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

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Design and Methods

Patients

Diagnostic specimens were sent to our laboratory from all eight Czech pediatric centers that treat children with acute leukemia. All patients were retrospectively re-evaluated; cases that failed to fulfill the definition criteria for ALL or AML were excluded and all exclusions are presented in the Results. Patients entered the study after their parents or guardians had signed informed consent. The study was approved by the institutional ethics committee.

The prednisone response (<1000 blasts/μL in peripheral blood after 1 week of prednisone pre-phase treatment and one dose of intrathecal methotrexate) was evaluated for all therapeutic protocols used for ALL except for the POG 9407 protocol.[1,2] Complete remission in AML protocols was defined according to the published CALGB criteria,[3] and in ALL protocols as described previously.[1,2] The French-American-British (FAB) leukemia classification has been described previously.[4,5] All event-free survival data used the duration of life between the date of diagnosis and the date of event (i.e., death, relapse or secondary malignancy) or the end of follow-up of cases in remission.

Sample processing

Samples were processed within 24 h of having been collected from patients. Sample preparation consisted of incubation for 15 min with monoclonal antibodies at the sample-to-monoclonal antibody volume ratios recommended by the manufacturers. Red blood cells were then lysed during incubation for 15 min with ammonium chloride, followed by 5 min of centrifugation (500 x g). The supernatant was discarded and the samples were resuspended in phosphate-buffered saline, and the data was acquired immediately. A Fix & Perm kit (An Der Grub Bioresearch, Austria) was used for intracellular staining. All events were acquired and stored in listmode files. A minimum of 20,000 events per tube were acquired at the time of diagnosis.

Treatment protocol selection

Non-infant children indicated to ALL-directed treatment were recruited into the following treatment protocols: ALL-BFM 1995[2] (for those diagnosed before November 1, 2002) or ALL IC BFM 2002[6] (for those diagnosed November 1, 2002 or later). Infants fulfilling the criteria for ALL-directed therapy were treated with one of the infant ALL protocols: POG 9407[7] (for those diagnosed before December 31, 1999), Interfant 99[1] (for those diagnosed from January 1, 2000 (some centers from 1999) to December 31, 2005) or Interfant 2006 (for those diagnosed from January 1, 2006 onwards). Patients who fulfilled the criteria for AML-directed therapy were treated according to the AML BFM 93 protocol[8] (for those diagnosed before October 31, some centers before December 31, 1998), the AML BFM 98 protocol[9] (for those diagnosed between November 1, 1998 to January 8, 2004) or the AML BFM 2004 protocol (for those diagnosed from January 9, 2004 onwards).

Monoclonal antibodies

CD20 (clone L27) and CD15 were purchased from BD Biosciences (San Jose, CA, USA). CD1a (clone BL6), CD2 (clone 39C1.5), CD3 (clone UCHT1), CD5 (clone BL1a), CD7 (clone 8H8.1), CD8 (clone B9.11), CD10 (clone ALB2), CD13 (clone SJ1D1), CD14 (clone RMO52), CD19 (clone J4.119), CD20 (clone B9E9), CD22 (clone SJ10.1H11), CD24 (clone ALB9), CD33 (clone D3HL60.251), CD64 (clone 22), CD65 (clone 88H7), CD117 (clone 95C3), MPO (clone CLB-MPO-1), TCRab (clone IP26A) and TCRgd (clone IMMU510) were purchased from Immunotech (Marseille, France). CD10 (clone SS2/36) and TdT (clone HT-6) were purchased from DAKO (Glostrup, Denmark). CD79a (clone ZL7-4) was purchased from Serotec. All reported molecules were assessed in two- to four-color fluorescence, consistently adhering to the same clone.

Genotype subsets

All cases who received ALL-directed therapy were categorized as *TEL/AML1*-positive, *BCR/ABL*-positive or *MLL/AF4*-positive if one of these fusion genes was positive by reverse transcription-polymerase chain reaction (RT-PCR), as described previously.[10-12] The remaining cases were separated into hyperdiploid (DNA index 1.16 - 1.6)[13] or non-hyperdiploid without listed gene fusions. Cases without information on DNA index are listed separately. Exceptional cases that fulfilled more than one subset or that could not be categorized because of missing information are mentioned whenever they could be includ-

ed in the analyses.

All three fusion genes were also investigated in T-ALL, and T-ALL patients are listed as a separate subset. AML cases and cases with MPAL treated with AML-directed therapy were investigated for the presence of *AML1/ETO*, *CBFB/MYH11* and *PML/RARA* fusion genes using RT-PCR.[14]

Other cytogenetic and molecular genetic investigations

Besides *MLL/AF4*, the presence of other *MLL* gene rearrangements (*MLL/AF6*, *MLL/AF9*, *MLL/AF10*, *MLL/ENL* and *MLL/ELL*) and the *BCR/ABL* breakpoint specification (minor or major *BCR/ABL* variant) were assessed using RT-PCR, as described previously.[12] Internal tandem duplication (ITD) and D835 activating mutations of the *FLT3* gene were investigated as described previously.[15,16] Cytogenetic analysis of G-banded chromosomes and fluorescence *in situ* hybridization (FISH) were carried out using already described conventional techniques.[17,18] FISH assays with appropriate centromeric and/or locus-specific DNA Vysis probes (Abbott Molecular, Abbott Park, IL, USA) were performed according to the manufacturer's instructions. Complex chromosomal aberrations were analyzed by mFISH/mBAND, using 24*X*Cyte and/or *X*Cyte color kit (MetaSystems GmbH, Altlussheim, Germany), respectively. Karyotypes were described according to the ISCN.[19]

Detection of immunoglobulin and T-cell receptor gene rearrangements

Mononuclear cells from diagnostic bone marrow samples were isolated using Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation. Genomic DNA was isolated using a QIAamp® DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany). Primers and protocols for the detection of immunoglobulin heavy chain (*IGH*) rearrangements, immunoglobulin light chain kappa deletions (*KDE*), T-cell receptor gamma (*TCRG*), T-cell receptor delta (*TCRD*) and T-cell receptor beta (*TCRB*) gene rearrangements have already been described.[20,21] Clonality of PCR products was confirmed using heteroduplex analysis.[20] Sequencing was performed in the ABI PRISM® 310 Genetic Analyzer with a BigDye™ Primer v3.0 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The variable (V), diversity (D) and joining (J) regions of the immunoreceptor gene were identified by comparing the sequences to GenBank sequences using the ImMunoGeneTics (IMGT) database (*http://imgt.cines.fr, IMGT, European Bioinformatics Institute, Montepellier, France*) and the IGBlast search (*http://www.ncbi.nlm.nih.gov/igblast/*, National Center for Biotechnology Information, Bethesda, MD, USA).

Statistics

The significance of all frequency comparisons was calculated using Fisher's exact test of a 2x2 contingency table. Survival analyses were computed in Statistica software (Statsoft, Tulsa, USA) and the Kaplan-Meier survival probabilities are shown. The survival comparisons were analyzed using the log-rank test and multivariate survival analyses were computed using a Cox regression model. Principal component analysis is used to lower data complexity while illustrating similarities and differences among cases. The information on all molecules is transformed into each dimension in a weighted manner so that the most significant differences and clusters are shown in a three-dimensional image.[22] Principal component analysis of the immunophenotypic data (composed of percentages of leukemic cells expressing CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD15, CD19, CD20, CD22, CD33, CD34, CD38, $CD45^{bright}$, $CD45^{dim}$, CD64, CD65, CD66c, CD117, HLA DR, NG2 and intracellular CD3, CD79a, IgM, TdT, and MPO) were performed using MultiExperiment Viewer (MEV) 4.3 software (Microarray software suite TM4, *http://www.tm4.org*).[23] Briefly, the data are transformed to vectors in multidimensional space so that the best separation of individual patients is achieved. Vectors comprise weighted information on all antigens. Two- and three-dimensional views represent a simplified view in which data leading to the most powerful separation are visible. Patients for whom there was significant contamination of the leukemic blast gate (greater than 30% non-malignant cells in the best gate by optical scatter) were excluded from the principal component analysis.

Results

Patients' characteristics

No patient had a preceding diagnosis of malignant disease or defined congenital bone marrow failure disease. Two patients were originally classified and treated as having AML (AML BFM 98) with a negative MPAL score, but they were excluded from this study, as their immunophenotype did not fulfill the definition of AML. The closest known subtype for both cases is NK/myeloid precursor acute leukemia.[24] Two other patients were originally classified as having ALL (non-MPAL according EGIL), but experienced a lineage switch[25-30] to myeloid leukemia during the first week of ALL treatment (prednisone prephase, both classified as prednisone poor responders) and both patients were excluded from further analyses. Thus, 107 patients with AML or with MPAL treated by AML-directed therapy and 582 patients with ALL or with MPAL treated by ALL-directed therapy were used for analyses. ALL-directed therapy consisted of the following protocols: ALL-BFM 1995[2] (n=334 patients), ALL IC BFM 2002[6] (n=224), Interfant 99[1] (n=13), Interfant 2006 (n=3) and POG 9407 (n=5). One infant died on the day of diagnosis prior to assignment to a protocol. AML treatment protocols included: AML BFM 93[8] (n=16), AML BFM 98[9] (n=69) and AML BFM 2004 (n=21). In addition, three children with Down's syndrome (two ALL, one AML) were not treated due to parents' refusal; these patients were included in the epidemiological analyses, but were excluded from the survival analyses. No patient's lineage category was changed following molecular genetics analysis.

Online Supplementary Table S1A. Key clinical and diagnostic information of the patients.

UPN	Lineages involved and treatment direction	Positive antigens relevant for treatment decision	Antigens leading to a positive MPAL score (EGIL)	Molecular genetics	Cytogenetics	Morphology – FAB classification	Age at diagnosis	Leukocyte count at diagnosis
B1	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+#79a+|10+24+#TdT+#MPO+β3+	TEL/AML1	47,XX,der(2)ins(5;2)(q?33;q?34)t(2;13;5;6)(q?34;q?12;q31;q?24),+21[15]/46,XX[5]	L2	6.2y	3.1
B2	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+|10+24+#TdT+|3+|15+β3+	TEL/AML1	tetraploidy[10]/46,XX [1]	L2	4.9y	10
B3	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+|10+24+#TdT+|3+|15+β3+	TEL/AML1	not available	L1	7.9y	39
B4	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+|10+24+#TdT+|3+β3+|117+	TEL/AML1	46,XY,del(12)(p?)[9]		7.1y	2.5
B5	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+|10+24+#TdT+|3+β3+|117+	TEL/AML1	46,XX,der(9)t(X;9)(?;p13),t(12;21)(p13;q22)[4]/45,X,-X,idem[16]	L1	5.5y	9.1
B6	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+|10+24+#TdT+|3+|15+β3+	TEL/AML1	46,XY[13]	L1	14y	7.3
B7	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+|10+24+#TdT+|3+β3+|117+	TEL/AML1	not available	L1	3.4y	4.6
B8	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+#β3+|10+24+#TdT+|13+|15+β3+	TEL/AML1	46,XX[20]	L1	6.1y	2.6
B9	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+|10+24+#TdT+|3+|15+β3+	TEL/AML1	46,XY[20]	L1	4.5y	4.5
B10	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+|10+24+#TdT+|5+|7+#MPO+|13+	BCR/ABL	45,XY,-7,t(9;22)(q34;q11)[9]/46,XY[1]	L2	9.1y	42
B11	My/B, treated as BCP ALL	CD19+*i*79a+	CD19+79a+*i*79a+|10+24+#TdT+#MPO+|13+β3+	BCR/ABL	46,XY,t(9;22)(q34;q11)[9]/46,XY[1]	L2	3.7y	101
B12	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+79a+#*IgM*+|10+#TdT+|13+β3+65+	hyperdiploid	57,XY,+X,+Y,+4,+der(6)del(6)(q22q24),+der(7)ins(7;11)(p12;q13?),+9,+10,+14,der(16)t(16;17)(p13.1;p12?),+18,+21,+21[12]	L1	17y	0.7
B13	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+#*IgM*+|10+24+#TdT+#MPO+|15+	(neg1)	not available	L1	2.9y	4
B14	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+#TdT+|13+β3+|17+|15+	(neg1)	46,XX[10]	L2	3.7y	14
B15	My/B, treated as BCP ALL	CD19+*i*79a+	CD19+*i*79a+*i*TdT+|13+65+|15+	(neg1)	not available	L1	4.3y	6
B16	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+|10+24+#TdT+|3+|15+33+	(neg1)	46,XX[10]	L1	2.4y	8.9
B17	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+24+#TdT+|13+β3+|117+|15+	(neg1)	46,XY,1qh+[20]	L1	1.8y	23
B18	My/B, treated as BCP ALL	CD19+22+	CD19+22+24+#TdT+#MPO+65+|15+	(neg1)	46,XX[17]	L1	3.2y	21
B19	My/B, treated as BCP ALL	CD19+*i*79a+	CD19+*i*79a+24+|7+β3+65+|15+	(neg1)	46,XX,der(3)t(3;11)(q13;q23),der(3)t(3;?)x2,del(11)(q23),der(19)t(3;19)(q13;?)[28]/46,XX[1]	L2	1d	209
B20	My/B, treated as BCP ALL	CD19+*i*79a+	CD19+*i*79a+24+#TdT+β3+65+|4+|15+64+	MLL/AF4	46,XX,t(4;11)(q21;q23)[1]/46,XX[8]	L2	1m	292
B21	My/B, treated as BCP ALL	CD19+*i*79a+	CD19+*i*79a+24+#TdT+β3+65+|5+	MLL/AF4	46,XY,ins(4;11)(q21;q23),del(11)(q23)[24]/46,XY[1]	L1	6m	230
B22	My/B, treated as BCP ALL	CD19+22+*i*79a+	CD19+22+*i*79a+24+#TdT+#MPO+|13+β3+65+|15+64+	MLL/AF4	46,XX,t(4;11)(q21;q23)[20]	undifferentiated or L2	24d	195
T1	My/T, treated as T ALL	CD7+*i*3+ *i*MPO low	CD7+*i*3+*i*TdT+5+#MPO+|13+β3+|117+	BCR/ABL	46,XY,t(9;22)(q34;q11)[18]	Myeloid or L2	17y	47
T2	My/T, treated as T ALL	CD7+*i*3+ *i*MPO-	CD7+*i*3+10+#TdT+5+β3+|117+|15+	(neg1)	46,XY,del(7q)[5]/46,XY[20]; deletion of both TEL alleles by FISH	L2	10y	0.6
T3	My/T/B, treated as T ALL	CD7+*i*3+ *i*MPO low, 19+*i*79a+	CD19+#79a+β3+#TdT+|TCRγδ+2+|5+8+|7+|13+β3+|117+|15+|	(neg1)	46,XY[15]	L2	17y	2.9
T4	My/T, treated as T ALL	CD7+*i*3+ *i*MPO-	CD7+*i*3+33+117+15+	(neg1)	46,XX [14]		4.9y	22
T5	My/T, treated as T ALL	CD7+*i*3+ *i*MPO-	CD7+*i*3+*i*TdT+2+|13+|117+|15+	(neg1), *FLT3*ITD	46,XY,t(6;14)(q?26;q?31)[25]	L1	15y	2.6
T6	My/T, treated as T ALL	CD7+*i*3+ *i*MPO-	CD7+*i*3+5+33+65+|117+	(neg1)	46,XY[12]	L2	16y	1.4
My1	My/T, treated as AML	CD13+33+117+*i*MPO >30%	*i*MPO+CD13+15+β3+|117+#79a+#β3+#TdT+2+|7+	(neg2)	46,XY[18]/47,XY,dup(2)(p?),+mar[4]	M2 or L2	9.7y	2.7
My2	My/T, treated as AML	CD13+33+117+*i*MPO >30%	*i*MPO+CD13+14+|15+β3+65+#β3+|10+2+5+|7+|	(neg2)	46,XY [20]	M4 or L2	2.3y	29
My3	My/T, treated as AML	CD13+33+65+*i*MPO> 30%	*i*MPO+13+33+|117+#β3+22+|7+	(neg2)	46,XY,del(5)(q14q33)[11]/46,XY[3]	M2, Auer rods	15y	17
My4	My/T, treated as AML	CD13+33+65+117+*i*MPO>30%	*i*MPO+CD13+15+β3+65+|117+|9+β3+|7+	(neg2), *FLT3*ITD	47,XX,+8[11]/46,XX,+8,-15,-21,+mar[2]/46,XX [1]	M1, Auer rods	13y	143

Complete remission in ALL and AML patients was evaluated at days 33 and 28 of the treatment, respectively; N/A (prot.): does not apply due to treatment protocol; N/A (e.d.): not evaluable due to early death; *Complete remission achieved only after imatinib in UPN B10. Molecular genetic codes: (neg1) non-hyperdiploid, *BCR-ABL*^{neg}, *MLL/AF4*^{neg} and *TEL/AML1*^{neg}; (neg2) *AML1/ETO*^{neg}, *CBFB/MYH11*^{neg}, *PML/RARA*^{neg}. In addition, *MLL/AF6*, *MLL/AF9*, *MLL/AF10*, *MLL/ENL* and *MLL/ELL* rearrangements were negative in all 32 patients, as determined by multiplex PCR; all cases were tested for *FLT3*-D835-activating mutations (all patients negative) and for *FLT3* internal tandem duplication (*FLT3-ITD*).

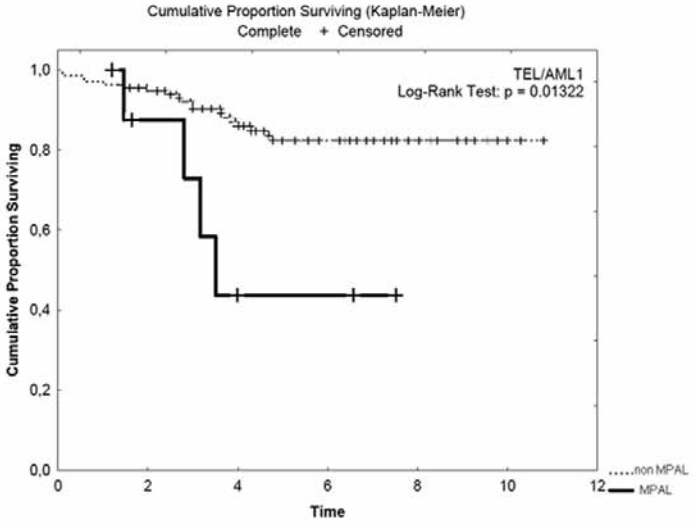
Online Supplementary Table S1B. Key clinical and diagnostic information of the patients.

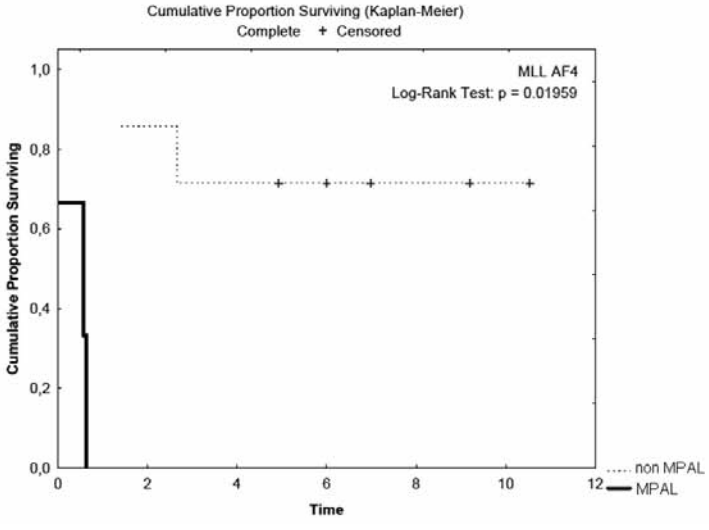
UPN	Frontline treatment	Risk group	Complete remission after induction treatment	Prednisone response	Day 15 BM	Outcome
B1	ALL BFM 95	intermediate	yes	good	M1	alive
B2	ALL IC BFM 2002	standard	yes	good	M1	alive
B3	ALL BFM 95	intermediate	yes	good	M1	alive
B4	ALL IC BFM 2002	intermediate	yes	good	M1	alive
B5	ALL IC BFM 2002	standard	yes	good	M2	relapsed, alive
B6	ALL IC BFM 2002	intermediate	yes	good	M2	alive
B7	ALL IC BFM 2002	standard	yes	good	M2	relapsed, alive after SCT
B8	ALL BFM 95	standard	yes	good	not available	relapsed, alive after SCT
B9	ALL BFM 95	standard	yes	good	not available	relapsed, alive after SCT
B10	ALL BFM 95, imatinib, MUD SCT	high	no*	good	M3	relapsed, died
B11	ALL BFM 95	high	yes	good	M3	relapsed, alive after SCT
B12	ALL BFM 95	intermediate	N/A (e.d.)	good	M2	early death (pancreatitis)
B13	ALL BFM 95	standard	yes	good	M1	alive
B14	ALL BFM 95	standard	yes	good	M1	alive
B15	ALL BFM 95	standard	yes	good	M1	alive
B16	ALL BFM 95	high	yes	poor	M2	alive
B17	ALL IC BFM 2002	intermediate	yes	good	M2	alive
B18	ALL BFM 95	intermediate	yes	good	M2	alive
B19	death before treatment started	N/A (e.d.)	N/A (e.d.)	N/A (e.d.)	N/A (e.d.)	early death
B20	Interfant 2003	high	yes	good	M1	relapsed, alive after SCT
B21	POG 9407	N/A (prot.)	yes	N/A (prot.)	not available	death in CR
B22	Interfant 2003	N/A (e.d.)	N/A (e.d.)	N/A (e.d.)	N/A (e.d.)	early death, disease progression
T1	ALL IC BFM 2002, imatinib (EsPhALL), MUD SCT	high	yes	poor	M3	alive
T2	ALL BFM 95	intermediate	yes	good	M2	relapsed, died
T3	ALL IC BFM 2002	intermediate	yes	good	M1	alive
T4	ALL IC BFM 2002	intermediate	yes	good	M2	alive
T5	ALL IC BFM 2002	intermediate	yes	good	M2	alive
T6	ALL BFM 95	intermediate	yes	good	M2	alive
My1	AML BFM 98, autologous SCT	high	no	N/A (prot.)	M2	relapsed, died
My2	AML BFM 2004 , MSD SCT	high	yes	N/A (prot.)	M1	alive
My3	AML BFM 98, MSD SCT	high	yes	N/A (prot.)	M1	relapsed, died
My4	AML BFM 98	high	no	N/A (prot.)	M2	relapsed, died

Complete remission in ALL and AML patients was evaluated at days 33 and 28 of the treatment, respectively; N/A (prot.): does not apply due to treatment protocol; N/A (e.d.): not evaluable due to early death; *Complete remission achieved only after imatinib in UPN B10. Molecular genetic codes: (neg1) non-hyperdiploid, *BCR-ABL*^{neg}, *MLL/AF4*^{neg} and *TEL/AML1*^{neg}; (neg2) *AML1/ETO*^{neg}, *CBFB/MYH11*^{neg}, *PML/RARA*^{neg}. In addition, *MLL/AF6*, *MLL/AF9*, *MLL/AF10*, *MLL/ENL* and *MLL/ELL* rearrangements were negative in all 32 patients, as determined by multiplex PCR; all cases were tested for *FLT3*-D835-activating mutations (all patients negative) and for *FLT3* internal tandem duplication (*FLT3-ITD*).

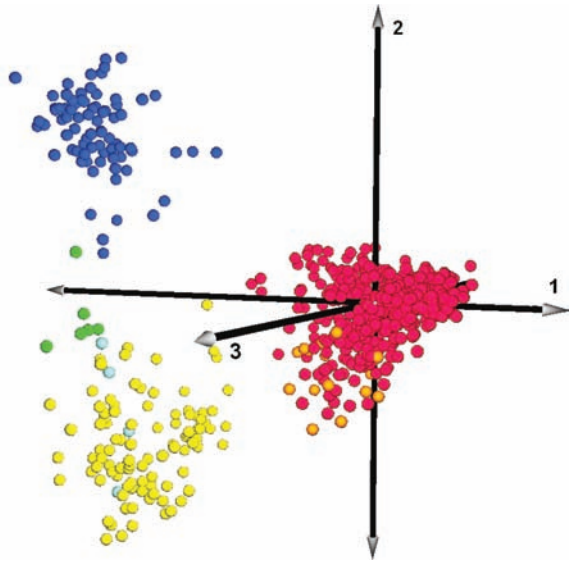
Online Supplementary Table S2. Immunoglobulin and T-cell receptor gene rearrangements in MPAL cases. Negative results are labeled '-'. Cases UPN B8, B9 and T6 were not analyzed due to insufficient material and are excluded from this Table. Abbreviations (neg1) and (neg2) are explained in Supplementary Table S1. Case UPN T2 was analyzed at relapse only, as there was insufficient material at the time of diagnosis.

UPN	Genotype	IGH VDJ	IGH DJ	IGK KDE	TCRγ VJ	TCRδ VDJ	TCRδ VD	TCRβ VDJ	TCRβ DJ	Total
B1	TEL/AML1	2	-	1	1	-	1	1	1	7
B2	TEL/AML1	1	-	2	1	-	-	1	1	6
B3	TEL/AML1	1	-	2	2	-		1	-	6
B4	TEL/AML1		1	1	1	-	-	-	-	3
B5	TEL/AML1	1	-	1	1	-	-	-	1	4
B6	TEL/AML1	2	-	-	3	-	1	1	-	7
B7	TEL/AML1	1	-	-	1	-	2	2	-	6
B10	BCR/ABL	2	-	-	-	-	-	-	1	3
B11	BCR/ABL	-	1	-	-	-	1	-	-	2
B12	hyperdiploid	1	-	-	1	-	-	-	-	2
B13	(neg1)	2	-	2	-	-	1	-	-	5
B14	(neg1)	2	-	-	-	-	1	-	1	4
B15	(neg1)	2	-	-	1	-	1	-	-	4
B16	(neg1)	2	-	-	-	-	-	-	-	2
B17	(neg1)	1	-	-	-	-	1	-	-	2
B18	(neg1)	1	-	-	-	-	1	-	-	2
B19	(neg1)	-	-	-	-	-	1	-	-	1
B20	MLL/AF4	-	2	-	1	-	2	-	-	5
B21	MLL/AF4	-	1	-	2	-	-	-	-	3
B22	MLL/AF4	1	-	-	-	-	-	-	-	1
T1	BCR/ABL	-	-	-	-	-	-	-	-	0
T2	(neg1)	-	1	-	3	1	1	-	-	6
T3	(neg1)	-	-	-	2	1	1	-	1	5
T4	(neg1)	-	-	-	-	-	-	-	-	0
T5	(neg1), FLT3ITD	-	-	-	-	-	-	-	-	0
My1	(neg2)	-	-	-	-	-	-	-	-	0
My2	(neg2)	-	-	-	-	-	-	-	-	0
My3	(neg2)	-	-	-	-	-	-	-	-	0
My4	(neg2), FLT3ITD	-	-	-	-	-	-	-	-	0

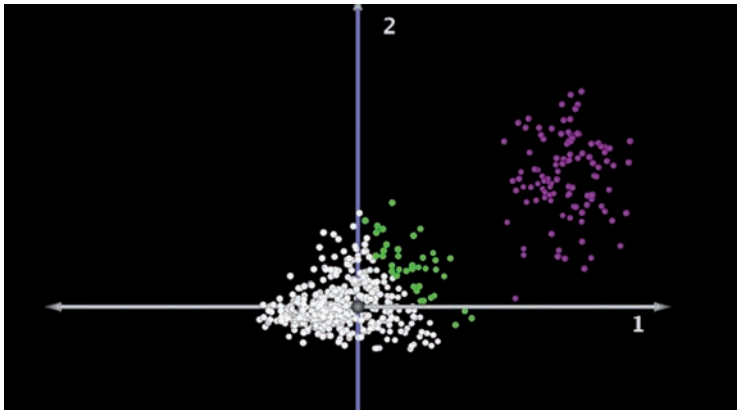




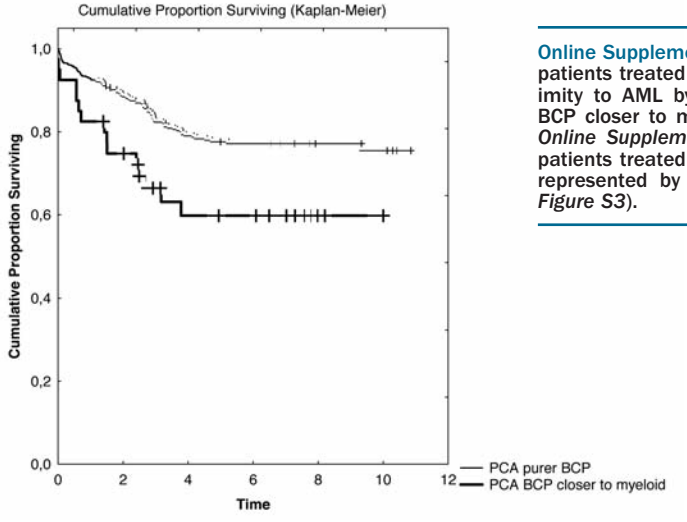
Online Supplementary Figure S1. Event-free survival of (A) *TEL/AML1*-positive or (B) *MLL/AF4*-positive patients. All patients received ALL-directed treatment. Bold line: AHL, thin line, non AHL. Time from diagnosis in years is on X-axes. Further details are described in the Patients, Statistics and Results sections.



Online Supplementary Figure S2. Principal component analysis of immunophenotype data. Each dot represents a patient and colors were selected based on the diagnostic immunophenotype classification. Red: non-mixed BCP-ALL, orange: MPAL treated as BCP-ALL, yellow: non-mixed AML, turquoise: MPAL (T/My) treated as AML, green: MPAL treated as T-ALL, blue: non-mixed T-AL.



Online Supplementary Figure S3. Immunophenotypic principal component analysis of cases that fulfilled treatment criteria for AML (violet) and BCP ALL treatment (white or green). Specimens from patients treated as having BCP ALL are shown in green if this principal component analysis showed a closer proximity to AML or in white (all others). The prognosis of these patients was analyzed and is shown in *Online Supplementary Figure S4*.



Online Supplementary Figure S4. Event-free survival of patients treated as having BCP ALL with a closer proximity to AML by principal component analysis ("PCA BCP closer to myeloid", represented by green dots in *Online Supplementary Figure S2*) compared to other patients treated as having BCP ALL ("PCA purer BCP", represented by white dots in *Online Supplementary Figure S3*).

Online Supplementary Movie 1. Rotation of *Online Supplementary Figure S2* in three-dimensional space.

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