RESEARCH ARTICLE

Clinical Significance of Novel Subtypes of Acute Lymphoblastic Leukemia in the Context of Minimal Residual Disease–Directed Therapy

Sima Jeha[1,2], John Choi[3], Kathryn G. Roberts[3], Deqing Pei[4], Elaine Coustan-Smith[5], Hiroto Inaba[1], Jeffrey E. Rubnitz[1], Raul C. Ribeiro[1], Tanja A. Gruber[1], Susana C. Raimondi[3], Seth E. Karol[1], Chunxu Qu[3], Samuel W. Brady[6], Zhaohui Gu[3], Jun J. Yang[7], Cheng Cheng[4], James R. Downing[3], Williams E. Evans[7], Mary V. Relling[7], Dario Campana[5], Charles G. Mullighan[3], and Ching-Hon Pui[1,2,3]

ABSTRACT

We evaluated clinical significance of recently identified subtypes of acute lymphoblastic leukemia (ALL) in 598 children treated with minimal residual disease (MRD)–directed therapy. Among the 16 B-cell ALL (B-ALL) and 8 T-cell ALL subtypes identified by next-generation sequencing, *ETV6–RUNX1*, high-hyperdiploid, and *DUX4*-rearranged B-ALL had the best 5-year event-free survival rates (95.0%–98.4%); *TCF3–PBX1*, PAX5-altered (PAX5alt), T-cell, early T-cell precursor (ETP), intrachromosomal amplification of chromosome 21 (iAMP21), and hypodiploid ALL intermediate rates (80.0%–88.2%); and *BCR–ABL1*, *BCR–ABL1*-like, *ETV6–RUNX1*-like, and *KMT2A*-rearranged ALL the worst rates (64.1%–76.2%). All but 3 of the 142 patients with day 8 blood MRD <0.01% remained in remission. Among new subtypes, intensified therapy based on day 15 MRD ≥1% improved outcome of *DUX4*-rearranged, *BCR–ABL1*-like, and *ZNF384*-rearranged ALL, and achievement of day 42 MRD <0.01% did not preclude relapse of PAX5alt, *MEF2D*-rearranged, and *ETV6–RUNX1*-like ALL. Thus, new subtypes including *DUX4*-rearranged, PAX5alt, *BCR–ABL1*-like, *ETV6–RUNX1*-like, *MEF2D*-rearranged, and *ZNF384*-rearranged ALL have important prognostic and therapeutic implications.

SIGNIFICANCE: Genomic analyses and MRD should be used together for risk-directed treatment of childhood ALL. Six recently described subtypes—*DUX4*-rearranged, PAX5alt, *BCR–ABL1*-like, *ETV6–RUNX1*-like, *MEF2D*-rearranged, and *ZNF384*-rearranged ALL—had prognostic and therapeutic significance with contemporary risk-directed treatment.

See related commentary by Segers and Cools, p. 294.

See related video from the AACR Annual Meeting 2021: https://vimeo.com/558556916

[1]Department of Oncology, St. Jude Children's Research Hospital and the University of Tennessee Health Science Center, Memphis, Tennessee. [2]Department of Global Pediatric Medicine, St. Jude Children's Research Hospital and the University of Tennessee Health Science Center, Memphis, Tennessee. [3]Department of Pathology, St. Jude Children's Research Hospital and the University of Tennessee Health Science Center, Memphis, Tennessee. [4]Department of Biostatistics, St. Jude Children's Research Hospital and the University of Tennessee Health Science Center, Memphis, Tennessee. [5]Department of Pediatrics, School of Medicine, National University of Singapore, Singapore. [6]Department of Computational Biology, St. Jude Children's Research Hospital and the University of Tennessee Health Science Center, Memphis, Tennessee. [7]Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital and the University of Tennessee Health Science Center, Memphis, Tennessee.

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S. Jeha, J. Choi, and K.G. Roberts contributed equally to this study as first authors.

Corresponding Authors: Ching-Hon Pui, Department of Oncology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105. Phone: 901-595-3335; Fax: 901-521-9005; E-mail: ching-hon.pui@stjude.org; and Charles G. Mullighan, Department of Pathology, St. Jude Children's Research Hospital, Memphis, TN 38105-3678. Phone: 901-595-3387; Fax: 901-595-5947; E-mail: Charles.mullighan@stjude.org

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INTRODUCTION

Childhood acute lymphoblastic leukemia (ALL) is one of the most curable cancers, with 5-year event-free survival rates exceeding 80% in many developed countries (1). Precise assessment of the early treatment response based on measurement of minimal residual disease (MRD) for risk-directed therapy has contributed significantly to this success (2). In randomized trials, MRD-directed treatment improved event-free survival by augmenting postremission therapy in patients with persistent MRD at the end of remission induction and by reducing treatment intensity in low-risk patients with rapid early clearance of MRD (3, 4). Accurate identification of patients with highly curable leukemia provides unique opportunities for further reduction in treatment intensity, thus decreasing the likelihood of short-term morbidity and mortality as well as long-term sequelae (4, 5). The relative risk of relapse among patients with early MRD clearance appears to differ among leukemia subtypes (6, 7). In the AIEOP-BFM 2000 study, for example, standard-risk patients who were MRD negative on days 33 and 78 of induction were randomized to receive reduced-intensity treatment in the delayed intensification phase, but this modification was successful only for patients with *ETV6–RUNX1* and those who were 1 to 6 years old (8).

Recent integrated genomic analyses, especially transcriptome sequencing, have identified several new subtypes of ALL, including *BCR–ABL1*-like, *DUX4*-rearranged, *ETV6–RUNX1*-like, *MEF2D*-rearranged, *PAX5*-altered (PAX5alt), and *ZNF384*-rearranged ALL (9–13). The clinical significance of some of these novel subtypes, however, is uncertain as they were identified retrospectively among selected patient cohorts that had received a variety of treatment regimens, the intensity of which was not consistently based on MRD levels (9–13). In this study, we evaluated the prognostic and therapeutic implications of all leukemia subtypes identifiable by genetic and transcriptomic analyses including nine B-cell (B-ALL) and eight T-cell ALL (T-ALL) subtypes not identifiable by conventional cytogenetic analysis among consecutive patients who had comprehensive genomic analyses and were treated on a contemporary risk-directed protocol based on well-recognized genetic abnormalities and MRD assessment at three time points during remission induction (14).

RESULTS

Risk Assignment and Genomic Classification

Of the 598 evaluable patients enrolled in St. Jude Total Therapy Study 16, 260 were classified to have low-risk, 280

Table 1. Treatment groups and clinical outcome according to leukemia subtypes

Subtype	N	Low risk (N = 260) N (%)	Standard risk (N = 280) N (%)	High risk (N = 58) N (%)	Transplant N	5-year EFS, % (95% CI)	5-year OS, % (95% CI)	5-year CRR, % (95% CI)
ETV6–RUNX1	128	111 (86.7)	17 (13.3)	0 (0.00)	0	98.4 (95.9–100)	99.2 (97.4–100)	0.8 (0.0–2.3)
Hyperdiploid	154	103 (66.9)	51 (33.1)	0 (0.00)	0	95.3 (91.2–99.4)	99.4 (97.8–100)	3.3 (0.1–6.5)
DUX4-rearranged	20	8 (40.0)	12 (60.0)	0 (0.00)	0	95.0 (84.2–100)	95.0 (84.2–100)	0
TCF3–PBX1[a]	17	1 (5.9)	14 (82.4)	2 (11.8)	2	88.2 (71.7–100)[a]	88.2 (71.7–100)[a]	0[a]
PAX5alt[b]	24	4 (16.7)	20 (83.3)	0 (0.00)	0	82.7 (65.3–100)	100 (100–100)	17.3 (1.5–33.1)
T-cell	94	0 (0.00)	79 (84.0)	15 (16.0)	11	81.3 (72.5–90.1)	88.2 (80.8–95.6)	12.0 (5.3–18.7)
ETP	10	0 (0.00)	0 (0.00)	10 (100)	6	80.0 (53.5–100)	77.1 (49.9–100)	20.0 (0.0–46.1)
iAMP21[c]	5	1 (20.0)[c]	4 (80.0)	0 (0.00)	0	80.0 (39.4–100)[c]	100 (100–100)[c]	20.0 (0.0–59.2)
Hypodiploid[d]	6	1 (16.7)	4 (66.7)	1 (16.7)	1	100 (100–100)[d]	100 (100–100)[d]	0[d]
BCR–ABL1	13	0 (0.00)	0 (0.00)	13 (100)	0	76.2 (51.9–100)	83.1 (60.8–100)	16.2 (0.0–37.7)
BCR–ABL1-like[e]	15	3 (20.0)	9 (60.0)	3 (20.0)	2	73.3 (47.0–99.6)	86.7 (66.1–100)	6.7 (0.0–19.9)
ETV6–RUNX1-like[f]	9[f]	2 (22.2)	7 (77.8)	0 (0.00)	0	66.7 (35.9–97.5)[f]	87.5 (66.1–100)[f]	22.2 (0.0–51.3)[f]
KMT2A-r	28	0 (0.00)	18 (64.3)	10 (35.7)	1	64.1 (43.9–84.3)	75.0 (56.0–94.0)	25.2 (8.7–41.7)
MEF2D-r[g]	3	1 (33.3)	2 (66.7)	0 (0.00)	0	66.7 (23.2–100)[g]	66.7 (23.2–100)[g]	33.3 (0.0–98.7)[g]
ZNF384-r[h]	7	0 (0.00)	7 (100)	0 (0.00)	0	100 (100–100)	100 (100–100)	0
NUTM1-r[h]	3	0 (0.00)	3 (100)	0 (0.00)	0	100 (100–100)	100 (100–100)	0
PAX5 P80R[h]	2	2 (100)	0 (0.00)	0 (0.00)	0	100 (100–100)	100 (100–100)	0
B other	60	23 (38.3)	33 (55.0)	4 (6.67)	2	86.3 (76.9–95.7)	93.3 (86.4–100)	10.3 (2.4–18.2)
Total	598	260 (43.5)	280 (46.8)	58 (9.70)	25	88.8 (85.9–91.7)	94.0 (91.8–96.2)	7.4 (5.3–9.6)

Abbreviations: CI, confidence interval; CRR, cumulative risk of any relapse; EFS, event-free survival; ETP, early T-cell precursor ALL; iAMP21, intrachromosomal amplification of chromosome 21; OS, overall survival.

[a]One standard-risk patient with day 42 MRD <0.01% relapsed at 5.7 years and was alive in second remission for 2.1 years, and two high-risk patients died of transplant-related toxicities at 0.6 and 2.4 years, respectively.

[b]Four patients with day 42 MRD <0.01% relapsed.

[c]One low-risk patient with day 42 MRD <0.01% relapsed at 3.4 years and remained in second remission for 5.6 years.

[d]One patient with day 42 MRD <0.01% developed secondary acute myeloid leukemia at 5.8 years, resulting in 7-year EFS of 75.0% (23.1–100).

[e]Two patients had treatment-related death, and one died of multiple secondary malignancies.

[f]Two standard-risk patients relapsed, and one low-risk patient developed secondary myelodysplastic syndrome.

[g]Two patients were alive in remission at 3.6 and 4.0 years, respectively, and one 12-year-old standard-risk patient with day 42 MRD <0.01% died of relapse at 2.9 years; data shown are 3-year results.

[h]Remission durations for the seven patients with *ZNF384*-rearranged ALL were 6.8, 7.8, 9.4, 9.7, 10.3, 11.1, and 11.5 years; for the three with *NUTM1*-rearranged ALL, 4.4, 4.7, and 7.0 years; and for the two with PAX5 P80R, 7.1 and 9.1 years, respectively.

standard-risk, and 58 high-risk ALL based on presenting clinical and biological features and MRD levels on days 15 and 42 of remission induction (Supplementary Fig. S1; Table 1). For B-ALL, genomic analyses identified 16 leukemia subtypes defined by recurring genetic alterations and distinct gene-expression profiles, 9 of which could not be reliably identified with conventional methods and required transcriptomic sequencing analysis for accurate identification: *BCL2/MYC*, *BCR–ABL1*-like, *DUX4*-rearranged, *ETV6–RUNX1*-like, *MEF2D*-rearranged, *NUTM1*-rearranged, PAX5alt, PAX5 P80R, and *ZNF384*-rearranged (Table 1; Supplementary Figs. S2–S4). The demographic characteristics, sequential MRD levels, treatment risk group, and clinical outcomes for patients with each leukemia subtype are provided in Supplementary Table S1. Most patients with *ETV6–RUNX1* or high-hyperdiploid ALL having low levels of MRD measured at three time points during remission induction (Fig. 1) were treated in the low-risk group, all patients with *BCR–ABL1* or early T-cell precursor (ETP) ALL in the high-risk group, and most patients with other subtypes in the standard-risk group (Table 1). "B other" comprised B-ALL cases that could not be classified by cytogenetic, genetic, or transcriptomic analyses.

Treatment Outcome by Leukemia Subtypes

The entire cohort of 598 patients had a 5-year event-free survival of 88.8% [95% confidence interval (CI), 85.9–91.7], overall survival of 94.0% (91.8–96.2), and cumulative risk of any relapse of 7.4% (5.3–9.6). Based on their highest event-free survival rates (Table 1; Fig. 2), *ETV6–RUNX1*, high-hyperdiploid, and *DUX4*-rearranged B-ALL were categorized as favorable

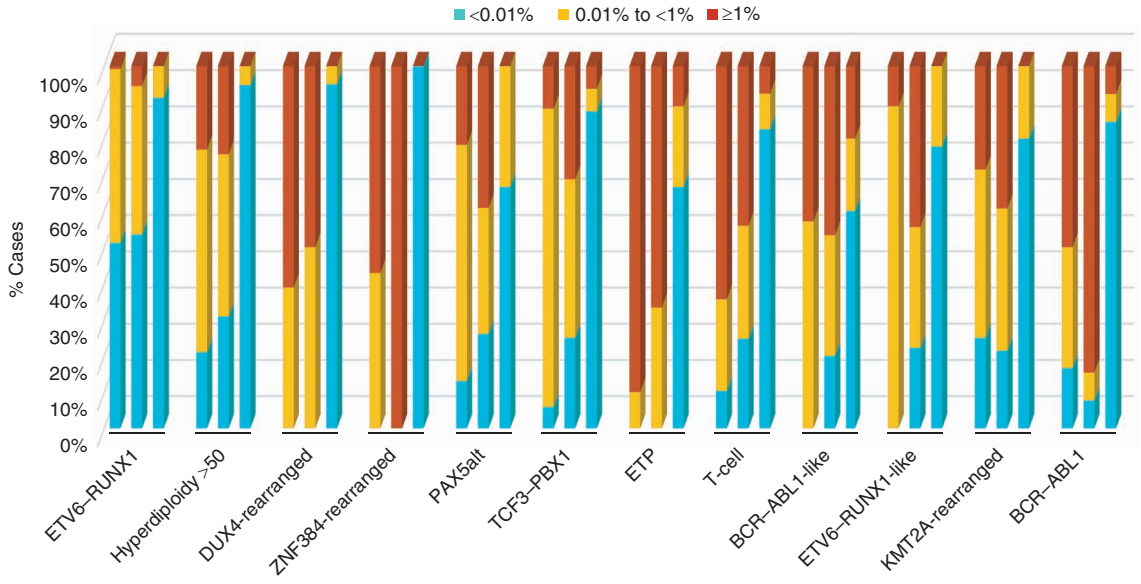


Figure 1. Sequential levels of MRD in blood on day 8 (left column), in bone marrow on day 15 (middle column), and in bone marrow on day 42 (right column) of remission induction for individual leukemia subtypes. Results are not shown for some subtypes because of small number and not for "B other" because it represents heterogeneous disease.

subtypes (Supplementary Fig. S5); these three subtypes also have the highest overall survival rates (Supplementary Fig. S6) and the lowest relapse rates (Table 1). Notably, only 13.3% of patients with *ETV6–RUNX1* abnormality and 33.1% of those with high hyperdiploidy but 60% of patients with *DUX4* rearrangement received standard-risk treatment, suggesting that MRD assessment improved the outcome of these patients by avoiding overtreatment or undertreatment.

BCR–ABL1, BCR–ABL1-like, *ETV6–RUNX1*-like, *KMT2A*-rearranged, and *MEF2D*-rearranged ALL had high levels of MRD (Fig. 1) and were categorized to be unfavorable subtypes because of their worst event-free survival rates (Table 1; Fig. 2). The remaining subtypes including *TCF3–PBX1*, PAX5alt, T-cell, ETP, intrachromosomal amplification of chromosome 21 (iAMP21), hypodiploid, *ZNF384*-rearranged, *NUTM1*-rearranged, and PAX5 P80R ALL were considered to have intermediate risk (Supplementary Fig. S5). The *BCL2/MYC* group was composed of only one case and therefore not included in downstream analyses.

Impact of Peripheral Blood MRD Levels on Day 8

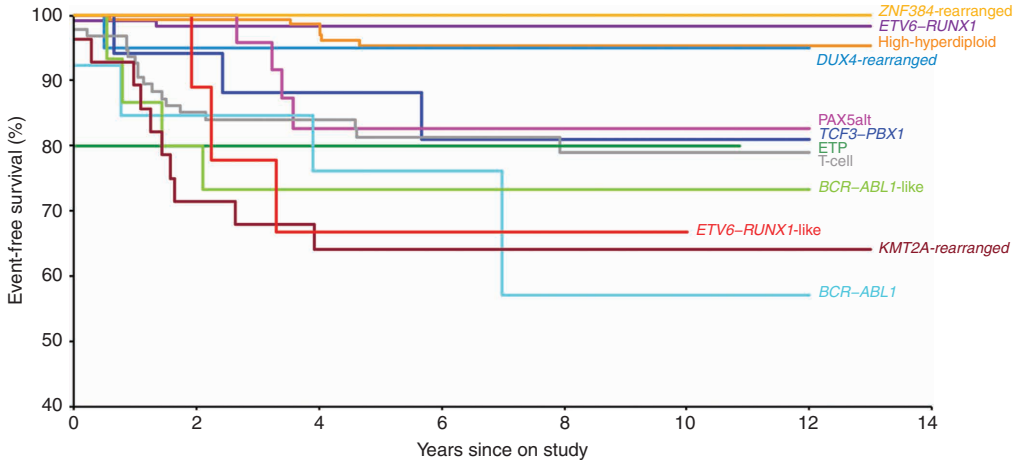
Day 8 MRD levels were <0.01% in 142 (24.8%) of the 572 patients with available data (Supplementary Table S2). Notably, all but three of these patients (two with *KMT2A*-rearranged and one with *TCF3–PBX1* ALL) remained in continuous complete remission. The proportion of patients with a day 8 MRD <0.01% ranged widely across leukemia subtypes, from 0% to 51.2% (Supplementary Table S2). The day 8 MRD finding did not correlate significantly with outcome within individual leukemia subtypes, except for high-hyperdiploid ALL. Among leukemia subtypes associated with the lowest risk of relapse, a day 8 MRD <0.01% was found in 51.2% of patients with *ETV6–RUNX1* and 21.1% of those with high-hyperdiploid ALL, but in none of those with *DUX4*-rearranged ALL.

Impact of Bone Marrow MRD Levels on Day 15

MRD levels on day 15 were <0.01% in 187 (31.7%), 0.01% to <1% in 226 (38.3%), and ≥1% in 177 (30.0%) of the 590 patients tested (Fig. 3A; Table 2). Overall, patients with a day 15 MRD ≥1% had significantly worse 5-year event-free survival and higher cumulative risk of relapse than those with lower or undetectable MRD levels ($P < 0.001$). However, high MRD on day 15 conferred a significantly poorer 5-year event-free survival only in cases with high-hyperdiploid ALL ($P = 0.05$) and B other ALL ($P < 0.001$), which consisted of heterogeneous diseases (Table 2). In patients with other leukemia subtypes, day 15 MRD ≥1% lacked prognostic impact, which could be due to treatment intensification triggered by this MRD finding and small number of patients in some subtypes. With standard-risk or high-risk treatment, relapse did not occur in any of the 36 patients with day 15 MRD ≥1% and *ETV6–RUNX1, DUX4*-rearranged, iAMP21, hypodiploid, *BCR–ABL1*-like, or *ZNF384*-rearranged ALL (Supplementary Table S3), again suggesting that subsequent intensification of treatment improved their outcome.

Impact of Bone Marrow MRD Levels on Day 42

Day 42 MRD levels were 0.01% to <1% in 60 (10.2%) of the patients and ≥1% in only 15 (2.6%; Fig. 3B; Table 3). Patients who attained a day 42 MRD <0.01% had a significantly better outcome than those with levels of 0.01% to <1%, who in turn fared better than patients with MRD ≥1% ($P < 0.001$). Among the 279 patients with favorable genotypes (*ETV6–RUNX1*, high hyperdiploidy, or *DUX4* rearrangement) who attained day 42 MRD <0.01%, 2 relapsed with a 5-year cumulative risk of relapse of 1.3% (0–2.8; Table 3). By contrast, of the 184 patients with intermediate-risk or unfavorable subtypes and day 42 MRD <0.01%, 20 including 4 with PAX5alt ALL and



Number at risk														
ZNF384-rearranged	7	7	7	7	7	7	7	7	6	5	5	3	1	
ETV6–RUNX1	128	127	127	126	121	102	87	75	61	52	38	29	12	1
High-hyperdiploid	154	154	153	153	147	124	99	83	65	54	38	22	14	2
DUX4-rearranged	20	20	19	19	18	15	14	11	8	7	6	4	2	
PAX5alt	24	24	24	24	23	16	14	13	12	9	7	6	2	
TCF3–PBX1	17	17	16	16	14	13	12	11	10	9	3	2	1	
ETP	10	8	8	8	8	6	6	5	4	3	2	2		
T-cell	94	92	88	79	77	69	59	52	41	34	25	15	5	
BCR–ABL1-like	15	15	13	12	11	9	7	5	5	5	4	1	1	
ETV6–RUNX1-like	9	9	9	8	7	5	5	3	3	3	3			
KMT2A-rearranged	28	27	25	20	19	17	13	10	9	9	5	3	2	1
BCR–ABL1	13	12	11	11	11	9	8	5	3	2	2	1	1	

Figure 2. Event-free survival for common leukemia subtypes. Note that there were only seven cases with *ZNF384*-rearranged ALL and nine with *ETV6–RUNX1*-rearranged cases. Results are not shown for some subtypes because of small number.

1 each with *BCL-ABL1*–like, *ETV6–RUNX1*-like, or *MEF2D*-rearranged ALL relapsed [9.5% (5.2–13.7), $P < 0.001$; Table 3].

Outcome of T-ALL Subgroups Segregated by Expression of Transcriptional Factors

Supplementary Table S4 summarizes the treatment risk groups, sequential MRD levels, and clinical outcome of various T-ALL subgroups. Most patients were treated in the standard-risk group, but higher proportions of patients in the HOXA and LMO1/2 subgroups were treated in the high-risk group due to day 42 MRD ≥1%. Patients in the HOXA and LMO1/2 groups also had high 5-year cumulative risk of relapse [25.1% (5.2–45.1) and 40% (0–89), respectively] and poor event-free survival [60.6% (37.1–84.1) and 60.0% (7.5–100), respectively]. In the HOXA group, there was no significant difference between the 9 patients with and the 12 without *KMT2A* rearrangement in 5-year cumulative risk of relapse [22.2% (0.0–51.2) vs. 27.8% (0.0–57.0), $P = 0.92$]. Notably, most subtype-defining genomic alterations observed in typical T-ALL cases were not identified in ETP ALL (Supplementary Table S5). There were no significant differences between T-ALL and ETP patients in 5-year event-free survival [81.3% (72.5–90.1) vs. 80.0% (53.5–100), $P = 0.86$)] or 5-year cumulative risk of relapse [12.0% (5.3–18.7) vs. 20.0% (0.0–46.1), $P = 0.49$], showing the impact of treatment intensification to abolish the historically poor prognostic significance of ETP in this study.

DISCUSSION

We demonstrate that genomic analyses coupled with MRD determination during remission induction have important prognostic and therapeutic implications. Our data indicate that patients with certain genetic ALL subtypes are almost always curable with conventional chemotherapy guided by early MRD assessment. In our study, 5-year overall survival for patients with *ETV6–RUNX1*-positive or high-hyperdiploid ALL exceeded 99% [99.2% (95% CI, 97.4–100) and 99.4% (97.8–100), respectively]. In the study by Lilljebjörn and colleagues (10), relapse was observed in 4 of 28 *DUX4*-rearranged patients, whereas in our study, despite elevated early MRD in 12 (60%) cases, the only adverse event in the *DUX4*-rearranged cohort was fatal sepsis, resulting in a 5-year event-free survival of 95.0% (84.2–100). MRD of less than 0.01% in peripheral blood on day 8 of induction treatment by itself identified a subgroup with an excellent outcome: Among the 142 patients with this early finding, only 3 (2 with *KMT2A*-rearranged and 1 with *TCF3–PBX1* ALL) relapsed. None of the 95 patients with either *ETV6–RUNX1* or high-hyperdiploid ALL who had a day 8 MRD <0.01% in blood and received low-risk therapy relapsed, suggesting that patients with these features should be considered for further treatment reduction in future trials. Our data, however, should not be interpreted to support treatment reduction in patients with other ALL subtypes even if they achieve a day 8 MRD <0.01%, as 39 of the 47 patients in this subgroup received standard- or high-risk therapy in our study.

The prognostic significance of MRD levels in peripheral blood on day 8 of induction has also been evaluated in other studies. Among patients who received Berlin–Frankfurt–Münster (BFM) backbone treatment regimens, the day 8 MRD result in blood after 1 week of pre-phase prednisone therapy and intrathecal methotrexate had little prognostic impact

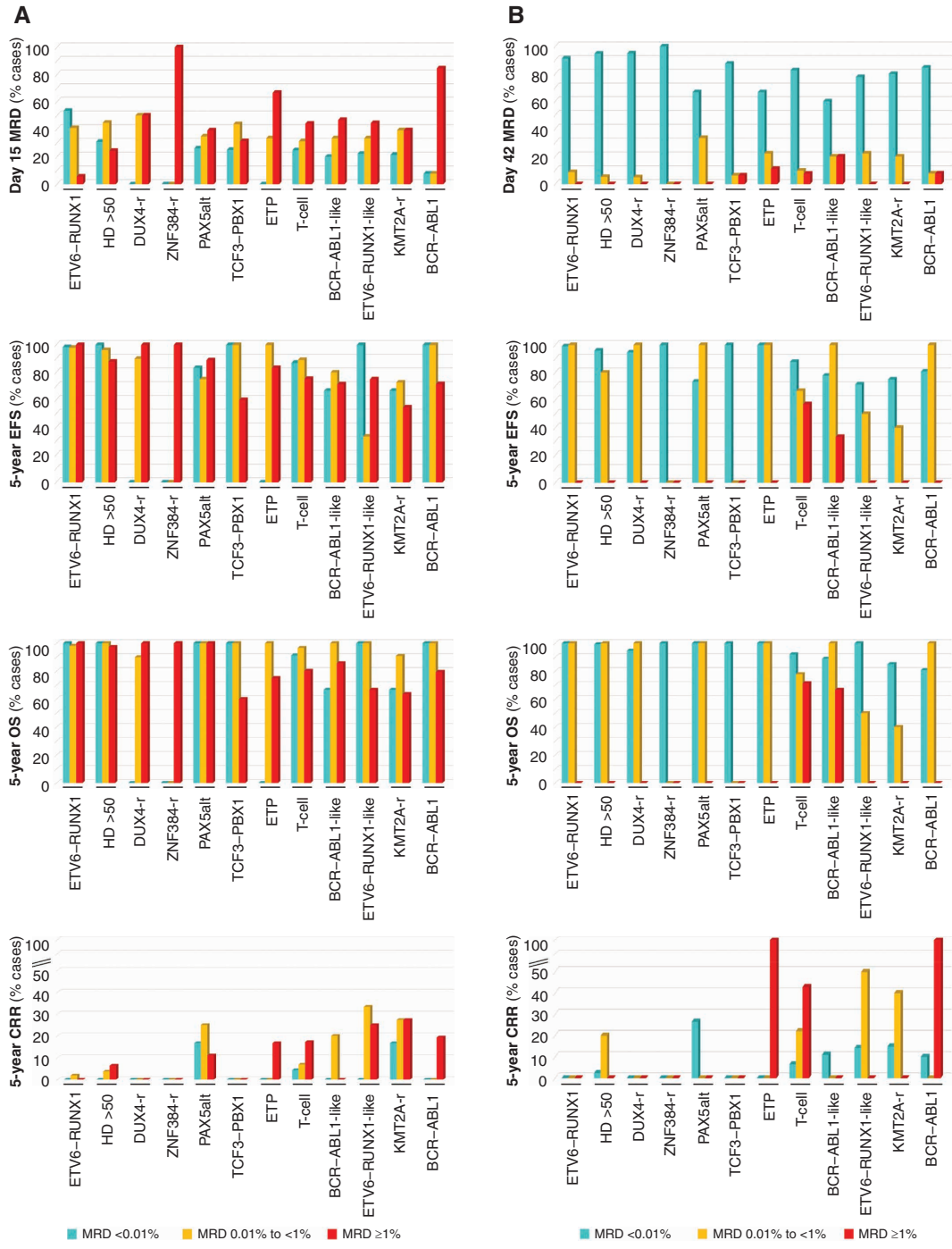


Figure 3. Treatment outcome based on leukemia cell subtype and MRD levels in bone marrow on day 15 (**A**) and day 42 (**B**). See Tables 2 and 3 for additional data. CRR, cumulative risk of any relapse; EFS, event-free survival; HD, hyperdiploidy; OS, overall survival.

(15–17). Among B-ALL patients treated in the COG P9900 protocols, however, a day 8 MRD ≤0.01% in blood after three- or four-drug induction plus intrathecal therapy was associated with a better event-free survival, while increasing levels of MRD at that time point were associated with a progressively worse outcome (18, 19). Because flow-cytometric measurements of MRD can be simplified when applied at early time points during remission induction therapy, particularly in peripheral blood (20), and a reduction in the intensity of remission induction therapy in low-risk patients was highly successful in a recent study (21), the day 8 MRD finding in blood could be used together with an uncomplicated genetic analysis (22) to identify low-risk patients for treatment reduction. This strategy would be especially effective in low- and

Table 2. Treatment outcome based on leukemia cell subtype and MRD in bone marrow at day 15 of induction

	Number of patients (%)			5-year EFS, % (95% CI)				5-year CRR, % (95% CI)			
	MRD <0.01%	MRD 0.01% to <1%	MRD ≥1%	MRD <0.01%	MRD 0.01% to <1%	MRD ≥1%	*P* value	MRD <0.01%	MRD 0.01% to <1%	MRD ≥1%	*P* value
ETV6–RUNX1	68 (53.5)	52 (40.9)	7 (5.51)	98.5 (95.0–100)	98.1 (93.8–100)	100 (100–100)	0.92	0	1.9 (0.0–5.7)	0	0.49
High-hyperdiploid	47 (30.9)	68 (44.7)	37 (24.3)	100 (100–100)	96.3 (90.8–100)	87.9 (76.1–99.7)	0.05	0	3.7 (0.0–8.9)	6.4 (0.0–15.2)	0.33
DUX4-rearranged	0 (0.00)	10 (50.0)	10 (50.0)	—	90.0 (68.8–100)	100 (100–100)	0.32	—	0	0	—
TCF3–PBX1	4 (25.0)	7 (43.8)	5 (31.3)	100 (100–100)	100 (100–100)	60.0 (22.8–97.2)	0.21	0	0	0	0.16
PAX5alt	6 (26.1)	8 (34.8)	9 (39.1)	83.3 (44.9–100)	75.0 (42.1–100)	88.9 (68.3–100)	0.75	16.7 (0.0–49.3)	25.0 (0.0–57.3)	11.1 (0.0–32.9)	0.75
T-cell	23 (24.7)	29 (31.2)	41 (44.1)	87.0 (70.5–100)	89.1 (76.8–100)	75.4 (61.1–89.7)	0.30	4.3 (0.0–12.9)	6.9 (0.0–16.3)	17.2 (5.4–29.0)	0.35
ETP	0 (0.00)	3 (33.3)	6 (66.7)	—	100 (100–100)	83.3 (50.0–100)	0.48	—	0	16.7 (0.0–49.3)	0.48
iAMP21	1 (20.0)	1 (20.0)	3 (60.0)	0	100 (100–100)	100 (100–100)	0.14	—	0	0	0.14
Hypodiploid	0 (0.00)	4 (66.7)	2 (33.3)	—	100 (100–100)	100 (100–100)	0.26	—	0	0	—
BCR–ABL1	1 (7.69)	1 (7.69)	11 (84.6)	100 (100–100)	100 (100–100)	71.6 (43.4–99.8)	0.52	0	0	19.3 (0.0–44.9)	0.56
BCR–ABL1-like	3 (20.0)	5 (33.3)	7 (46.7)	66.7 (23.2–100)	80.0 (39.4–100)	71.4 (34.0–100)	0.87	0	20.0 (0.0–59.2)	0	0.46
ETV6–RUNX1-like	2 (22.2)	3 (33.3)	4 (44.4)	100 (100–100)	33.3 (0.0–70.9)	75.0 (32.5–100)	0.38	0	33.3 (0.0–100)	25.0 (0.0–74.0)	0.66
KMT2A-rearranged	6 (21.4)	11 (39.3)	11 (39.3)	66.7 (29.1–100)	72.7 (44.5–100)	54.5 (22.4–86.6)	0.60	16.7 (0.0–49.3)	27.3 (0.0–55.0)	27.3 (0.0–55.2)	0.94
MEF2D-rearranged	1 (33.3)	1 (33.3)	1 (33.3)	—	—	0	0.37	—	—	—	0.37
ZNF384-rearranged	0 (0.00)	0 (0.00)	7 (100)	—	—	100 (100–100)	—	—	—	0	—
NUTM1-rearranged	3 (100)	0 (0.00)	0 (0.00)	100 (100–100)	—	—	—	0	—	—	—
PAX5 P80R	1 (50.0)	1 (50.0)	0 (0.00)	100 (100–100)	100 (100–100)	—	—	0	0	—	—
B other	21 (35.6)	22 (37.3)	16 (27.1)	100 (100–100)	95.5 (86.1–100)	62.5 (40.0–85.0)	<0.001	0	0	31.3 (7.6–54.9)	0.10
Total	187 (31.7)	226 (38.3)	177 (30.0)	95.1 (91.2–99.0)	92.5 (88.6–96.4)	79.0 (72.3–85.7)	<0.001	2.2 (0.1–4.4)	5.6 (2.5–8.7)	13.5 (8.3–18.7)	<0.001

Abbreviations: CRR, cumulative risk of any relapse; EFS, event-free survival.

middle-income countries to decrease the rates of induction death and treatment abandonment.

In this study, MRD measured in bone marrow on day 15 of remission induction was useful to identify patients with a poor early response who may have otherwise been regarded as having low-risk ALL for treatment intensification. Thus, none of the 7 *ETV6–RUNX1* and 10 *DUX4*-rearranged patients, and only 2 of the 37 high-hyperdiploid patients who received standard-risk treatment because of MRD ≥1% on day 15, subsequently relapsed. Treatment intensification based on MRD ≥1% on day 15 also appeared to be beneficial for patients with intermediate-risk or unfavorable genetic subtypes. With standard- or high-risk treatment, relapse did not occur in any patient with iAMP21, *ZNF384*-rearranged, hypodiploid <44, or *BCR–ABL1*-like ALL and a day 15 MRD ≥1%. Notably, achievement of undetectable (<0.01%) MRD on day 42 did not preclude subsequent relapse in patients with intermediate-risk or unfavorable subtypes, including *TCF3–PBX1*, PAX5alt, T-cell, iAMP21, *BCR–ABL1,* *BCR–ABL1*-like, *ETV6–RUNX1*-like, *KMT2A*-rearranged, or *MEF2D*-rearranged ALL. It is possible that more sensitive MRD assays, such as deep sequencing analysis, could identify patients at a higher risk of relapse among those with a negative MRD finding according to the most widely used cutoff of 0.01% (23, 24). If

Table 3. Treatment outcome based on leukemia cell subtype and MRD in bone marrow at day 42 (end of induction)

	Number of patients			5-year EFS, % (95% CI)				5-year CRR, % (95% CI)			
	MRD <0.01	MRD 0.01% to <1%	MRD ≥1%	MRD <0.01%	MRD 0.01% to <1%	MRD ≥1%	*P* value	MRD <0.01%	MRD 0.01% to <1%	MRD ≥1%	*P* value
ETV6-RUNX1	115 (91.3)	11 (8.73)	0 (0.00)	99.1 (97.1-100)	100 (100-100)	—	0.76	0	0	—	—
High-hyperdiploid	145 (94.8)	8 (5.23)	0 (0.00)	96.0 (92.1-99.9)	80.0 (48.6-100)	—	0.20	2.5 (0.0-5.4)	20.0 (0.0-59.2)	—	0.07
DUX4-rearranged	19 (95.0)	1 (5.00)	0 (0.00)	94.7 (83.3-100)	100 (100-100)	—	0.82	0	0	—	—
TCF3-PBX1[a]	14 (87.5)	1 (6.25)	1 (6.25)	100 (100-100)[a]	0	0	0.006	0[a]	—[a]	—[a]	1.00
PAX5alt[b]	16 (66.7)	8 (33.3)	0 (0.00)	73.4 (48.7-98.1)	100 (100-100)	—	0.12	26.6 (3.1-50.0)	0	—	0.11
T-cell	76 (82.6)	9 (9.78)	7 (7.61)	87.8 (79.6-96.0)	66.7 (33.0-100)	57.1 (20.4-93.8)	0.05	6.6 (1.0-12.2)	22.2 (0.0-51.3)	42.9 (2.6-83.1)	0.01
ETP	6 (66.7)	2 (22.2)	1 (11.1)	100 (100-100)	100 (100-100)	0	0.02	0	0	100	0.02
iAMP21	5 (100)	0 (0.00)	0 (0.00)	80.0 (39.4-100)	—	—	—	20.0 (0.0-59.2)	—	—	—
Hypodiploid	5 (83.3)	1 (16.7)	0 (0.00)	100 (100-100)	100 (100-100)	—	0.48	0	0	—	—
BCR-ABL1	11 (84.6)	1 (7.69)	1 (7.69)	80.8 (56.3-100)	100 (100-100)	0	0.14	10.1 (0.0-30.0)	0	100 (100-100)	0.09
BCR-ABL1-like[c]	9 (60.0)	3 (20.0)	3 (20.0)	77.8 (50.6-100)	100 (100-100)	33.3 (0.0-86.6)	0.13	11.1 (0.0-33.1)	0	0	0.76
ETV6-RUNX1-like[d]	7 (77.8)	2 (22.2)	0 (0.00)	71.4 (37.9-100)	50.0 (1.0-99.0)	—	0.48	14.3 (0.0-42.7)	50.0 (0.0-100)	—	0.28
KMT2A-rearranged	20 (80.0)	5 (20.0)	0 (0.00)	75.0 (53.8-96.2)	40.0 (0.0-82.9)	—	0.11	15.0 (0.0-31.1)	40.0 (0.0-91.0)	—	0.15
MEF2D-rearranged[e]	3 (100)	0 (0.00)	0 (0.00)	66.7 (23.2-100)	—	—	—	33.3 (0-98.7)	—	—	—
ZNF384-rearranged	7 (100)	0 (0.00)	0 (0.00)	100 (100-100)	—	—	—	0	—	—	—
NUTM1-rearranged	3 (100)	0 (0.00)	0 (0.00)	100 (100-100)	—	—	—	0	—	—	—
PAX5 P80R	2 (100)	0 (0.00)	0 (0.00)	100 (100-100)	—	—	—	0	—	—	—
B other	49 (83.1)	8 (13.6)	2 (3.39)	93.6 (86.2-100)	62.5 (31.9-93.1)	50 (1.0-99.0)	0.002	2.2 (0.0-6.6)	37.5 (1.1-73.9)	50 (0.0-100)	<0.001
Total	512 (87.2)	60 (10.2)	15 (2.6)	92.6 (89.9-95.3)	78.9 (66.7-91.1)	40.0 (12.8-67.2)	<0.001	4.4 (2.5-6.2)	16.1 (6.2-25.9)	40.0 (13.9-66.1)	<0.001

Abbreviations: CRR, cumulative risk of any relapse; EFS, event-free survival.

[a]Among patients with *TCF3-PBX1* ALL, one with day 42 MRD <0.01% relapsed at 5.7 years, and two with positive MRD died of transplant-related toxicities at 0.6 and 2.4 years, respectively.

[b]Of the 16 PAX5alt patients with day 42 MRD <0.01%, 4 relapsed [2 hematologic and 2 central nervous system (CNS) relapses].

[c]Of the nine *BCR-ABL1*-like patients with day 42 MRD <0.01%, one developed CNS relapse.

[d]Of the seven *ETV6-RUNX1*-like patients with day 42 MRD <0.01%, one had hematologic relapse, and another developed myelodysplastic syndrome.

[e]Of the three patients with *MEF2D*-rearranged ALL and day 42 MRD <0.01%, one 12-year-old patient with standard-risk disease relapsed and died at 2.9 years, and the other two patients were alive in remission at 3.6 and 4.0 years, respectively; data shown are 3-year results.

so, such patients might be considered as candidates for novel targeted therapies (25, 26).

Our study suggests that several newly identified genotypes might be prognostically relevant in the context of contemporary risk-directed treatment. Conceivably, *DUX4*-rearranged ALL (9, 10) could join *ETV6–RUNX1* and high-hyperdiploid ALL as one of the most favorable subtypes. Although none of our 20 patients with this feature relapsed, it should be noted that 12 of them received standard-risk therapy because of day 15 MRD >1%. Patients with PAX5alt ALL, commonly classified

as having high-risk ALL by NCI criteria because of presenting age above 10 years or leukocyte count above $100 \times 10^3/\mu L$, had a 5-year event-free survival of 71.5% ± 7.0% when treated in the Children's Oncology Group AALL0232 protocol for high-risk ALL (13). In our study, 2 of the 24 patients with PAX5alt ALL developed hematologic relapse and two central nervous system (CNS) relapse, with a 5-year event-free survival of 82.7% (65.3–100). Although they had a day 42 MRD <0.01%, all four relapsed patients were treated with standard-risk therapy because of unfavorable presenting clinical features (age >10 years in two patients, leukocyte count $225 \times 10^3/\mu L$ in one) or a poor early treatment response (day 15 MRD >1% in one). Hence, we consider this subtype to have an intermediate risk of relapse.

In the first report of *ETV6–RUNX1*-like ALL, 2 of the 10 patients relapsed (10). Among our nine patients with this genotype, seven were treated with standard-risk therapy, two of whom relapsed (one with a day 42 MRD <0.01%) and two were treated with low-risk therapy, one of whom developed myelodysplastic syndrome. Likewise, both of our relapsed *MEF2D*-rearranged and iAMP21 patients had a day 42 MRD <0.01%; both genotypes have been associated with an increased risk of relapse (11, 27, 28). Notably, our relapsed patient with *MEF2D*-rearranged ALL was also treated with standard-risk therapy. Thus, an MRD <0.01% at the end of induction does not ensure high curability of patients with several recently identified genetic subtypes, even in the context of contemporary risk-directed therapy. Additional studies of a larger number of patients are needed to confirm our findings and to determine whether patients with these subtypes can benefit from additional molecularly targeted therapy, immunotherapy, or both.

Transcriptome sequencing analyses in this study identified patients with three other uncommon subtypes: *ZNF384*-rearranged, *NUTM1*-rearranged, and PAX5 P80R ALL. Our previous study suggested that, despite expression of B- and myeloid lineage markers, *ZNF384*-rearranged cases should be treated as ALL based on the similarity of their genomic landscape to that of B-ALL (29). In two small series, these patients had 5-year event-free survival rates of 50% to 83% (11, 30). All seven cases in this study remained in remission for 6.8 to 11.5 years, but they were all treated with standard-risk therapy owing to a day 15 MRD >1%. *NUTM1*-rearranged ALL is a rare B-ALL subtype, and while all seven patients reported in one series were in continuous remission, four received treatment for intermediate- to high-risk ALL (31). In this study, all three *NUTM1*-rearranged patients were in remission after standard-risk treatment. PAX5 P80R is a recently identified B-ALL subtype with a 5-year event-free survival of 75.0% ± 7.0% in the eight patients treated in the Children's Oncology Group AALL0232 study, and 50.0% ± 17.7% in the six patients treated in the St. Jude Total Therapy studies (13). For these reasons, we believe that all three subtypes have an intermediate-risk prognosis—an impression requiring confirmation.

Several of the novel subtypes have immunophenotypic features suggestive of the diagnosis: CD2 and CD371 positivity in *DUX4*-rearranged ALL (32), CD10 negativity and CD28 positivity in *MEF2D*-rearranged ALL (27), and aberrant myeloid antigen expression in *ZNF384*-rearranged ALL (29). With the exception of CD371, none of the other features are specific for the associated subtypes, and some level of genomic analysis is required for accurate diagnosis. Moreover, *ZNF384* rearrangement defines a broader entity comprising B-progenitor ALL (such cases may have aberrant myeloid marker expression, but not myeloperoxidase) and B/myeloid mixed phenotype acute leukemia (myeloperoxidase positive; ref. 29).

T-ALL can be divided into subtypes by gene-expression profiling or by mutated functional pathway; some cases had rare ABL class fusions (e.g., *NUP214–ABL1*) that may respond to a tyrosine kinase inhibitor (26). Unlike B-ALL, T-ALL lacks consensus genetic classification with prognostic or therapeutic significance. Inconsistently, *NOTCH1* and *FBXW7* mutations were associated with favorable prognosis, whereas Ras mutation, *PTEN* mutation, and lack of biallelic *TRG* rearrangement (as a surrogate for immature, early T-cell precursor ALL) were associated with unfavorable prognosis (26). An important future study will be comprehensive consideration of gene expression, sequence, and structural cohorts in adequately powered studies of uniformly treated T-ALL to examine the interaction of subtype and secondary mutations and outcome in T-ALL. In the Children's Oncology Group AALL0434 study, based on the expression of various transcription factors and event-free survival, T-ALL cases were grouped into low-risk (*NKX2*, *HOXA, TAL2*, and *TLX1*), intermediate-risk (*LMO2-LYL1*, *TLX3*, and *TAL1*), and high-risk (*LMO1/2*, *ABL1*, and *KMT2A*-rearranged) categories (26, 33). In this study, we could confirm the poor prognosis of patients in the LMO1/2 subgroup, but our patients in the HOXA group (with or without *KMT2A* rearrangement) had a high cumulative risk of relapse resulting in low event-free survival. Additional studies are needed to determine the prognosis of patients with HOXA expression.

Together, our results suggest that both systematic genomic analyses and MRD measurements are required to accurately stratify children with ALL into risk groups and tailor their therapy accordingly. We have adopted this approach in our current Total Therapy Study 17. Our data showing poor prognosis of several newly identified subtypes of B-ALL despite very intensive therapy emphasize the need to expand the application of immunotherapy and novel mutation-, fusion gene–, or pathway-directed treatments to leukemia variants resistant to conventional treatment. Because of the small number of patients studied, additional studies are needed to evaluate the prognostic and therapeutic relevance of *ETV6–RUNX1*-like, *ZNF384*-rearranged, and *MEF2D*-rearranged B-ALL, and T-ALL with HOXA expression.

METHODS

Patients and Risk Classification

From October 29, 2007, to March 26, 2017, 598 eligible patients ages between 0.12 and 18.9 years (median, 6.04) with newly diagnosed ALL were enrolled in Total Therapy Study 16 (ClinicalTrials.gov Identifier NCT00549848) at St. Jude Children's Research Hospital (14). The trial protocol was approved by the institutional review board and is available in the Supplementary Information. The study was conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from the parents or guardians and assent from the patients, as appropriate.

The diagnosis of ALL was based on the immunophenotypic and genetic characteristics of the leukemic cells (14). Genomic classification was based on cytogenetics: FISH for *ETV6–RUNX1, TCF3–PBX1,*

BCR–ABL1, and *KMT2A* rearrangement and transcriptome sequencing [RNA sequencing (RNA-seq)] where available ($n = 502$; ref. 13). Details for genomic classification are provided in Supplementary Figs. S2–S4. MRD levels were determined by flow cytometry (14, 34) in blood samples on day 8 and in bone marrow samples on days 15 and 42 (the end of remission induction); a negative MRD was defined as a level <0.01%.

Patients with B-ALL between 1 and 10 years and with a blood leukocyte count at presentation $<50 \times 10^3/\mu L$, DNA index ≥1.16 (high hyperdiploidy), or *ETV6–RUNX1* fusion were provisionally classified as having low-risk ALL. Those with MRD ≥1% on day 15 of induction or 0.01% to <1% on day 42 were classified as having standard (intermediate)-risk ALL. Patients with the *BCR–ABL1* or ETP ALL, infants with *KMT2A* rearrangement, and any patients with day 42 MRD ≥1% (regardless of provisional classification) or persistent MRD during the consolidation phase were classified as having high-risk ALL. The remaining patients, including those with *TCF3–PBX1*, hypodiploidy with fewer than 44 chromosomes, T-ALL, testicular leukemia, or a CNS-3 status (≥5 leukocytes/μL of cerebrospinal fluid with blasts or cranial palsy) at diagnosis were considered to have standard-risk ALL.

Transcriptome Sequencing (RNA-seq)

RNA-seq was performed on 502 samples using TruSeq library preparation and HiSeq 2000/2500 or NovaSeq 6000 sequencers (Illumina). All sequence reads were paired-end, and sequencing was performed using (35) total RNA and stranded RNA-seq [100 base-pair (bp) reads] or (36) polyA-selected mRNA (100 bp reads). Sequencing reads were mapped to the GRCh37 human genome reference by STAR (ref. 1; version 2.4.2a) through the suggested two-pass mapping pipeline. Gene annotation downloaded from the Ensembl website (http://www.ensembl.org/) was used for STAR mapping and the following read-count evaluation. All the samples were sequenced with RefSeq coding region covered with 30-fold coverage ≥15% (median ± standard deviation, 37.2% ± 7.5%). CICERO (36, 37) and FusionCatcher (38, 39) were used to detect fusions, and all the reported rearrangements were manually reviewed to keep the reliable ones. Due to the complexity of *DUX4* rearrangements, some of the *DUX4* fusions were manually rescued by checking the aligned reads within the Integrative Genomics Viewer browser (40).

To evaluate gene-expression levels from RNA-seq, read count for each annotated gene was calculated by HTSeq package (41), and gene-expression level normalization and differential expression analysis were carried out by DESeq2 Bioconductor R package (42). To evaluate the digital gene-expression levels, regularized log-transformed (rlog) value was calculated by DESeq2 (Supplementary Table S6). ComBat function in sva R package (43) was used to correct the batch effect introduced by different library preparation strategies and sequencing lengths. Prediction Analysis of Microarrays (PAM; ref. 44) was used to identify subgroups with distinct gene-expression profiles as reported previously (13). R package Rtsne was used to map the samples to two-dimensional t-distributed stochastic neighbor embedding (tSNE) plot to visualize clusters. Genomic data are publicly available and have been deposited in the European Genome-phenome Archive (accessions EGAS00001000447, EGAS00001000654, EGAS00001001923, EGAS00001002217, EGAS00001003266, EGAS00001004739, and EGAS00001005084).

Treatments

Remission induction started with prednisone, vincristine, daunorubicin, and PEG-asparaginase (Supplementary Table S7). After 2 weeks of induction, patients with a day 15 MRD ≥1% were given an additional dose of PEG-asparaginase on day 15. Subsequent induction therapy between days 22 and 35 consisted of prednisone, vincristine, cyclophosphamide, cytarabine, and thiopurine. Patients with *BCR–ABL1* ALL ($n = 10$) or ABL class fusion ($n = 3$) received dasatinib from the diagnosis of the genotype (generally on day 22) until the end of all treatment. Upon hematopoietic recovery, MRD was measured on day 42, followed by consolidation therapy with high-dose methotrexate, mercaptopurine, and triple intrathecal therapy (Supplementary Table S7). All patients received antimetabolite-based continuation therapy for 120 weeks with two reinduction treatments and pulses of dexamethasone and vincristine, while standard-risk or high-risk patients received additional PEG-asparaginase, doxorubicin, high-dose cytarabine, and cyclophosphamide plus cytarabine drug pair (Supplementary Table S7). All patients received triple intrathecal chemotherapy for CNS-directed treatment with the number of doses based on presenting characteristics and CNS status (Supplementary Table S7). Allogeneic hematopoietic cell transplantation was an option for patients with high-risk leukemia.

Main Outcomes and Measures

The primary objective of the study was to determine the prognostic and therapeutic implications of leukemia subtypes, especially the novel subtypes, among patients who had comprehensive genomic analyses and sequential MRD determination during remission induction for risk-directed treatment.

Statistical Analysis

The primary endpoint was event-free survival, and secondary endpoints were overall survival and cumulative risk of relapse. Event-free survival was defined as the time from diagnosis of ALL until the date of induction failure (≥5% blasts in bone marrow), relapse, death in remission from any cause, the development of a second cancer, or the date of last contact (all event-free survivors). Event-free and overall survival rates were estimated by the Kaplan–Meier method and compared by the log-rank test. Cumulative risk of relapse was estimated according to the method of Kalbfleisch and Prentice (45) and compared with Gray's test (46); death in remission and the development of secondary neoplasms were regarded as competing events. The 95% CI was computed by using the asymptotic normality approximation; a nonparametric method was applied if the sample size was small. All reported *P* values were two-sided and not adjusted for multiple comparisons. MRD levels at each time point were categorized into three groups (<0.01%, 0.01%–<1%, and ≥1%) and regarded as unordered in the analysis. Outcome data updated on June 2, 2020, were used in all analyses; 88.7% of the survivors had been seen within 1 year. The median follow-up time for the 557 patients who were alive at the time of analysis was 7 years (interquartile range 5 years; range, 1.1–7.2 years). All statistical analyses were based on intent to treat and done with SAS software (version 9.4) and R version 3.3.0.

Authors' Disclosures

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Disclaimer

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Authors' Contributions

S. Jeha: Resources, supervision, investigation, project administration, writing–review and editing. **J. Choi:** Data curation, validation, investigation, methodology, writing–review and editing. **K.G. Roberts:** Resources, data curation, investigation, writing–review and editing. **D. Pei:** Data curation, formal analysis, methodology, writing–review and editing. **E. Coustan-Smith:** Resources, data curation, formal analysis, supervision, validation, investigation, methodology, writing–review and editing. **H. Inaba:** Resources, investigation, writing–review and editing. **J.E. Rubnitz:** Resources, investigation, writing–review and editing. **R.C. Ribeiro:** Resources, investigation, writing–review and editing. **T.A. Gruber:** Resources, investigation, writing–review and editing. **S.C. Raimondi:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **S.E. Karol:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **C. Qu:** Resources, data curation, formal analysis, funding acquisition, investigation, writing–original draft, writing–review and editing. **S.W. Brady:** Conceptualization, resources, data curation, formal analysis, funding acquisition, investigation, writing–original draft, writing–review and editing. **Z. Gu:** Conceptualization, resources, data curation, formal analysis, funding acquisition, investigation, writing–original draft, writing–review and editing. **J.J. Yang:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, writing–original draft, writing–review and editing. **C. Cheng:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **J.R. Downing:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **W.E. Evans:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **M.V. Relling:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **D. Campana:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **C.G. Mullighan:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **C.-.H Pui:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing.

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