-week prednisolone monotherapy (1), *in vitro* cellular drug resistance profile (2, 3), minimal residual disease (MRD) (4), gene expression profile (5), and presence of BCR-ABL genes re-arrangement (6). The speed of blast clearance during therapy is a major prognostic factor of outcome in childhood ALL (7). Blast count in the peripheral blood on day 8, or in the bone marrow on day 15 and day 33, have been widely used to deliver risk-directed therapy. Another approach to measure the speed of leukemia clearance is the detection of minimal residual disease during induction therapy or at days 33 and 78 of antileukemic therapy. *In vitro* measurements of drug resistance (called, individual tumor resistance testing, ITRT) in leukemia cells obtained at diagnosis, have been of prognostic significance in the prediction of clinical outcome in selected groups of patients (8). The high cost of obtaining data is, however, a disadvantage for standard use of some factors. Thus, the number of reports comparing the prognostic value of ITRT and MRD is limited (8-11).

In order to estimate the predictive impact of ITRT and MRD, we investigated the potential of peripheral blood (PB) and bone marrow (BM) findings at diagnosis (day 0) and early follow-up time points (day 8, day 15, and day 33). In the current analysis, we focused especially on comparison of results of ITRT determined at diagnosis and the presence of residual disease, as measured by flow cytometry at day 15 of induction therapy.

The overall objective of the study was to assess and compare the prognostic role of MRD and ITRT in childhood ALL. In particular, the objectives included the analysis of the prognostic impact of residual disease (MRD) at day 15 of induction therapy; ITRT; correlation of MRD and ITRT, and multivariate analysis of the prognostic role of MRD, ITRT, initial factors and initial therapy response in the risk of relapse.

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Key Words: Minimal residual disease, drug resistance, individualized tumor response testing, acute lymphoblastic leukemia, flow cytometry, pediatric, early response.

Table I. *Patients' characteristics and overall treatment results with respect to therapy protocol.*

Characteristics	Protocol		*p*-Value
	ALL-BFM-90	ALL-IC-2002	
Patients, n	36 (20 M, 16 F)	51 (27 M, 24 F)	
Age (median, range), years	5.1 (1.9-17.0)	5.4 (1.5-17.9)	0.749
PGR/PPR	34/2	47/4	0.519
M1/M2/M3	30/4/2	42/3/6	0.690
pDFS	0.715±0.068	0.727±0.059	0.623
Mean survival (95% CI), years	11.4 (10.4-12.7)	7.2 (6.6-7.9)	

PGR: Prednisolone good-responder; PPR: prednisolone poor-responder; M1/M2/M3: bone marrow response at day 15 (see text); pDFS: probability of disease-free-survival; M: male; F: female.

Patients and Methods

Patients. A total of 87 children, aged 1-18 years, diagnosed with pre-B-ALL, treated either with ALL-BFM-90 (12) or ALL-IC-2002 (13) protocols were included in the study (Table I). Standard, intermediate, and high-risk group criteria were specifically characterized in each therapy protocol. The median follow-up was 9.8 years (range=0.2-12.5 years).

Drugs. The following drugs were used: prednisolone (Jelfa, Jelenia Gora, Poland; 0.0076-250 μg/ml), dexamethasone (Jelfa; 0.00018-6 μg/ml), vincristine (Gedeon Richter, Budapest, Hungary; 0.019-20 μg/ml), L-asparaginase (Medac, Hamburg, Germany; 0.0032-10 IU/ml), daunorubicin (Rhone-Poulenc Rorer, Paris, France; 0.0019-2 μg/ml), doxorubicin (Pharmacia Italia S.p.A., Milan, Italy; 0.031-40 μg/ml), etoposide (Bristol-Myers Squibb, Sermoneta, Italy; 0.048-50 μg/ml), and cytarabine (Upjohn, Puurs, Belgium; 0.24-250 μg/ml).

Individual tumor response testing. ITRT (cellular *in vitro* drug resistance) was tested by 3-4,5-dimethylthiazol-2-yl-2,5-difenyl tetrazolium bromide (MTT) assay, as described previously (14). The drug concentration that was inhibitory to 50% of the cells (IC_{50}) was calculated from the dose-response curve and was used as a measure of *in vitro* drug resistance in each sample. The relative resistance (RR) between groups analyzed for each drug was calculated as the ratio of the mean values of IC_{50} of the respective groups for this drug. Only patients who had a successful MTT assay at diagnosis and bone marrow flow cytometric analysis at day 15, for the presence of residual disease, were included in the study. All patients reached remission by day 33.

Combined ITRT profile (PVA score). Results obtained for all samples were calculated also according to percentile values of *in vitro* cytotoxic concentrations for each drug. Reference values for combined *in vitro* resistance profile for prednisolone, vincristine and L-asparaginase (PVA score) were determined based on the division of all IC_{50} values into three equal groups; the cut-off values were created by the 33rd and 67th percentile (2, 3). All patients were re-assessed according to PVA score with values from 3 up to 9. For the purpose of this study, all children were classified as *in vitro* chemosensitive (with PVA score below 6) or *in vitro* chemoresistant (with PVA score 6 or above).

Residual disease. Peripheral blood was assessed for leukemia cells by morphology at days 0 and 8, while bone marrow was analyzed by morphology and flow cytometry at days 0 and 15. Early bone marrow response by day 15 was assessed as M1, blast percentage <5%; M2, blast percentage 5-<25%; and M3, blast percentage 25% or higher. Bone marrow lymphoblasts, assessed by flow cytometry at day 15 (BML15) were analyzed using a cut-off value at 0.5% blasts. Leukemia-associated immunophenotypes were studied as described previously (7). Antibodies were purchased from BD Biosciences (San Jose, CA, USA), DAKO/DakoCytomation (Glostrup, Denmark), and Beckman/Coulter/Immunotech (Marseille, France). At least two selected combinations were applied during follow-up for MRD detection for each patient. Analyses were performed on EPICS XL (Coulter, Miami, FL, USA) or Cytomics FC500 (Beckman-Coulter, Miami, FL, USA) flow cytometers.

Statistical analysis. Baseline characteristics of all patients were summarized using descriptive statistics. Differences between groups were measured by chi-square test, Fisher exact test and Mann-Whiney *U*-test. Results of antileukemic therapy were presented as probability of disease-free survival (pDFS). The median survival of this patient group was estimated with a 95% confidence interval (CI). Kaplan–Meier curves were used to summarize disease-free survival in univariate analysis. Cox proportional hazards regression model was used to correlate each potential prognostic factor with survival in univariate analysis. All factors with $p<0.1$ in univariate analysis were then fitted together in multivariate analysis, and dropped one at a time in a backward stepwise manner using the likelihood ratio test at a 0.05 level until all factors in the model were significant. A final check was made to ensure that no excluded factors would improve the fit.

Results

Prognostic impact of residual disease (MRD) at day 15 of induction therapy. Basic patients characteristics and overall treatment results are presented in Table I. No differences in pDFS between patients treated with the specific therapy protocol were found. No difference in pDFS were observed between *in vivo* prednisolone-good-responder (PGR) and prednisolone-poor-responder (PPR), nor between children aged less than and older than 6 years (data not shown). The

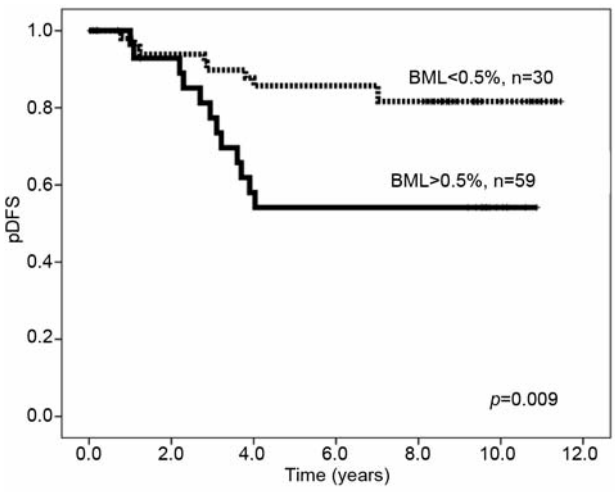

Figure 1. *Prognostic impact of residual disease as shown by bone marrow lymphoblasts (BML) at day 15 of induction therapy.*

overall pDFS was 0.721±0.052 and the mean survival was 9.1 (95% CI=8.2-9.9) years. No differences in pDFS was found between patients treated with either the ALL-BFM-90 or ALL-IC-2002 protocol (Table I). Patients with BML15<0.5% had pDFS=0.816±0.055, while those with BML15>0.5% had pDFS=0.542±0.098 (log-rank test, p=0.009, Figure 1). The risk of relapse in BML15-positive patients was 3.0-fold higher (95% CI=1.3-7.1, p=0.013).

Prognostic impact of ITRT. pDFS was significantly better for patients with sensitive ITRT profile to: PVA score (1.00±0.00 *vs*. 0.61±0.06, p=0.0009), prednisolone (0.94±0.04 *vs*. 0.51±0.08, p=0.0002), vincristine (0.84±0.06 *vs*. 0.61±0.08, p=0.035), daunorubicin (0.89±0.05 *vs*. 0.54±0.08, p=0.002), and L-asparaginase (0.84±0.06 *vs*. 0.59±0.08, p=0.009). In multivariate analysis in a Cox model, the prognostic value was retained only for ITRT, for PVA score (p=0.009, OR=0.03, 95% CI=0.01-0.8), prednisolone (p=0.013, OR=0.08, 95% CI=0.01-0.6) and daunorubicin (p=0.024, OR=0.05, 95% CI=0.01-0.4).

Prognostic impact of correlation of MRD and ITRT. Patients with MRD-positive status at day 15 (BML15>0.5%) had higher ITRT for the following drugs: prednisolone (p=0.030, RR=2.2; Mann-Whitney *U*-test), daunorubicin (p=0.005, RR=2.1), L-asparaginase (p=0.029, RR=3.2), and etoposide (p=0.021, RR=4.0), while no differences were found for the other drugs (Table II). In multivariate logistic regression, significant impact on development of BML15>0.5% was found for daunorubicin (p=0.035, OR=0.33) and etoposide (p=0.048, OR=0.14).

Multivariate analysis of prognostic role of MRD, ITRT, initial factors and initial therapy response to the risk of relapse. In multivariate analysis in the Cox model for relapse risk, three factors had predictive value: BML15>0.5% (p=0.010), PVA score (p=0.012) and ITRT for prednisolone (p=0.012), while age, *in vivo* PPR at day 8, BM response at day 15, and BCR–ABL re-arrangement had no significant value (Table III). No significant prognostic factor was found when patients were analyzed in subgroups determined by the two therapy protocols.

Discussion

Therapy of childhood ALL is based on a risk factor-adapted strategy. Current treatment protocols are based on the following risk factors: age, immunophenotype, initial WBC, presence of BCR–ABL rearrangements, and initial response to therapy determined usually as a response at day 8, 15 and 33 (13). Over the past decade, several new potent risk factors have been determined, but their role has not yet been established. The three most important factors recently under investigation include: MRD, ITRT and gene expression profile. Due to relatively high costs or laborious procedure, there have been no studies comparing the prognostic value of all three of these factors, there are only several studies comparing any two of these factors.

In this study, we compared the prognostic value of ITRT profile and bone marrow MRD in 87 children with pre-B/common-ALL with long-term follow-up. We have shown that patients with a low level of MRD have a significantly better long-term outcome. The same finding was determined for three factors related to ITRT: lymphoblast sensitivity to prednisolone, to daunorubicin and combined profile of sensitivity to prednisolone, vincristine and L-asparaginase (PVA score). The prognostic value of these factors was already well-documented in previous studies, with the possible exception of a prognostic role of ITRT for daunorubicin (2, 15-18). Our study aimed at the comparison of these factors. In a long-term single-center analysis of children with precursor-B-ALL, we showed that the value of these factors is comparable when analyzed by multivariate analysis. Thus, the results of this study may suggest that new prognostic factors, such as ITRT and MDR have equivalent value. It is also possible that phenomena of ITRT and MRD correspond to certain biological properties of leukemia cells, common for response to therapy.

In multivariate logistic regression analysis, we found that initial *in vitro* drug resistance to prednisolone, L-asparaginase, daunorubicin, and etoposide contributed to the presence of residual blasts at day 15. This is additional evidence that both factors are strongly related to each other.

Currently, there are reports originating from only two study groups, which combine and compare analysis of ITRT

Table II. *Individual tumor response testing (ITRT) profile with respect to bone marrow residual disease by day 15.*

ITRT profile	Median IC_{50} (range)		RR	*p*-Value
	BML15<0.5%	BML15>0.5%		
Prednisolone	2.36, 0.04–>250 (n=30)	5.12, 0.15–>250 (n=57)	2.2	0.030
Dexamethasone	0.87, 0.06–>6 (n=26)	0.91, 0.01–>6 (n=57)	1.0	0.235
Vincristine	0.48, 0.03–9.58 (n=30)	1.38, 0.02–14.38 (n=57)	2.9	0.156
L-asparaginase	0.08, 0.01–3.59 (n=30)	0.26, 0.05–>10 (n=57)	3.2	0.029
Daunorubicin	0.21, 0.03–>2 (n=30)	0.44, 0.02–1.66 (n=57)	2.1	0.005
Doxorubicin	0.43, 0.25–1.8 (n=24)	0.69, 0.13–>8 (n=57)	1.6	0.809
Etoposide	0.83, 0.05–>50 (n=30)	3.36, 0.05–>50 (n=57)	4.0	0.021
Cytarabine	0.31, 0.01–>10 (n=20)	0.91, 0.06–>10 (n=57)	2.9	0.055
PVA score	4, 3–9 (n=30)	5, 3–9 (n=57)	1.3	0.681

BML15: Percentage of bone marrow blast at day 15; IC_{50}: inhibitory drug concentration to 50% of cells; n: number of patients; RR: relative resistance; PVA: combined ITRT profile to prednisolone, vincristine and L-asparaginase.

Table III. *Uni- and multivariate analysis of prognostic factors for probability of disease-free survival (pDFS).*

Factor	Characteristic	Univariate analysis		Multivariate analysis	
		OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
BML15	<0.5% (n=30)	0.32 (0.15-0.82)	0.013	0.33 (0.14-0.75)	0.010
	>0.5% (n=57)	1		1	
PVA score	Sensitive (n=43)	0.17 (0.05-0.50)	0.001	0.23 (0.07-0.72)	0.012
	Resistant (n=44)	1		1	
ITRT for prednisolone	Sensitive (n=43)	0.08 (0.02-0.37)	0.001	0.17 (0.04-0.73)	0.018
	Resistant (n=44)	1		1	
ITRT for daunorubicin	Sensitive (n=43)	0.27 (0.01-1.3)	0.068	0.52 (0.07-30.5)	0.953
	Resistant (n=44)	1		1	
Age	<6 years (n=45)	0.48 (0.19-1.12)	0.080	0.72 (0.23-1.75)	0.271
	>6 years (n=42)	1		1	
Prednisolone response day 8	Good (n=81)	0.79 (0.32-1.96)	0.128		
	Poor (n=6)	1			
BM response day 15	M1 (n=72)	0.21 (0.02-1.89)	0.164		
	M2/M3 (n=15)	1			
BCR-ABL gene rearangement	Negative (n=85)	0.95 (0.23-3.17)	0.865		
	Positive (n=2)	1			

BML15: Percentage of bone marrow blast at day 15; n: number of patients; PVA; combined ITRT profile to prednisolone, vincristine and L-asparaginase; ITRT: individual tumor response testing; BM: bone marrow; M1/M2/M3: bone marrow response at day 15 (see text); OR: odds ratio.

and MRD: Danish/Dutch (9) and Nordic (8, 11) study groups. Schmiegelow *et al*. presented a study on a group of 34 children with precursor-B-ALL, both at day 15 and at the end of induction correlated with *in vitro* resistance to prednisolone but not to vincristine or doxorubicin (9). On the other hand, in the study of Lonnerholm *et al*., of 230 children with B-cell precursor ALL, the risk of any relapse was correlated to *in vitro* vincristine and doxorubicin resistance (11). Both groups measured the levels of MRD by PCR. Our study confirmed the correlation of MRD and *in vitro* resistance to prednisolone, and also to L-asparaginase, daunorubicin and etoposide, but not to vincristine and doxorubicin. It underlines a unique role of prednisolone in the therapy of pediatric ALL.

In conclusion, this study provides evidence that ITRT and flow cytometric analysis of bone marrow at day 15 of induction therapy, have comparable prognostic impact in childhood ALL. The predictive value of these two factors is much stronger than that of other risk factors, such as poor initial response to therapy, determined classically as PPR by day 8 or lack of remission by the end of induction therapy by day 33.

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