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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

Absolute count of leukemic blasts in cerebrospinal fluid as detected by flow cytometry is a relevant prognostic factor in children with acute lymphoblastic leukemia

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Abstract

Background Usually, central nervous system (CNS) involvement in acute lymphoblastic leukemia (ALL) is diagnosed by cytomorphology (CM) of cerebrospinal fluid (CSF) on cytospin slides. Multicolor flow cytometry (MFC) provides the opportunity to detect low numbers of leukemia cells undetectable by CM. The present study aimed at evaluating the clinical significance of MFC for the diagnosis of CNS involvement at initial manifestation of childhood ALL.

Methods In 155 children with ALL, CSF samples were studied in parallel by CM and MFC. Patients were treated according to protocol ALL-MB-2008 for childhood ALL. The prognostic impact of the leukemia burden in CSF was determined categorizing the findings as positive/negative. In addition, the absolute blast cell count per 1 ml of CSF was studied as a continuous variable.

Results CSF positivity was significantly more frequent using MFC compared with CM (35.3% vs. 15.3% of patients). The outcome of MFC-positive and MFC-negative patients was not different in clinically relevant patient risk groups—CNS1, standard and intermediate-risk groups. Using the quantitative approach, at the threshold level of 20 blasts per ml of CSF, patients could be divided into two groups with a significantly different outcome, irrespective of the clinical risk group, the type of CNS-directed therapy, and the CNS status determined by CM.

Conclusions Our data do not support the concept of re-stratification and modification of therapy based on qualitative CSF investigation by MFC. However, MFC is a highly sensitive technique of CSF investigation improving the definition of CNS involvement in childhood ALL, and quantitative measurement of blast cells in CSF, if well-organized, can be a useful additional tool for stratification of patients in clinical trials.

Keywords Acute lymphoblastic leukemia · Central nervous system · Cerebrospinal fluid · Flow cytometry · Immunophenotype

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Introduction

Central nervous system (CNS) involvement at diagnosis of childhood acute lymphoblastic leukemia (ALL) is one of the major causes of treatment failure (Burger et al. 2003; Pui et al. 2015; Hunger and Mullighan 2015). However, correct and timely diagnosis of CNS disease is still challenging (Del Principe et al. 2014b; Crespo-Solis et al. 2012). Leukemia cells can easily be detected by conventional cytomorphology (CM) on cytospin slides. The specificity of this method is near 100%, but the sensitivity is rather low (Del Principe et al. 2014b; Crespo-Solis et al. 2012). It was shown earlier that low levels of leukemia cells, undetectable by CM, could be found using more sensitive techniques such as multicolor flow cytometry (MFC) (Levinsen et al. 2016; Dass et al. 2017; Del Principe et al. 2014a; Cancela et al. 2017). Nevertheless, the prognostic value of these MFC-detectable blasts in the cerebrospinal fluid (CSF) is still unclear (Cancela et al. 2017; Martinez-Laperche et al. 2013; Ranta et al. 2015). The aim of the present study was to evaluate the clinical significance of MFC application for the diagnosis of CNS involvement at initial manifestation of childhood ALL.

Patients and methods

Patients

The study was approved by the Ethics Committees of the Ural State Medical University and Regional Children's Hospital 1. Informed consent for the collection and investigation of samples was obtained from patients' parents or legal guardians.

Between December 2008 and November 2014, 169 consecutive children with newly diagnosed ALL were enrolled in trial ALL-MB 2008 of the MB (Moscow–Berlin) study group in a single institution. Diagnostic immunophenotyping, chromosomal and genetic analyses were performed by standard techniques. Minimal residual disease (MRD) was measured by MFC on day 36 (end of remission induction).

Treatment protocol and risk group assignment

All patients were initially allocated to risk groups according to stratification criteria as defined in the protocol (Supplementary table 1). Risk group allocation was re-evaluated on day 36 of induction therapy: patients not having achieved a complete remission (CR) were shifted to the high-risk group.

The treatment is shown in Supplementary table 2. In brief, all patients received induction therapy followed by three consolidation courses and maintenance therapy. Randomizations are marked in Supplementary table 2.

Preventive CNS therapy consisted of intrathecal injections with three drugs (triple intrathecal therapy—TIT) (number of injections according to risk group and randomization arm, see Supplementary table 2). In ImRG patients, preventive CNS therapy was randomized: cranial radiation therapy (crRT) was performed in 27 patients, and 56 children received TIT only.

High-risk non-T-cell ALL patients received identical induction therapy. Thereafter, they were treated according to the chemotherapy regimen designed for high-risk patients in trial ALL-BFM-2000: six blocks of intensive therapy followed by protocol II plus cranial irradiation (only three patients received crRT) and maintenance therapy (Conter et al. 2010). Three patients not in CR on day 36 were allocated to allogeneic stem cell transplantation after they had achieved CR during the HRG chemotherapy.

CNS involvement investigation

CSF samples were investigated by microscopy of cytospin slides and by MFC. White blood cells (WBC) were counted in a Fuchs–Rosenthal chamber. CNS status was classified by CM as CNS1 (no blast cells in CSF), CNS2 (<5 WBC/µl CSF with blast cells), or CNS3 (≥ 5 WBC/µl CSF with leukemia cells or other signs of CNS involvement) (Burger et al. 2003).

Flow cytometric CSF investigation was performed in at least 1 ml of fluid using four to six color antibody combinations based on the immunophenotype of leukemic cells as determined at the initial diagnostic bone marrow investigation. For nearly all BCP-ALL patients, a CD19/CD10/CD34/CD45 set was sufficient to obtain adequate results. For T-cell ALL, a combination of CD7/CD3/CD5/CD2/CD99/CD45 was most appropriate. According to the rules of cytometric rare event analysis (Hedley and Keeney 2013), at least 30 clustered cells with leukemia-associated immunophenotype and appropriate light-scatter properties were required to define MFC positivity qualitatively (yes/no). Immunophenotype patterns of leukemic cells were comparable in bone marrow and CSF (Fig. 1). To convert dichotomic MFC results to continuous variables, we calculated the absolute blast count in 1 ml of CSF using the WBC count assessed in the Fuchs-Rosenthal chamber and the percentage of leukemia cells among all nucleated cells as detected by MFC. In the case of MFC positivity, cytometric CSF investigations were performed at all consecutive lumbar puncture time points until MFC negativity was achieved.

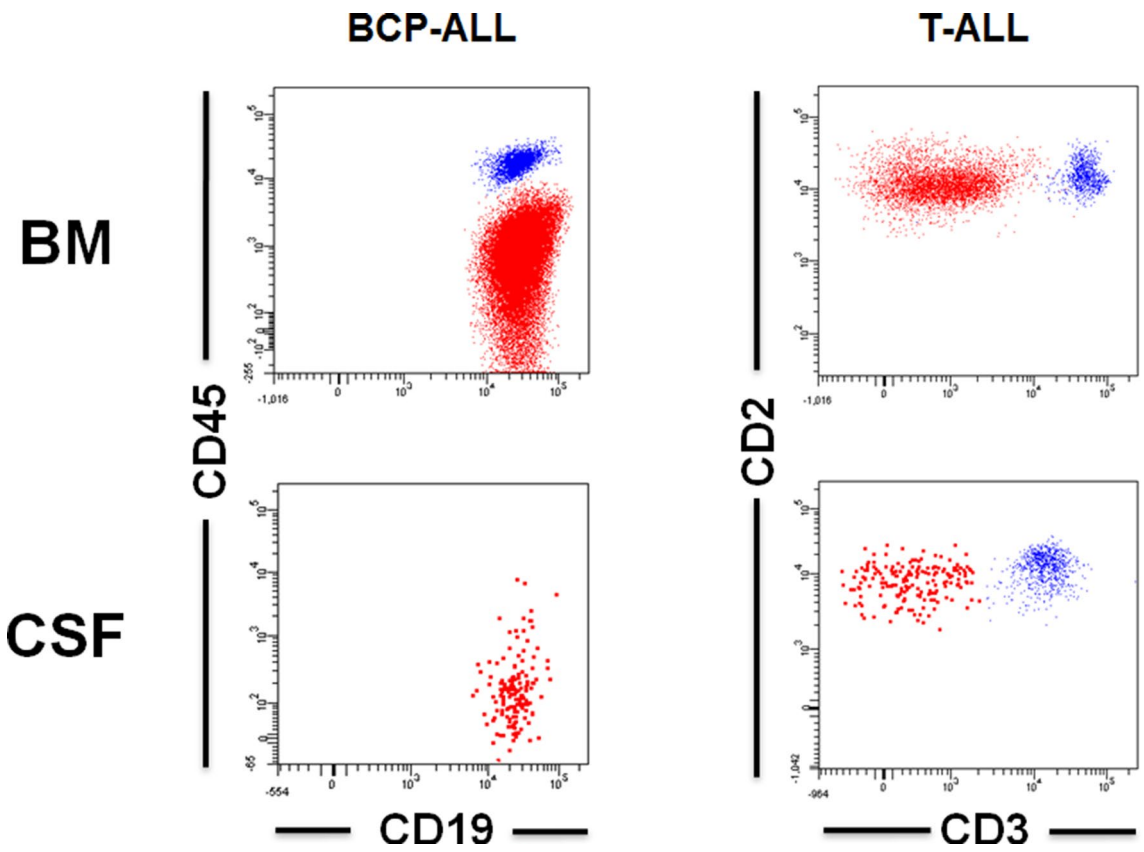

Fig. 1 Correspondence of immunophenotypic patterns of leukemic cells (painted red) in bone marrow and CSF in BCP-ALL (left column, only CD19-positive cells are shown) and T-lineage ALL (right column, only CD7-positive cells are shown). Residual lymphocytes of the respective lineage are painted blue

Statistics

Yates corrected Chi square test was used for qualitative comparison, and the Mann–Whitney *U* test for quantitative comparison. Differences in outcome between groups were compared calculating the cumulative incidence of relapse (CIR), estimated adjusting for competing risks of the other pertinent events. Comparison of CIR between groups was performed using Gray's test. Median time of follow-up was 5 years. Receiver operator characteristic (ROC) analysis (Zweig and Campbell 1993) was applied to test whether quantitative MFC data allowed better discrimination of patients having experienced a relapse from those who remained in remission. Significance of discrimination was calculated by area under the curve (AUC). The absolute blast cell count with the best sum of sensitivity and specificity predicting poor outcome was set as the threshold level (TL) for further conversion of continuous quantitative values to dichotomic variables marked as "high" and "low". Competing-risks regression was used to analyze the prognostic impact of the blast cell count in CSF in a one-step multivariate model that included the most frequently used conventional prognostic features. The proportional hazard assumption was tested with 0.05 significance level.

Results

Fourteen patients were excluded due to a traumatic lumbar puncture with contamination of blast cells, inadequate quality of the CSF sample, or non-compliance with the regulations of the treatment protocol. Finally, 65 girls and 90 boys aged 1–16 years (median age 4 years) were eligible for analysis. B-cell precursor ALL (BCP-ALL) was diagnosed in 134 patients (86.5%), while the remaining 21 patients (13.5%) had T-lineage ALL. The standard-risk group (SRG) consisted of 59 patients, the intermediate-risk group (ImRG) of 87 patients, and the high-risk group (HRG) of 9 patients.

The outcome of the 155 studied patients was comparable with the outcome of all 3312 patients enrolled in the ALL-MB 2008 trial: 7-year event-free survival 0.80 ± 0.04 and 0.81 ± 0.01, respectively, $p = 0.7817$. In 155 patients of the study group, 26 relapses were diagnosed (CIR 0.20 ± 0.04).

CNS3 status was found in five patients (3 patients with BCP-ALL and 2 with T-cell ALL). These patients were excluded from further analysis due to obvious evidence of CNS leukemia. In all of them, CSF positivity was assessed by both, CM and MFC. CNS2 was diagnosed in 23 of the remaining 150 patients (15.3%), while 127 patients (84.7%) had CNS1. By MFC, CSF involvement was detected in 53 (35.3%) out of 150 studied children. In 32 CNS1 patients, leukemic blasts were found by MFC only. Thus, the diagnostic sensitivity of MFC was significantly higher ($p < 0.001$) than conventional CM. The absolute blast cell count in 1 ml of CSF positive by both methods was higher than in samples that were positive by MFC only (median = 418, range 8-158171 and median = 34, range 5–2762 respectively, $p < 0.001$).

Relapses occurred in 23 of 150 patients with CNS1 and CNS2. In 13 of these patients (56.5%), leukemia cells had initially been detected in CSF by MFC, but CNS2 was diagnosed in 8 (34.8%) children only. No isolated CNS relapses were observed, and in only five patients CNS recurrence was seen in combination with bone marrow relapse. All five patients were initially CNS positive by MFC, although in only two of them blast cells were also detected by CM.

Qualitative results obtained with both methods, CM and MFC, were used for the calculation of CIR (Fig. 2). CIR was significantly higher in 23 children with CNS2 than in 127 patients with CNS1: CIR 0.36 ± 0.10 and 0.14 ± 0.03, respectively ($p = 0.005$). In 53 MFC-positive patients the CIR was 0.25 ± 0.06 compared to 0.13 ± 0.04 in 97 children who were MFC negative ($p = 0.017$). Nevertheless, MFC failed to disclose an impact on prognosis in clinically relevant patient risk groups—CNS1, SRG, and ImRG (Fig. 3).

Using ROC analysis, it was possible to discriminate patients with a different probability of relapse by the absolute blast cell count as measured by MFC in a defined volume of CSF (Fig. 4). Despite the relatively low AUC value of 0.66, a significant difference between patients with and without relapse was found at the TL of 20 cells/ml of CSF (Fig. 4a). Analyzing CIR using this TL, the difference between patients with low/negative counts (< 20/ml, $n = 114$, CIR 0.12 ± 0.04) and high counts (≥ 20/ml, $n = 36$, CIR 0.34 ± 0.08) was statistically significant ($p = 0.001$) (Fig. 4b). Patient characteristics of these groups are shown in Table 1. Patients with higher blast cell counts were more frequent in ImRG than in SRG, but the distribution among the randomization arms was not significantly different. Discriminating patients at this TL, significant differences were also seen between patients within the clinically relevant patient risk groups mentioned above (Fig. 5). In 18 patients of group CNS1 with high counts, CIR was 0.28 ± 0.11 compared

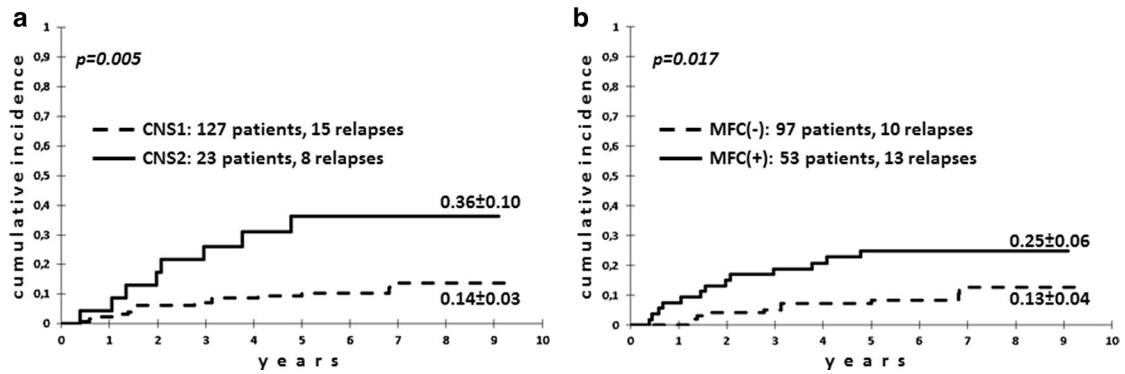

Fig. 2 Cumulative incidence of relapse by CNS status (**a**) and MFC positivity (**b**)

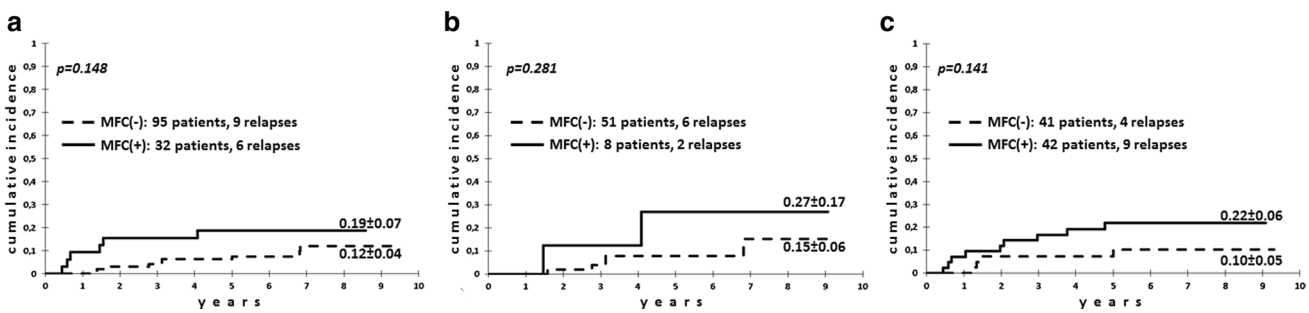

Fig. 3 Cumulative incidence of relapse according to MFC positivity in patients with CNS1. (**a**) SRG patients (**b**) and ImRG patients (**c**)

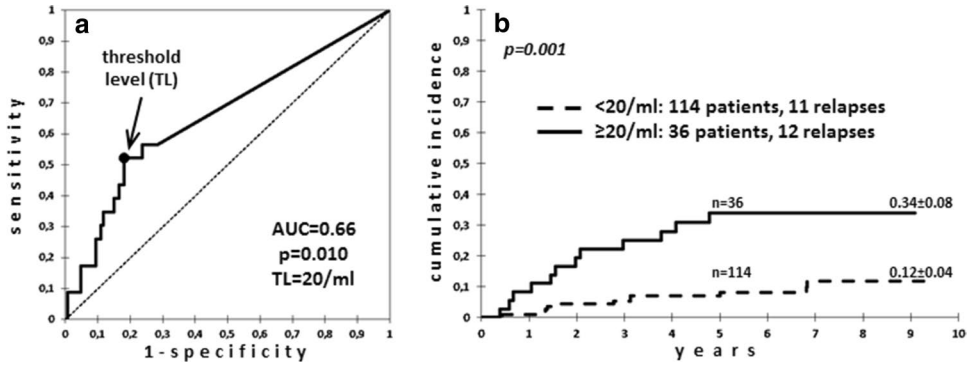

Fig. 4 ROC curve for correlation between relapse probability and absolute blast count in CSF (**a**), arrow indicates the threshold level (TL) with the optimal ability for discrimination of patients with different outcomes (20 blasts per 1 ml); application of this TL for CIR investigation in the studied patients (**b**)

Table 1 Patient characteristics by absolute blast cell count in CSF measured by MFC

	Blasts in CSF < 20/ml ($n=114$)		Blasts in CSF ≥ 20/ml ($n=36$)		p
	n	%	n	%	
Sex (m/f)	70/44	61/39	16/20	44/56	0.109
Age (< 10/≥10 years)	92/22	81/19	26/10	72/28	0.396
WBC (< 30/≥ 30×10^9/L)	86/28	75/25	21/15	58/42	0.077
t(12;21) y/n	23/91	20/80	5/31	14/86	0.549
Immunophenotype (B/T)	103/11	90/10	28/8	78/22	0.091
Risk group					
SRG/ImRG/HRG	54/54/6	47/47/6	5/29/2	14/81/5	0.001
Randomization arms during remission induction in SRG[a]					
PEG + DNR–/PEG + DNR +/PEG –DNR +	19/15/20	35/28/37	3/0/2	60/0/40	0.421
Randomization arms during remission induction in ImRG[a]					
PEG +/PEG –	28/26	52/48	15/14	52/48	0.826

[a]Randomization scheme is shown in Supplementary table 2. Eight patients, allocated to HRG, were not randomized

with 0.12 ± 0.04 in 109 patients with low/negative counts ($p=0.024$). Out of 5 SRG patients with high MFC positivity, 2 children experienced a relapse (CIR 0.47 ± 0.25), significantly inferior compared to 6 out of 54 MFC-low/negative SRG patients (CIR 0.14 ± 0.06, $p=0.043$). MFC-low/negative ImRG patients ($n=54$) had a better outcome than patients with high MFC positivity ($n=29$): CIR 0.10 ± 0.04 and 0.28 ± 0.08, respectively, $p=0.034$.

The prognostic impact of a high CNS leukemia cell count was not affected by the type of CNS-directed treatment (Fig. 6). Among 120 patients having received TIT only, MFC was low/negative in 92 patients (CIR 0.13 ± 0.04); in contrast, 28 children with high MFC positivity displayed a CIR of 0.33 ± 0.09 (Fig. 6a, $p=0.007$). This difference was also found in 30 ImRG and HRG patients having received crRT: CIR 0.04 ± 0.04 versus 0.37 ± 0.17 for 22 children with low/negative MFC and 8 patients with high MFC positivity, respectively (Fig. 6b, $p=0.025$). In 36 patients with a high blast cell count detected by MFC, the outcome was similar irrespective of crRT (Fig. 6c): CIR 0.37 ± 0.17 and 0.33 ± 0.09 for patients with and without crRT, respectively ($p=0.653$).

In multivariate analysis, high blast cell count in CSF retained its prognostic significance if adjusted for conventional prognostic features and stratification parameters applied in trial ALL-MB 2008, even if MRD response on day 36 was included in the model (Table 2).

Cytometric follow-up measurements of the CSF burden were performed in 32 of 53 patients with initial MFC positivity. Any detectable level of leukemia cells at the next lumbar puncture was associated with a worse outcome

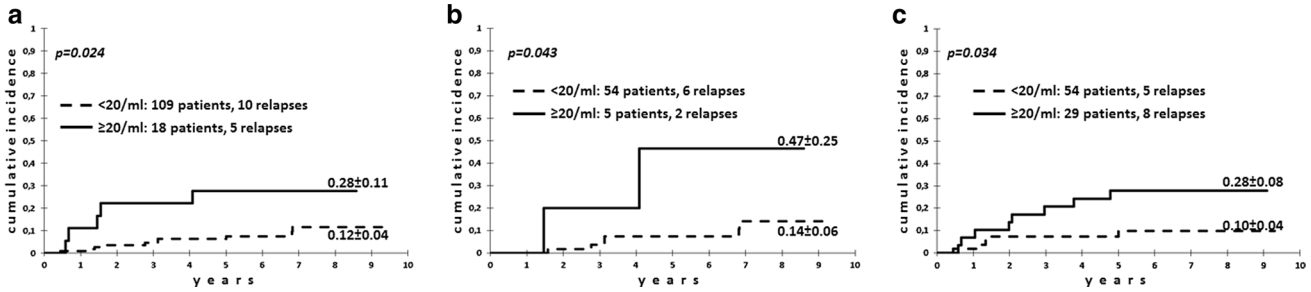

Fig. 5 Cumulative incidence of relapse by absolute blast cell count in CSF in patients with CNS1 (**a**), SRG patients (**b**) and ImRG (**c**)

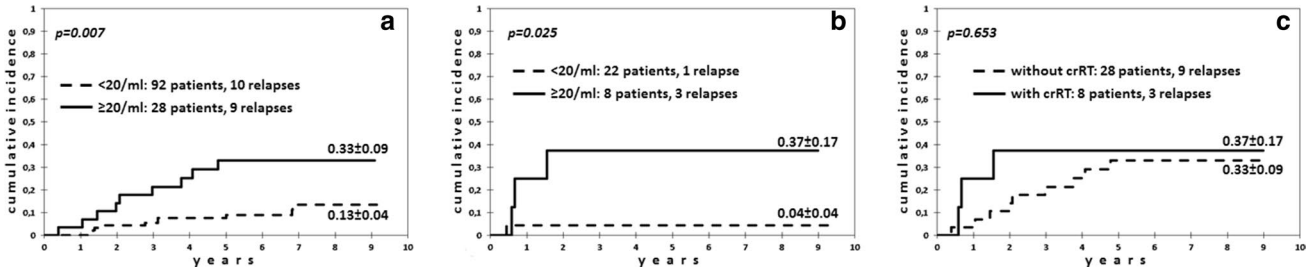

Fig. 6 Cumulative incidence of relapse by absolute blast cell count in CSF in patients who received only TIT (**a**) and who received also crRT (**b**) as well as CIR by crRT in patients with high CSF blast cell count (**c**)

Table 2 Multivariate analysis of the prognostic impact on risk of relapse by conventionally used prognostic features, criteria applied in protocol MB 2008, and MRD response following induction therapy

Covariate	Number of patients	Number of relapses	Hazard ratio	95% CI	*p*
Sex					
Male	85	11	1		0.140
Female	65	12	2.18	0.78–6.08	
Age at diagnosis					
< 10 years	120	18	1		0.480
≥ 10 years	30	5	0.56	0.11–2.80	
WBC count at diagnosis					
$< 30 \times 10^9$/L	105	12	1		0.450
≥ 30 and $< 100 \times 10^9$/L	30	5	1.67	0.40–6.89	0.004
$\geq 100 \times 10^9$/L	15	6	9.21	2.05–41.32	
Day 8 glucocorticoid response in blood (< 1000/µl)					
Good	140	19	1		0.400
Poor	10	4	0.32	0.02–4.49	
Day 15 BM response (by CM)					
< 10% blasts	130	17	1		0.880
≥ 10% blasts	20	6	0.82	0.06–11.12	
Spleen enlargement below costal margin					
< 4 cm	84	13	1		0.150
≥ 4 cm	66	10	0.44	0.15–1.35	
ALL lineage					
BCP-ALL	131	17	1		0.950
T-ALL	19	6	0.95	0.14–6.30	
Absolute blast count in CSF as measured by MFC					
< 20/ml	114	11	1		0.018
≥ 20/ml	36	12	4.65	1.30–16.60	
Day 36 MRD (MFC)					
< 0.1%	123	12	1		0.002
≥ 0.1%	27	11	7.43	2.16–25.56	

in comparison to children in whom the blasts were rapidly eliminated after the start of treatment (Fig. 7): CIR 0.50 ± 0.16 for slow responders ($n = 10$) and 0.29 ± 0.10 for rapid responders ($n = 22$), respectively, $p = 0.173$. This difference was significant when only patients with high initial MFC positivity (≥ 20 cells/ml) were considered: CIR 0.71 ± 0.17 for MFC positive ($n = 7$) and 0.33 ± 0.12 for MFC negative ($n = 16$) respectively, $p = 0.027$.

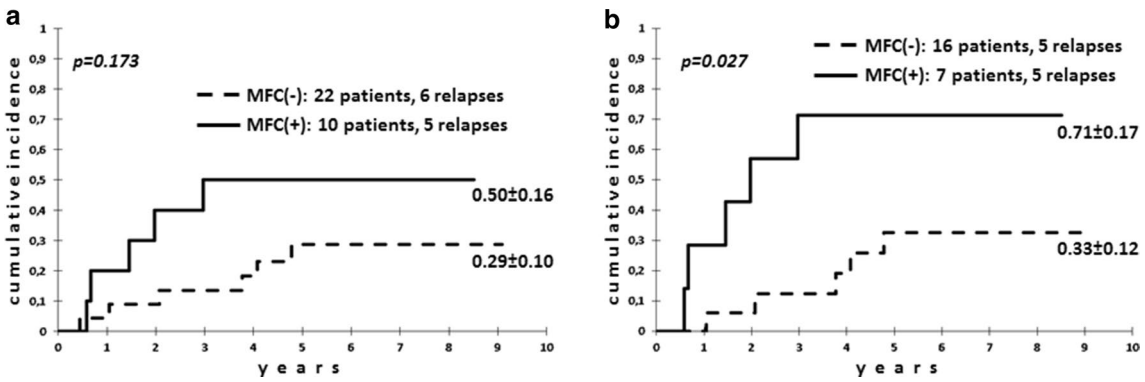

Fig. 7 Cumulative incidence of relapse by MFC positivity at the second time point of lumbar puncture in patients with detectable blasts in initial CSF sample (**a**) and in patients with high initial MFC positivity (≥ 20 per ml) (**b**)

Discussion

The central nervous system represents a sanctuary in which leukemia cells are protected from and may thus escape systemic chemotherapy and therefore a potential source of origin of relapse. Before the introduction of effective preventive CNS therapy, up to 60% of patients with ALL experienced their first relapse in the CNS. The reported data here demonstrate that even in the absence of isolated CNS relapses, the CIR in patients with high CSF positivity (as detected by MFC) was inferior compared with patients in whom no significant CNS involvement was found. This suggests that leukemia cells in CNS, even at a submicroscopic level, may have a substantial effect on the origin of a relapse—not only isolated CNS relapse. This supports the assumption that CNS and bone marrow relapses are competing events depending on the effectivity of therapy in different compartments. A similar situation has been described earlier for submicroscopic BM involvement in patients with clinically "isolated" extramedullary relapses (Hagedorn et al. 2007). Recently, it was shown that ALL cells with a specific marker expression at diagnosis of ALL have a high propensity to invade the CNS and may thus facilitate relapses originating in the CNS.(van der Velden et al. 2016). Therefore, precise detection and characterization of leukemia cells in CSF are crucial to gain more insight into the pathogenesis of leukemia and to develop better and more specific CNS-directed therapies.

Currently, CNS involvement in ALL remains underdiagnosed; this is confirmed by autopsy findings of CNS infiltration in patients who were considered to have leukemia restricted to the bone marrow only (Del Principe et al. 2014b). First attempts to improve the sensitivity and specificity of CSF cytomorphology were made more than 25 years ago by the implementation of a terminal deoxynucleotidyl transferase (TdT) immunofluorescence assay. Since that time, rapid development of MFC allowed complex immunophenotype analysis of both normal and leukemic cells in the CSF (Hooijkaas et al. 1989). However, flow cytometric CSF investigation still harbors several methodological difficulties (de Graaf et al. 2011). Due to the paucity and very rapid degradation of cells, CSF specimens have to be processed within few hours after sampling. In this context, some authors recommend the use of fixative media (Greig et al. 2014; de Jongste et al. 2014). Moreover, immunophenotype analysis of benign and malignant cells in CSF is subjective and requires experienced and skilled laboratory staff (de Graaf et al. 2011). Therefore, implementation of MFC as a routine method to assess CNS involvement, especially in multicenter trials, is only reasonable if relevant consequences can be drawn for clinical decisions.

In the current study, MFC yielded a more than twofold higher CSF positivity compared to CM. This data agrees with the results of previous studies having reported a frequent presence of low levels of leukemic blasts in CSF of ALL patients (Dass et al. 2017; Del Principe et al. 2014a; Levinsen et al. 2016). In the investigated cohort of 150 children without obvious signs of CNS leukemia, the outcome of MFC-positive patients was significantly worse compared with patients in whom MFC was negative. Nevertheless, the clinical use of these qualitative results (positive/negative) is doubtful. Within the group of SR patients and within children with CNS1, CIR differences between MFC-positive and MFC-negative patients were not significant. However, according to the ALL-MB 2008 stratification system, only these patients might have been upstaged and thus have received more intensive therapy in the case of CNS involvement. Likewise, in ImRG patients, an impact of MFC on prognosis could not be confirmed.

In pediatric oncology/hematology, MFC is being used as a quantitative technology. Therefore, it seems reasonable to make an attempt to convert results of cytometric CSF investigation into relative or absolute numbers of leukemia cells. In bone marrow studies, especially for monitoring of

minimal residual disease, the percentage of leukemic blasts among normal cells is always used to predict the outcome (Coustan-Smith et al. 2000; Basso et al. 2009; Dworzak et al. 2002). For CSF, this procedure is not fully reliable as the "normal" background in this body fluid may be very variable. Thus, it seems more appropriate to use the absolute blast cell count, recalculated from the percentage of leukemia cells among all nucleated cells similarly to BM in several MRD studies (Dworzak et al. 2002). After conversion to the absolute blast cell count, it was possible to determine a prognostically relevant threshold level. In patients with more than 20 leukemic cells per 1 ml of CSF, the relapse incidence was significantly higher compared to children with lower or negative MFC results. Moreover, in contrast to qualitative data, this quantitative approach remained a significant predictor of relapse in multivariate analysis as well as in all clinically relevant risk subgroups as shown above. The prognostic impact of quantitative MFC data remained significant independent of the type of CNS-directed therapy. Furthermore, application of a quantitative threshold level increases the reliability of the test because it allows dealing with values higher than MFC borderline sensitivity.

Nevertheless, precise assessment of the absolute blast cell count in CSF is more difficult than the distinction between positive and negative only, because it requires two different technologies. Both cytometric results and counting WBC may be affected or confounded by sample quality and shipping conditions. Thus, the application of this technology in multicenter trials requires immediate sample shipping and expert-level laboratory staff.

Conclusion

MFC is a highly sensitive technique of CSF investigation improving the definition of CNS involvement in childhood ALL. In the present study, quantitative measurement of leukemia cells in CSF by MFC allowed the discrimination of patients into groups with lower or higher cell counts with statistically significantly different outcomes. Nevertheless, the relatively small number of investigated patients from a single center is not adequate to draw definitive conclusions concerning therapeutic consequences for future large-scale trials. The results should be confirmed in a larger number of patients; further, i to reliably measure the absolute blast cell count, the logistics of shipping CSF samples from regional centers to reference laboratories have to be established. MFC investigation of CSF, if well-organized, can be a useful additional tool for stratification of patients in clinical trials.

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Compliance with ethical standards

Conflict of interest The authors declare no relevant conflicts of interests.

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