Prognosis of children with mixed phenotype acute leukemia treated on the basis of consistent immunophenotypic criteria

Ester Mejstrikova,[1-2] Jana Volejnikova,[1-2] Eva Fronkova,[1-2] Katerina Zdrahalova,[1-2] Tomas Kalina,[1-2] Jaroslav Sterba,[3] Yahia Jabali,[4] Vladimir Mihal,[5] Bohumir Blazek,[6] Zdena Cerna,[7] Daniela Prochazkova,[8] Jiri Hak,[9] Zuzana Zemanova,[10] Marie Jarosova,[11] Alexandra Oltova,[3] Petr Sedlacek,[2] Jiri Schwarz,[12] Jan Zuna,[1-2] Jan Trka,[1-2] Jan Stary,[2] and Ondrej Hrusak[1-2]

[1]CLIP-Childhood Leukemia Investigation Prague [2]Department of Pediatric Hematology and Oncology, Charles University, 2nd Faculty of Medicine and University Hospital Motol, Prague; [3]Departments of Pediatric Oncology and Medical Genetics, University Hospital, Brno; [4]Department of Pediatrics, Regional Hospital, Ceske Budejovice; [5]Department of Pediatrics, Faculty of Medicine Palacky University and University Hospital, Olomouc; [6]Department of Pediatrics, Teaching Hospital Ostrava; [7]Department of Pediatrics, University Hospital Pilsen; [8]Department of Pediatrics, Masaryk Hospital Usti nad Labem; [9]Department of Pediatrics, Faculty of Medicine Hradec Kralove; [10]Center of Oncocytogenetics, Institute of Clinical Biochemistry and Laboratory Diagnostics, General University Hospital and 1st Faculty of Medicine, Charles University, Prague; [11]Department of Hemato-oncology, Faculty of Medicine Palacky University and University Hospital, Olomouc, and [12]Institute of Hematology and Blood Transfusion, Prague, Czech Republic

ABSTRACT

Background
Mixed phenotype acute leukemia (MPAL) represents a diagnostic and therapeutic dilemma. The European Group for the Immunological Classification of Leukemias (EGIL) scoring system unambiguously defines MPAL expressing aberrant lineage markers. Discussions surrounding it have focused on scoring details, and information is limited regarding its biological, clinical and prognostic significance. The recent World Health Organization classification is simpler and could replace the EGIL scoring system after transformation into unambiguous guidelines.

Design and Methods
Simple immunophenotypic criteria were used to classify all cases of childhood acute leukemia in order to provide therapy directed against acute lymphoblastic leukemia or acute myeloid leukemia. Prognosis, genotype and immunoglobulin/T-cell receptor gene rearrangement status were analyzed.

Results
The incidences of MPAL were 28/582 and 4/107 for children treated with acute lymphoblastic leukemia and acute myeloid leukemia regimens, respectively. In immunophenotypic principal component analysis, MPAL treated as T-cell acute lymphoblastic leukemia clustered between cases of non-mixed T-cell acute lymphoblastic leukemia and acute myeloid leukemia, while other MPAL cases were included in the respective non-mixed B-cell progenitor acute lymphoblastic leukemia or acute myeloid leukemia clusters. Analogously, immunoglobulin/T-cell receptor gene rearrangements followed the expected pattern in patients treated as having acute myeloid leukemia (non-rearranged, 4/4) or as having B-cell progenitor acute lymphoblastic leukemia (rearranged, 20/20), but were missing in 3/5 analyzed cases of MPAL treated as having T-cell acute lymphobastic leukemia. In patients who received acute lymphoblastic leukemia treatment, the 5-year event-free survival of the MPAL cases was worse than that of the non-mixed cases (53±10% and 76±2% at 5 years, respectively, P=0.0075), with a more pronounced difference among B lineage cases. The small numbers of MPAL cases treated as T-cell acute lymphoblastic leukemia or as acute myeloid leukemia hampered separate statistics. We compared prognosis of all subsets with the prognosis of previously published cohorts.

Conclusions
Simple immunophenotypic criteria are useful for therapy decisions in MPAL. In B lineage leukemia, MPAL confers poorer prognosis. However, our data do not justify a preferential use of current acute myeloid leukemia-based therapy in MPAL.

Key words: pediatric acute lymphoblastic leukemia, pediatric acute myeloid leukemia, mixed phenotype leukemia, biphenotypic leukemia, aberrant antigens, cytometry, acute hybrid leukemia

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Correspondence: Ondrej Hrusak/Ester Mejstrikova, Dept. of Pediatric Hematology Oncology, V uvalu 84, 150 06 Praha 5, Czech Republic. E-mail: ondrej.hrusak@lfmotol.cuni.cz/ester.mejstrikova@lfmotol.cuni.cz

The online version of this article has a Supplemetary Appendix.

Introduction

In most cases of acute leukemia, immunophenotyping unambiguously identifies the primary lineage [myeloid, B-cell precursor (BCP) or T lineage] of the leukemic cells. However, there are uncommon cases that are difficult to assign to one lineage; these patients are diagnosed as having mixed phenotype acute leukemia (MPAL, otherwise known as acute biphenotypic or hybrid leukemia). MPAL account for 3-5% of all cases of acute leukemia.[1] The designation of MPAL describes one of three conditions: acute leukemia with two distinct leukemic blast populations, each belonging to a different lineage;[2] acute leukemia with an early switch from one lineage to another;[3,4] and acute leukemia with marked expression of aberrant molecules (those that are physiologically expressed in a different lineage).[5-7] Since aberrant molecules are frequently expressed by leukemic blasts,[8-11] there is a need to distinguish patients with typical less prominent aberrant expression from patients with "genuine MPAL".

The European Group for the Immunological Classification of Leukemias (EGIL) has created a scoring system that assigns score points to major antigens. Cases exceeding a given threshold for myeloid and at least one lymphoid lineage are considered MPAL.[12,13] To our knowledge, this has been the only standard international definition of MPAL until recently. In studies carried out in a single institution, an alternative definition of MPAL was applied which emphasized solely the antigens with the highest EGIL score points: cytoplasmic expression of CD79a, IgM, CD3, and myeloperoxidase (MPO).[1,14]

Recently, a new World Health Organization (WHO) classification of acute leukemia listed several molecular genetic subtypes of acute myeloid leukemia (AML), subtypes of B precursor acute lymphoblastic leukemia (ALL) also separated by molecular genetics, T lymphoblastic leukemia, four subsets of MPAL and acute undifferentiated leukemia. Typical immunophenotypic findings are reviewed for each entity. Clear-cut distinctions between strong and weak expression as well as percentage requirements for positivity (most importantly, positivity for MPO) are yet to be stated. Standardized categorizing into groups for different types of treatment (myeloid- or lymphoid-directed) was not in the scope of this WHO classification.[15-18] In consequence, in the routine clinical setting, there is no consensus on the strategy to assign patients with MPAL to either lymphoid- or myeloid-directed treatment. In clinical practice, treatment decisions may be based on morphology (which is frequently ambiguous), immunophenotypic details (with undetermined weight of the molecules which may conflict with each other), cytogenetic or molecular genetic data (which are usually unavailable at the very beginning of treatment, and often show no typical gene fusion) and other findings. The resulting treatment heterogeneity complicates the search for recommended treatments, especially in MPAL.

Here we present information on a population-based cohort of children who received treatment on the basis of standard, simple immunophenotypic criteria. Special attention is paid to the MPAL cases. Although the EGIL scoring system for MPAL has been appraised many times during the last decade, little is known about its prognostic significance. The primary aim of this study was to elucidate the effect of lymphoid-directed treatment in MPAL cases that fulfill the definition for ALL and to determine whether available myeloid-directed approaches in these patients are advisable.

Design and Methods

Patients

All Czech patients under 18 years of age with newly diagnosed primary acute leukemia and with a centrally investigated immunophenotype between September 1996 and August 2006 entered this study. Details on the patients, processing of samples, treatment protocol selection, monoclonal antibodies, genotype subsets, other cytogenetic and molecular genetic investigations, detection of immunoglobulin and T-cell receptor gene rearrangements and statistics are provided in the *Online Supplementary Design and Methods* section.

Treatment-determining molecules and EGIL scoring for mixed phenotype acute leukemia

Expression of all molecules was assessed by binding fluorochrome-labeled monoclonal antibodies to the leukemic blast population using standard flow cytometry.[11] BCP-ALL treatment was administered in cases with positive expression of two of the following: CD19, CD22 and CD79a. T-ALL treatment was administered in cases with positive expression of CD7 and CD3 together with MPO negativity or positivity in fewer than 30% blasts. AML-directed treatment was administered in cases with positive expression of two of the following: CD13, CD33, CD65, CD117 and MPO, together with the absence of ALL-defining criteria. MPAL was defined using the EGIL criteria using scores for particular antigens in three lineages.[12,13] B lineage EGIL score points were: 2.0 (CD79a and CD22), 1.0 (CD10, CD19 and CD20) and 0.5 (TdT and CD24). T lineage EGIL score points were: 2.0 (CD3, T-cell receptor αβ and T-cell receptor γδ), 1.0 (CD2, CD5, CD8 and CD10) and 0.5 (TdT, CD1a and CD7). Myeloid lineage EGIL score points were: 2.0 (MPO), 1.0 (CD13, CD33, CD65 and CD117) and 0.5 (CD14, CD15 and CD64). The EGIL score for a particular lineage was calculated as the sum of the points that corresponded to each positive antigen. Cases were considered to be MPAL if the sum of EGIL scores was greater in both myeloid and at least one of the lymphoid lineages.

Intracellular (MPO and TdT), intracellular and/or membrane (CD79a, CD22 and CD3) and membrane (all other antigens) expression of antigens was considered both for the definition of ALL/AML and for the MPAL EGIL score. Positivity for each antigen was defined as 20% or more of the gated blasts cells carrying the antigen, unless otherwise specified. CD64 was assessed only in cases in which the sum of scores for MPO, CD13, CD14, CD15, CD33, CD65 and CD117 was equal to 2.0. IgM was assessed only in cases that otherwise fulfilled the definition of BCP-ALL.

Flow cytometric findings of all patients were interpreted by an experienced specialist. Each interpretation included a diagnostic conclusion that assigned the respective patient to non-mixed acute leukemia (BCP-ALL, T-ALL or AML) or to MPAL (treated as BCP-ALL, T-ALL or AML). Molecular and cytogenetic testing was performed instantly for all MPAL patients and all patients who were reclassified due to an early switch between ALL and AML or because of other laboratory findings.

The leukemic blast expression of all treatment-determining molecules as well as of MPAL-scoring molecules was confirmed by simultaneous labeling, as recommended.[13] Patients with non-mixed EGIL scores who fulfilled other MPAL definitions (i.e., an early switch from ALL to AML or vice versa or those with simultaneous presence of significant AML and ALL populations at diagnosis) are listed separately and were omitted from all comparisons of patients with MPAL and non-mixed acute leukemia.

Results

Patients' characteristics

In total, 693 patients were diagnosed between September 1996 and August 2006 with primary acute leukemia. Of these, 109, 501 and 83 fulfilled the criteria for treatment with AML, BCP-ALL, and T-ALL regimens, respectively. Of these, four patients were excluded. Overall, the study patients represent approximately 98% of all children diagnosed and treated with acute leukemia during this period in the Czech Republic. Exclusion characteristics and other details are specified in the *Online Supplementary Results* section.

Frequency of a positive mixed phenotype acute leukemia score in leukemia subtypes

Table 1 summarizes the frequency of MPAL among acute leukemias. The frequency ratio between non-mixed acute leukemias and their MPAL counterparts did not differ in patients treated as having AML and ALL or in BCP and T-ALL. Among ALL-treated patients, MPAL was more frequent in *BCR/ABL*-positive cases (P=0.043). Within the BCP-ALL genotypes, MPAL was more frequent in cases of *MLL/AF4*-positive acute leukemia (P=0.0073) and less frequent in hyperdiploid acute leukemia (P=0.037). Although *TEL/AML1* was the most frequent genotype within BCP-MPAL (41%), its frequency was not significantly different from that in non-mixed ALL (28%, P>0.05).

In addition to genotype-defining chromosomal fusions, we searched for additional *MLL* translocations (*MLL/AF6, MLL/AF9, MLL/AF10, MLL/ENL* and *MLL/ELL*) and found that all MPAL patients were negative. In addition, although two patients carried *FLT3*-ITD, all other MPAL patients were negative for *FLT3*-ITD and for *FLT3*-D835 activating mutations.

Prognosis of mixed phenotype acute leukemia

The total 5-year event-free survival rates of children treated as having ALL and AML were 75±1.9% and 47±4.9%, respectively. Among patients receiving ALL-directed treatment, those with MPAL had a significantly lower event-free survival rate than those with non-mixed acute leukemia (53±10% and 76±2% at 5 years, respectively, P=0.0075, Figure 1A). This difference was more pronounced in cases treated as BCP-ALL (45±11% *versus* 77±2.1% at 5 years, P=0.00083, Figure 1B). The prognosis of cases treated as T-ALL is shown in Figure 1C (the difference was not compared statistically because of the low numbers). Although the numbers of patients within genotype subsets were relatively small, MPAL was correlated with a poorer prognosis among *TEL/AML1*-positive (P=0.013) and *MLL/AF4*-positive acute leukemia (P=0.019) (*Online Supplementary Figure S1A-B*). The prognosis of MPAL was not significantly different within *BCR/ABL*-positive or non-hyperdiploid acute leukemias without listed fusions (P>0.05). In a proportional hazard (Cox) regression analysis, positive MPAL scores correlated with a worse outcome, even when BCP-ALL-treated patients were stratified by genotype (P=0.0018). In addition, when both EGIL score and genotype subset were treated as independent variables in a multivariate analysis, EGIL score correlated with prognosis with a higher degree of significance than genotype (P=0.0019 and P=0.016, respectively).

When only patients treated following the ALL-IC BFM 2002 or ALL BFM 95 protocols were selected from the BCP-ALL-treated cohort, the difference in outcome seen among patients on all BCP-ALL protocols remained statistically significant (event-free survival at 5 years 55±13% *versus* 78±2.1% in MPAL and non-mixed BCP-ALL, respectively, P=0.043). Among non-infant patients with BCP-ALL who received the ALL-IC BFM 2002 or ALL BFM 95 protocls, the prognosis of MPAL and non-mixed cases remained significantly different (P=0.044).

Treated with ALL BFM 95 and ALL-IC BFM 2002 protocols (in which the first 15 days of therapy are identical), 13.6% of the MPAL patients had very poor blast clearance (M3 bone marrow morphology) at day 15 (comparable to that of non-MPAL patients, 11.8%). On the other hand, the percentages of M1 and M2 were different in MPAL (36.4% and 50%, respectively) from those in non-MPAL (64.1% and 24%, respectively). Overall, the differences in day 15 clearance of blasts were statistically significant between MPAL and non-MPAL cases (n=529, P=0.02, χ^2 test). No difference in the peripheral blood response to prednisone on day 8 was noted between MPAL and non-MPAL cases (P=0.78, χ^2 test).

The prognosis of patients treated with AML protocols is shown in Figure 1D. Although our diagnostic criteria separated MPAL cases with T lineage antigens to receive either ALL or AML-directed treatment, other studies may merge them. We, therefore, analyzed the prognosis of patients who fell into one of these categories, finding that

Table 1. Incidence of MPAL, defined by EGIL score, in subsets of acute leukemia.

Type of acute leukemia and treatment direction	Non MPAL n. of patients	%	MPAL n. of patients	%
ALL	554[b]	95	28[a]	4.8
T ALL	77	93	6[a]	7.2
B precursor ALL	477[b]	96	22	4.4
TEL/AML1	133	94	9	6.3
hyperdiploid	112	99	1	0.88
BCR/ABL	13	81	3[a]	19
MLL/AF4	7	70	3	30
non-hyperdiploid ALL without listed fusions	193[b]	97	6	3
ALL without listed fusions, DNA index unknown	19	95	1	5
AML	103	96	4	3.7

[a]*Includes one* BCR-ABL-positive *TALL.* [b]*Includes one case with unknown* TEL/AML1, *but excludes two cases (one early switch from ALL to AML and one case of bilineal acute leukemia) with MPAL defined by means other than EGIL score. The type of acute leukemia was defined in a standard manner as described in the Design and Methods section.*

Table 2. Immunoglobulin and T-cell receptor gene rearrangements in cases of MPAL.

MPAL treated as	Patients investigated for *IGTCR* rearrangements n	Patients with at least one rearranged *IGTCR* locus n	% investigated
BCP ALL	20	20	100%
T ALL	5	2	40%
AML	4	0	0%

Summary data on all three MPAL subsets are shown. Details are in the Online Supplementary Results..

the 5-year event-free survival rate was 58±16%.

The prognostic impact of the EGIL score was compared with the importance of each antigen separately in patients who received a BCP-ALL treatment. No single antigen conferred an unfavorable prognosis with a higher significance than the EGIL score. At a 20% cut-off value, CD33, CD65, MPO and CD13 correlated with significantly worse prognosis (P=0.0039, P=0.0042, P=0.017 and P=0.03, respectively), while CD14, CD15 and CD117 did not correlate with prognosis (P>0.05 in all instances, *data not shown*). The myeloid antigens did not correlate with T-ALL prognosis (P>0.05 in all instances, *data not shown*). The prognostic significance of CD64 was not analyzed statistically, as it was only assessed in a subset with a higher number of other myeloid antigens. Multivariate analysis of continuous data confirmed the significance of CD33 and CD65 only, in line with our previous observation that CD33 has a strong adverse prognostic impact.[10]

Unsupervised immunophenotype subset analysis

Unsupervised assessment of our immunophenotype data using principal component analysis divided the cases into three major categories (details in the *Online Supplementary Design and Methods and Results* sections). These categories corresponded to our classification of assignment to BCP-ALL, T-ALL and AML therapy (*Online Supplementary Figure S2*). Notably, principal component analysis revealed several cases with an intermediate immunophenotype between the AML and T-ALL clusters. All these cases were classified as MPAL treated by T-ALL protocols. In a three-dimensional view (*Online Supplementary Data*), these MPAL cases were clearly separated from both AML and non-mixed T-ALL clusters. The MPAL cases treated as AML were not different from the non-mixed AML cases in the principal component analysis. Although MPAL cases treated as BCP-ALL were placed eccentrically within the BCP-ALL cluster, they could not be distinctly separated from non-mixed BCP-ALL cases.

These results prompted us to ask whether "proximity to AML," as defined by the unsupervised principal component analysis, had a greater prognostic impact than EGIL-scored "phenotypic ambiguity". We, therefore, selected the 40 BCP-ALL cases whose immunophenotype had the closest similarity to AML in the three-dimensional principal component analysis plot. Ten of these cases also had mixed EGIL scores. Although the prognosis of these 40 cases was significantly poorer (5-year event-free survival rate 60±8.2%) than that of the remaining patients who received BCP-ALL treatment (n=440, 5-year event-free survival rate 78±2.1%; P=0.0097, this difference was less prominent than if the EGIL score was used for BCP-ALL categorization.

Immunoglobulin and T-cell receptor gene clonality

All MPAL cases treated as BCP-ALL contained the typical clonal immunoglobulin (*IG*) and/or T-cell receptor (*TCR*) gene rearrangements (Table 2). All but one of these cases contained clonal *IG* rearrangements, whereas "cross-lineage" *TCR* rearrangements were less common, with a frequency that was similar to that in previously reported cohorts of non-mixed ALL.[19] MPAL *TEL/AML1* cases had more rearrangements than other MPAL cases treated as BCP-ALL (P=0.003, Mann-Whitney). In contrast, three of five MPAL cases treated as T-ALL were negative for all rearrangements in *IG/TCR* genes. None of the four MPAL patients treated as having AML had the investigated *IG/TCR* rearrangements.

Discussion

Prognostic relevance of mixed phenotype acute leukemia; pros and pitfalls of existing definitions

In this study, all 689 patients (including the cases with

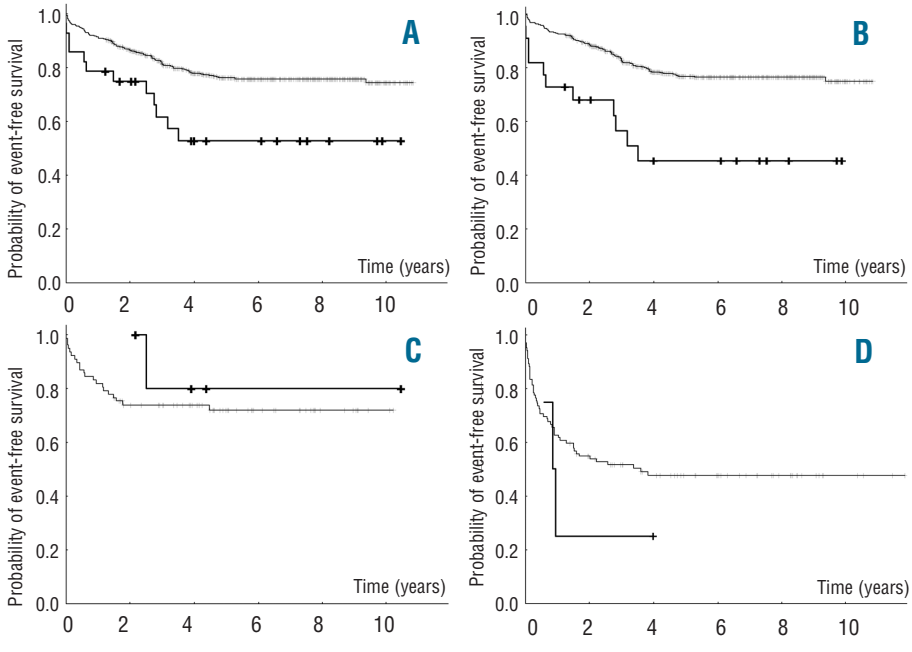

Figure 1. Treatment results of 693 cases of acute leukemia. Event-free survival of EGIL score-positive MPAL (bold lines) compared to other cases (thin lines). (A) Patients treated as having ALL (P=0.0075), (B) Patients treated as having BCP-ALL (P=0.00083), (C) Patients treated as having T-ALL, (D) Patients treated as having AML (differences in panels 1C and 1D not analyzed statistically due to low numbers).

positive EGIL scores) were assigned, for the purposes of therapy, to either one of the ALL lineages or to AML. Although the cases of MPAL were of special diagnostic interest, the simple treatment guidelines could be applied to all of them and led to the outcomes discussed below. The original proposal of the EGIL group identified MPAL by simultaneous findings characteristic of AML and ALL. This proposal did not, therefore, consider cases presenting just with B and T lineage ALL (without myeloid) molecule expression as MPAL.[12,13] Remarks on the potential overdiagnosis of B/T MPAL[18] thus address a misinterpretation of the original EGIL proposal rather than the proposal itself. Like any scoring system, the EGIL system may be criticized for misclassification as a result of the strict application of arbitrary criteria. The presented population-based analysis of EGIL-scored MPAL should aid the objective evaluation of this scoring system. Although no studies on the incidence of MPAL defined according to the WHO classification have been published, the EGIL scoring system for MPAL appears to depict a broader subset of patients than that of the newly proposed WHO classification. In order to make categorization reproducible, details of the WHO criteria should be specified, such as the exact distinction between strong and weak antigen expression and cut-off values for MPO positivity. The WHO definition uses fewer parameters than the EGIL scoring system; although it combines results from cytometry and cytochemistry, it may prove to be simpler (Table 3).

The statistical power of MPO expression in the EGIL scoring system in which it has 2 points, is the same as or even greater than in other systems, including the WHO classification.[1,18] However, MPO-positive B lymphoid malignancies which responded to ALL treatment have been reported.[20,21] This is in line with our treatment recommendations, in which high MPO expression can override lymphoid criteria only in T lineage leukemias.

Immunoglobulin and T-cell receptor gene rearrangements

The spectrum of *IG/TCR* gene rearrangements in MPAL treated as BCP-ALL or AML corresponded with the expected spectrum in the respective acute leukemia lineages. All MPAL patients who were given BCP-ALL treatment presented with at least one *IG* or *TCR* clonal rearrangement. It has previously been established that BCP-ALL lymphoblasts (like non-malignant B cells) can rearrange both *TCR* genes and *IG* genes.[19,22,23] As in our previous study on BCP-ALL,[24] MPAL *TEL/AML1*-positive cases had more rearrangements than other MPAL cases treated as BCP-ALL, which is indicative of *RAG1* upregulation. As with the majority of AML cases,[25] all MPAL cases assigned to AML therapy were negative for *IG/TCR* gene rearrangements. Interestingly, although other studies and our data (*not shown*) have identified *TCR* rearrangements in the majority of cases of T-ALL,[26] only two of five cases of MPAL cases treated as T-ALL had *TCR* rearrangements; these numbers are, however, too low for statistical analysis.

Unsupervised immunophenotype analysis, principal component analysis

In the principal component analysis of immunophenotype, the main differences were found among three major subsets (T-ALL, BCP-ALL and AML). As no cases were misplaced into different immunophenotype clusters, our principal categorization to AML, T-ALL and BCP-ALL therapy arms appears to respect general immunophenotype patterns. Interestingly, principal component analysis placed mixed T-ALL cases in a borderline zone between T-ALL and AML. No cases were found in the analogous borderline space between the BCP-ALL and AML clusters or between T-ALL and BCP-ALL. MPAL cases that were identified by their EGIL score within BCP-ALL and AML,

Table 3A. Definitions or characteristics of BCP ALL, T ALL and AML.

Reference	Type of acute leukemia	Logical operators	Conditions to be fulfilled				
Borowitz *et al.* (WHO)	BCP ALL	almost always:	CD19^{pos}	*i*CD22^{pos}	*i*CD79a^{pos}		
		always:	MPO^{neg}				
	T ALL	most often:	*(i)*CD3^{pos}				
Arber *et al.* (WHO)	AML	≥1 of:	≥20% myeloblasts[a]	≥20% promonocytes[a]	t(8;21)^{pos}	inv(16)^{pos} or t(16;16)^{pos}	t(15;17)^{pos}
Bene *et al.* (EGIL)	BCP ALL	≥2 of:	CD19^{pos}	*(i)*CD22^{pos}	*(i)*CD79a^{pos}		
	T ALL	single criterion:	*(i)*CD3^{pos}				
	AML	≥2 of:	CD13^{pos}	CD33^{pos}	CD65^{pos}	CD117^{pos}	*i*MPO^{pos}
This study[b,c]	BCP ALL	≥2 of:	CD19^{pos}	*(i)*CD22^{pos}	*(i)*CD79a^{pos}		
	T ALL	all 3 of:	CD7^{pos}	*(i)*CD3^{pos}	iMPO<30%		
	AML	≥2 of:	CD13^{pos}	CD33^{pos}	CD65^{pos}	CD117^{pos}	*i*MPO^{pos}
		and	BCP ALL criteria not met				
		and	T ALL criteria not met				
Behm *et al.* (SJCRH)[d]	BCP ALL	all of:	CD19^{pos}	*i*CD3^{neg}	MPO^{neg}		
		and ≥1 of:	CD22^{pos}	*i*CD79a^{pos}	iIgM^{pos}		
	T ALL	all of:	CD7^{pos}	*i*CD3^{pos}	CD22^{neg}	MPO^{neg}	
	AML	both:	*i*CD3^{neg}	*i*IgM^{neg}			
		and ≥1 of	CD22^{neg}	*i*CD79a^{neg}			
		and ≥1 of	MPO^{pos}	NSE^{pos}			

iIntracellular antigen staining (in cytochemistry, the fact that the reaction occurs intracellularly is disregarded). (i)Intracellular or membrane antigen staining. [a]By morphology (children with fewer than 30% blasts may be classified as having a myelodysplastic syndrome if further criteria are met). [b]Used for treatment decisions also in MPAL. [c]Positivity cut-off in this study is 20% of gated malignant cells. [d]Saint Jude Children's Research Hospital (SJCRH) criteria are used to distinguish non-MPAL cases with My+ ALL or Ly+ AML.

respectively, did not form a distinct cluster. This implies that the EGIL score does not reflect a difference in the overall immunophenotype within BCP-ALL and AML.

Myeloid-associated molecular genetics in mixed phenotypic acute leukemia

In T-ALL patients, Paietta *et al.* found an association between the expression of CD117 and *FLT3*-activating mutations.[27] Since most of our patients with CD117-positive MPAL treated as ALL were negative for *FLT3*-ITD and for *FLT3*-D835 activating mutations, our data contradict the original findings, in accordance with a case reported later.[28] Thus, *FLT3*-ITD and *FLT3*-D835-activating mutations are not causally involved in the pathogenesis of MPAL and our data do not support the general use of FLT3 inhibitors in MPAL. In one patient (T5), we identified a translocation 46,XY,t(6;14)(q?26;q?31), which may correspond to the previously published t(6;14)(q25;q32). The result of fluorescent *in situ* hybridization with painting (WCP6 and WCP14) and locus-specific probes (LSI IgH Dual Color Break Apart) confirmed the reciprocal translocation without rearrangement of the *IGH* gene which was translocated to the long arm of chromosome 6. This translocation was repeatedly found in MPAL patients with myeloid and T lineage components, based on antigen expression or bilinearity. The gene on chromosome 14 involved in this translocation appears to be *BCL11B*, which encodes the BCL11B protein, a transcription repressor with an important role in T-cell development.[29,30] Additionally, the same patient presented with a *FLT3*-ITD. Since published studies did not investigate *FLT3*, we cannot conclude whether there is a causal relationship between t(6;14) and *FLT3*-ITD. Another translocation, t(8;12)(q13;p13), leading to an *ETV6-NCOA2* fusion gene often associated with MPAL with myeloid and T lineage components[31] was not found in this cohort (*data not shown*).

Therapeutic implications

As recently noted, the low incidence of MPAL precludes large trials with separate analyses of subcohorts.[32] Thus, a statistical analysis of the prognosis of MPAL treated with T-ALL regimens is not available. Considering our diagnostic data only, one may speculate that all MPAL cases with a T lineage component, including those who received a T-ALL-directed therapy in this study, are biologically close to AML, given the findings of the principal component analysis and the low frequency of *TCR* rearrangements. However, patients with these MPAL appear to have a relatively good outcome after ALL-directed treatment, as presented here (Figure 1C). Rubnitz *et al.* reported, for a similar cohort of patients, a good response to ALL-directed treatment even among those who failed to achieve complete remission after initial AML-directed treatment.[1]

Quite the opposite situation is present in patients who fulfilled our criteria to be treated with BCP-ALL therapy. In these patients, both the immunophenotype-based principal component analysis and *IG/TCR* rearrangements are similar between MPAL and non-mixed BCP-ALL. However, the prognosis of these patients is significantly poorer. As there have been no studies conducted on the prognostic impact of uniformly treated MPAL defined by EGIL scores, the data presented can only be compared with those of studies using different definitions of "phenotypic ambiguity". A recent study on MPAL, which was defined using slightly different criteria and in which some patients were treated with AML-directed therapy, demonstrated a 5-year event-free survival rate of 36±16% for

Table 3B. Definitions of MPAL.

Reference	Type of MPAL	Logical operators	Conditions to be fulfilled				
Borowitz *et al.* (WHO)	MPAL My/B	both:	My characteristics	B characteristics			
	MPAL My/T	both:	My characteristics	T characteristics			
	MPAL B/T	both:	B characteristics	T characteristics			
	B characteristics	CD19 at least weak and ≥2 of:	CD19^{strong}	*i*CD22^{strong}	CD79^{astrong}	CD10^{strong}	
	T characteristics	single criterion	*(i)*CD3^{pos}				
	My characteristics		MPO^{pos}				
		or ≥2 of:	NSE^{pos}	CD11c^{pos}	CD14^{pos}	CD64^{pos}	lysozyme^{pos}
Behm *et al.* (SJCRH)	MPAL My/B	both:	MPO^{pos}	CD22^{pos}			
		and ≥1 of:	CD19^{pos}	*i*CD79a^{pos}			
	MPAL My/T	both:	MPO^{pos}	*i*CD3^{pos}			
	MPAL B/T	must be fulfilled:	*i*CD3^{pos}				
		and iIgM or ≥2 of:	*i*IgM^{pos}	iCD79a^{pos}	CD22^{pos}		
Bene *et al.* (EGIL)	MPAL My/B	both:	B score >2	My score >2			
	MPAL My/T	both:	T score >2	My score >2			
	B score	2 points per antigen	*(i)*CD79a^{pos}	*i*IgM^{pos}	iCD22^{pos}		
		1 point per antigen	CD19^{pos}	CD10^{pos}	CD20^{pos}		
		0.5 points per antigen	iTdT^{pos}	CD24^{pos}			
	T score	2 points per antigen	*(i)*CD3^{pos}	TCRαβ^{pos}	TCRγδ^{pos}		
		1 point per antigen	CD2^{pos}	CD5^{pos}	CD8^{pos}	CD10^{pos}	
		0.5 points per antigen	iTdT^{pos}	CD7^{pos}	CD1a^{pos}		
	My score	2 points per antigen	*i*MPO$^{pos[a]}$				
		1 point per antigen	CD13^{pos}	CD33^{pos}	CD65^{pos}	CD117$^{pos[b]}$	
		0.5 points per antigen	CD14^{pos}	CD15^{pos}	CD64^{pos}		

Abbreviations are explained in Table 3A. [a]The original paper also suggested a provisional inclusion of lysozyme positivity with a score of 2.0 points – this proposal was not addressed in the subsequent publications of the EGIL group. Lysozyme was not considered in the presented study. [b]This is the updated weight of CD117, the original proposal suggested a lower score. References to Tables 3A and 3B: (12-18, 38, 39)

B/My MPAL cases.[1] This outcome is no better than that conferred by the standardized approach presented here with a 5-year event-free survival rate of 45±11% (Figure 1B) for mixed BCP-ALL. Our cohort of MPAL comprised cases with known prognostically important genotypes. However, these cases cannot explain the poor prognosis of the whole MPAL group, as MPAL had a worse outcome both within the respective subsets themselves (see *TEL/AML1*-positive and *MLL/AF4*-positive survival curves in *Online Supplementary Figure S1A-B*) and in a stratified Cox regression analysis. The observed prognostic significance was striking in *TEL/AML1*-positive acute leukemia, since this subset of ALL typically correlates with myeloid antigens CD13 and CD33[9,33] and a non-specific positivity of the myeloid EGIL score could, therefore, be expected. Yet, our data proved that *TEL/AML1*-positive cases classified as MPAL according to the EGIL criteria have a significantly worse prognosis compared to the rest of the *TEL/AML1*-positive group and that the prognostic impact of "phenotypic ambiguity" is greater than that of other factors at diagnosis.[34,35]

Very recently, a study was published on adult MPAL treated with several lymphoid- or myeloid-directed approaches.[36] The authors showed that this heterogeneous approach led to a very unfavorable outcome (median disease-free survival, 5 months), significantly worse than that for both AML and ALL. Interestingly, recalculation of the data on achievement of complete remission showed a superior initial response to therapy in patients treated with corticosteroids (14/16) compared to those treated with myeloid-directed chemotherapeutics alone (1/5, Fisher's P=0.011).

The inferior prognosis of MPAL patients among those who fulfilled the criteria for treatment with BCP-ALL-therapy obviously urges the search for better treatment for these patients. Collectively, the available data do not support the general use of contemporary AML-directed treatment for MPAL fulfilling T-ALL or BCP-ALL criteria. The recommendation arising from the presented data is to start lymphoid-based treatment in MPAL patients who fulfill the criteria for the definition of B- or T-lineage acute leukemias. Urgent (cyto)genetic investigations should be requested and their results should be taken in consideration (especially *BCR/ABL* status, since kinase-inhibitor-containing protocols may be available, or ALL- or AML-associated gene fusions). Treatment response should be carefully monitored at appropriate times and a switch to myeloid-directed treatment or a protocol combining lymphoid and myeloid elements (such as the Interfant protocol for infant ALL) should be considered in patients with an inadequate response to the remission induction therapy.

Rubnitz *et al.* concluded that MPAL *per se* is not an indication for stem cell transplantation.[1] In the presented cohort, stem cell transplantation was indicated in ALL patients only in the case of no response or in those with high-risk *BCR/ABL*-positive ALL. Only two of 28 MPAL patients treated as having ALL have, therefore, been transplanted in first complete remission. Our data support the notion of Rubnitz *et al.* that stem cell transplantation is not recommended for patients with a good molecular response to induction treatment. Levels of minimal residual disease in cases of MPAL treated as ALL can be detected by *IG/TCR* rearrangements (as can be deduced from Table 2). Flow cytometry can identify residual MPAL cells, which are, by definition, immunophenotypically distinct from normal cells. Nonetheless, immunophenotypic shifts may still hamper the distinction of minimal residual disease from a non-malignant background in regenerative bone marrow.[37] In AML, the presented therapy protocols recommend stem cell transplantation only in high-risk cases with an HLA-matched sibling donor. The low number of patients in the presented cohort precludes the possibility of making a conclusive recommendation for MPAL cases treated as AML.

Authorship and Disclosures

EM performed the immunophenotyping and designed and wrote part of the paper, JV screened the patients for *IG TCR* rearrangements, EF performed *IG TCR* sequencing and *FLT3* molecular genetics and related analyses, KZ analyzed part of the clinical data, TK performed part of the immunophenotype analyses, JSte, YJ, VM, BB, ZC, DP and JH were responsible for treatment and clinical data, PS performed hematopoietic stem cell transplants, ZZ, MJ and AO performed cytogenetic analyses, JZ was responsible for determining *MLL* translocations, JSc performed *AML1/ETO*, *CBFB/MYH11* and *PML/RARA* analyses, JT was responsible for molecular genetics in ALL, JSta was responsible for treatment in Prague and for nationwide coordination, OH designed the study, performed principal component analysis, statistics and part of the immunophenotyping and wrote the paper.

The authors reported no potential conflicts of interest.

References

1. Rubnitz JE, Onciu M, Pounds S, Shurtleff S, Cao X, Raimondi SC, et al. Acute mixed lineage leukemia in children: the experience of St. Jude Children's Research Hospital. Blood. 2009;113(21):5083-9.
2. Weir EG, Ali Ansari-Lari M, Batista DA, Griffin CA, Fuller S, Smith BD, et al. Acute bilineal leukemia: a rare disease with poor outcome. Leukemia. 2007;21(11):2264-70.
3. Bierings M, Szczepanski T, van Wering ER, Willemse MJ, Langerak AW, Revesz T, et al. Two consecutive immunophenotypic switches in a child with immunogenotypically stable acute leukaemia. Br J Haematol. 2001;113(3):757-62.
4. Krawczuk-Rybak M, Zak J, Jaworowska B. A lineage switch from AML to ALL with persistent translocation t(4;11) in congenital leukemia. Med Pediatr Oncol. 2003;41(1):95-6.
5. Killick S, Matutes E, Powles RL, Hamblin M, Swansbury J, Treleaven JG, et al. Outcome of biphenotypic acute leukemia. Haematologica. 1999;84(8):699-706.
6. Matutes E, Morilla R, Farahat N, Carbonell F, Swansbury J, Dyer M, et al. Definition of acute biphenotypic leukemia. Haematologica. 1997;82(1):64-6.
7. Owaidah TM, Al Beihany A, Iqbal MA, Elkum N, Roberts GT. Cytogenetics, molecular and ultrastructural characteristics of biphenotypic acute leukemia identified by the EGIL scoring system. Leukemia. 2006;20(4):620-6.
8. Lo Coco F. Hybrid phenotypes and lineage promiscuity in acute leukemia. Haematologica. 1991;76(3):215-25.
9. Hrusak O, Porwit-MacDonald A. Antigen expression patterns reflecting genotype of acute leukemias. Leukemia. 2002;16(7):1233-58.
10. Mejstrikova E, Kalina T, Trka J, Stary J, Hrusak O. Correlation of CD33 with poorer prognosis in childhood ALL implicates a potential of anti-CD33 frontline therapy. Leukemia. 2005;19(6):1092-4.
11. Kalina T, Vaskova M, Mejstrikova E, Madzo J, Trka J, Stary J, et al. Myeloid antigens in childhood lymphoblastic leukemia: clinical data point to regulation of CD66c

distinct from other myeloid antigens. BMC Cancer. 2005;5(1):38.
12. Bene MC, Bernier M, Casasnovas RO, Castoldi G, Knapp W, Lanza F, et al. The reliability and specificity of c-kit for the diagnosis of acute myeloid leukemias and undifferentiated leukemias. The European Group for the Immunological Classification of Leukemias (EGIL). Blood. 1998;92(2): 596-9.
13. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia. 1995;9(10): 1783-6.
14. Campana D, Behm FG. Immunophenotyping of leukemia. J Immunol Methods. 2000;243(1-2):59-75.
15. Borowitz MJ, Chan JKC. T lymphoblastic leukemia/lymphoma. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Geneva: WHO Press; 2008. p. 176-8.
16. Borowitz MJ, Chan JKC. B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Geneva: WHO Press; 2008. p. 171-5.
17. Borowitz MJ, Chan JKC. B lymphoblastic leukaemia/lymphoma, not otherwise specified. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Geneva: WHO Press; 2008. p. 168-70.
18. Borowitz MJ, Béné M-C, Harris NL, Porwit A, Matutes E. Acute leukemias of ambiguous lineage. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Geneva: WHO Press; 2008. p. 150-1.
19. Szczepanski T, Beishuizen A, Pongers-Willemse MJ, Hahlen K, Van Wering ER, Wijkhuijs AJ, et al. Cross-lineage T cell receptor gene rearrangements occur in more than ninety percent of childhood precursor-B acute lymphoblastic leukemias: alternative PCR targets for detection of minimal residual disease. Leukemia. 1999;13(2):196-205.
20. Wright S, Chucrallah A, Chong YY, Kantarjian H, Keating M, Albitar M. Acute lymphoblastic leukemia with myeloperoxidase activity. Am J Hematol. 1996;51(2): 147-51.
21. Rytting ME, Kantarjian H, Albitar M. Acute lymphoblastic leukemia with Burkitt-like morphologic features and high myeloperoxidase activity. Am J Clin Pathol. 2009;132(2):182-5; quiz 306.
22. Krejci O, Prouzova Z, Horvath O, Trka J, Hrusak O. Cutting edge: TCR delta gene is frequently rearranged in adult B lymphocytes. J Immunol. 2003;171(2):524-7.
23. Fronkova E, Krejci O, Kalina T, Horvath O, Trka J, Hrusak O. Lymphoid differentiation pathways can be traced by TCR delta rearrangements. J Immunol. 2005;175(4): 2495-500.
24. Zuna J, Krejci O, Madzo J, Fronkova E, Sramkova L, Hrusak O, et al. TEL/AML1 and immunoreceptor gene rearrangements-which comes first? Leuk Res. 2005;29(6): 633-9.
25. Boeckx N, Willemse MJ, Szczepanski T, van der Velden VH, Langerak AW, Vandekerckhove P, et al. Fusion gene transcripts and Ig/TCR gene rearrangements are complementary but infrequent targets for PCR-based detection of minimal residual disease in acute myeloid leukemia. Leukemia. 2002;16(3):368-75.
26. Szczepanski T, van der Velden VH, Raff T, Jacobs DC, van Wering ER, Bruggemann M, et al. Comparative analysis of T-cell receptor gene rearrangements at diagnosis and relapse of T-cell acute lymphoblastic leukemia (T-ALL) shows high stability of clonal markers for monitoring of minimal residual disease and reveals the occurrence of second T-ALL. Leukemia. 2003;17(11): 2149-56.
27. Paietta E, Ferrando AA, Neuberg D, Bennett JM, Racevskis J, Lazarus H, et al. Activating FLT3 mutations in CD117/KIT(+) T-cell acute lymphoblastic leukemias. Blood. 2004;104(2):558-60.
28. Van Vlierberghe P, Meijerink JP, Stam RW, van der Smissen W, van Wering ER, Beverloo HB, et al. Activating FLT3 mutations in CD4+/CD8- pediatric T-cell acute lymphoblastic leukemias. Blood. 2005;106 (13):4414-5.
29. Wakabayashi Y, Watanabe H, Inoue J, Takeda N, Sakata J, Mishima Y, et al. Bcl11b is required for differentiation and survival of alphabeta T lymphocytes. Nat Immunol. 2003;4(6):533-9.
30. MacLeod RA, Nagel S, Drexler HG. BCL11B rearrangements probably target T-cell neoplasia rather than acute myelocytic leukemia. Cancer Genet Cytogenet. 2004;153(1):88-9.
31. Strehl S, Nebral K, Konig M, Harbott J, Strobl H, Ratei R, et al. ETV6-NCOA2: a novel fusion gene in acute leukemia associated with coexpression of T-lymphoid and myeloid markers and frequent NOTCH1 mutations. Clin Cancer Res. 2008;14(4): 977-83.
32. Nachman J. Apples and oranges: mixed lineage acute leukemia. Blood. 2009;113(21): 5036.
33. Baruchel A, Cayuela JM, Ballerini P, Landman-Parker J, Cezard V, Firat H, et al. The majority of myeloid-antigen-positive (My+) childhood B-cell precursor acute lymphoblastic leukaemias express TEL-AML1 fusion transcripts. Br J Haematol. 1997;99(1):101-6.
34. Stams WA, Beverloo HB, den Boer ML, de Menezes RX, Stigter RL, van Drunen E, et al. Incidence of additional genetic changes in the TEL and AML1 genes in DCOG and COALL-treated t(12;21)-positive pediatric ALL, and their relation with drug sensitivity and clinical outcome. Leukemia. 2006; 20(3):410-6.
35. Loh ML, Goldwasser MA, Silverman LB, Poon WM, Vattikuti S, Cardoso A, et al. Prospective analysis of TEL/AML1-positive patients treated on Dana-Farber Cancer Institute Consortium Protocol 95-01. Blood. 2006;107(11):4508-13.
36. Xu XQ, Wang JM, Lu SQ, Chen L, Yang JM, Zhang WP, et al. Clinical and biological characteristics of adult biphenotypic acute leukemia in comparison with that of acute myeloid leukemia and acute lymphoblastic leukemia: a case series of a Chinese population. Haematologica. 2009;94(7):919-27.
37. Mejstrikova E, Fronkova E, Kalina T, Omelka M, Batinic D, Dubravcic K, et al. Detection of residual B precursor lymphoblastic leukemia by uniform gating flow cytometry. Pediatr Blood Cancer. 2010;54(1):62-70.
38. Behm FG. Immunophenotyping. In: Pui CH, editor. Childhood Leukemias. Cambridge: Cambridge University Press; 2006. p. 150-209.
39. Hasle H. Myelodysplastic and myeloproliferative disorders in children. Curr Opin Pediatr. 2007;19(1):1-8.