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LYMPHOID NEOPLASIA

Monitoring of childhood ALL using *BCR-ABL1* genomic breakpoints identifies a subgroup with CML-like biology

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Key Points

- Combination of Ig/TCR and *BCR-ABL1* genomic approach for MRD monitoring in childhood ALL reveals patients with CML-like disease.
- Monitoring ALL using *BCR-ABL1* genomic breakpoint is feasible and enables the most specific and sensitive MRD quantification.

We used the genomic breakpoint between *BCR* and *ABL1* genes for the DNA-based monitoring of minimal residual disease (MRD) in 48 patients with childhood acute lymphoblastic leukemia (ALL). Comparing the results with standard MRD monitoring based on immunoglobulin/T-cell receptor (Ig/TCR) gene rearrangements and with quantification of *IKZF1* deletion, we observed very good correlation for the methods in a majority of patients; however, >20% of children (25% [8/32] with minor and 12.5% [1/8] with major-*BCR-ABL1* variants in the consecutive cohorts) had significantly (>1 log) higher levels of *BCR-ABL1* fusion than Ig/TCR rearrangements and/or *IKZF1* deletion. We performed cell sorting of the diagnostic material and assessed the frequency of *BCR-ABL1*-positive cells in various hematopoietic subpopulations; 12% to 83% of non–ALL B lymphocytes, T cells, and/or myeloid cells harbored the *BCR-ABL1* fusion in patients with discrepant MRD results. The multilineage involvement of the *BCR-ABL1*-positive clone demonstrates that in some patients diagnosed with *BCR-ABL1*-positive ALL, a multipotent hematopoietic progenitor is affected by the *BCR-ABL1* fusion. These patients have *BCR-ABL1*-positive clonal hematopoiesis resembling a chronic myeloid leukemia (CML)–like disease manifesting in "lymphoid blast crisis." The biological heterogeneity of *BCR-ABL1*-positive ALL may impact the patient outcomes and optimal treatment (early stem cell transplantation vs long-term administration of tyrosine-kinase inhibitors) as well as on MRD testing. Therefore, we recommend further investigations on CML-like *BCR-ABL1*-positive ALL. (*Blood.* 2017;129(20):2771-2781)

Introduction

The *BCR-ABL1* fusion gene, resulting from the reciprocal translocation t(9;22)(q34;q11), is a hallmark of chronic myeloid leukemia (CML) and is also present in a subset of acute lymphoblastic leukemia (ALL). According to the genomic breakpoint within the *BCR* gene, there are 2 common variants of the fusion, major (M) and minor (m) *BCR-ABL1*, that encode the $p210^{BCR\text{-}ABL1}$ and $p190^{BCR\text{-}ABL1}$ proteins, respectively.[1] Almost all patients diagnosed with CML carry the major-*BCR-ABL1*, whereas minor-*BCR-ABL1*-positive CML is very rare (~1% cases).[2] In contrast, the minor-*BCR-ABL1* is prevalent in ALL, particularly in children.[3] Generally, *BCR-ABL1*-positive leukemia is rare among children; *BCR-ABL1* fusion occurs in 2% to 4% of pediatric ALL cases,[4,5] and CML represents 2% to 4% of all leukemia diagnosed in childhood.[6]

The overall survival of *BCR-ABL1*-positive pediatric ALL has improved significantly since the introduction of tyrosine kinase inhibitors (TKIs) into treatment protocols.[3,7-9] Despite this improvement, *BCR-ABL1*-positive ALL has remained a high-risk subgroup with an unfavorable outcome. Therefore, the ongoing studies on childhood ALL aim to find an optimal chemotherapy backbone to the TKI treatment and to reduce the number of patients undergoing stem cell transplantation (SCT).[3,9] In pediatric CML, treatment standardization and balancing the survival advantage vs side effects of long-lasting TKI administration are the most important tasks[10-12]; in the advanced phases of CML, SCT is still considered the treatment of choice.[13,14]

One of the tools that might enable finding optimal treatments for both *BCR-ABL1*-positive ALL and CML is minimal residual disease (MRD) monitoring. Two targets are routinely used for polymerase chain reaction (PCR)–based MRD monitoring in *BCR-ABL1*-positive ALL, clonal immunoglobulin/T-cell receptor (Ig/TCR) gene rearrangements, and *BCR-ABL1* transcript levels. We have previously compared

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Table 1. Clinical and outcome data of the patients analyzed for MRD

UPN	DG	Age (y)	BCR-ABL1 variant	Initial treatment protocol	Initial WBC	Initial CNS involvement	TKI	SCT	Relapse (mo)	Death (mo)	Status	Follow-up (mo)	BCR-ABL1 vs Ig/TCR MRD
A0752	ALL	5	Major	ALL BFM 95	360 000	No	Frontline	CR2	5	—	CR2	177	Discordant
A1071	ALL	11	Minor	ANZCCSG Study 7	530 000	No	Frontline	CR1	16	30	Exitus	—	Concordant
A1861	ALL	5	Minor	ANZCHOG Study 8	1400	No	Frontline	CR1	—	—	CR1	144	Concordant
A1862	ALL	4	Minor	ANZCHOG Study 8	19 700	No	Frontline	CR1	—	—	CR2	128	Concordant
A2184	ALL	13	Minor	ANZCHOG Study 8	28 100	No	Frontline	CR1	22	—	CR2	104	Concordant
A2504	ALL	10	Minor	ANZCHOG Study 8	8500	No	Frontline	CR1	55	—	CR2	126	Concordant
A4643	ALL	14	Minor	ANZCHOG Study 8	6000	No	Frontline	CR1	—	—	CR1	93	Concordant
A5017	ALL	4	Major	ANZCHOG Study 8	917 000	Yes	Frontline	CR1	—	—	CR1	88	Concordant
A5020	ALL	7	Minor	ANZCHOG Study 8	148 000	No	Frontline	No	40	41	Exitus	—	Concordant
A5219	ALL	5	Minor	COG AALL0031	17 100	No	Frontline	CR1	—	—	CR1	70	Discordant
A5295	ALL	14	Major	Individualized therapy	321 500	No	Frontline	CR1	33	55	Exitus	—	Concordant
A5444	ALL	8	Minor	ANZCHOG Study 8	59 400	No	Frontline	No	—	—	CR1	63	Concordant
A5659	ALL	11	Minor	COG AALL0232	14 800	No	Frontline	No	—	—	CR1	51	Concordant
A5751	ALL	11	Minor	AIEOP-BFM ALL 2009	4200	No	Frontline	No	46	—	CR2	46	Concordant
A5925	ALL	10	Major	COG AALL1131	267 800	No	Frontline	CR1	—	8	Exitus	—	Concordant
A6002	ALL	15	Minor	Individualized therapy	337 400	No	Frontline	CR1	—	—	CR1	36	Concordant
A6036	ALL	4	Minor	COG AALL1112/CA180372	9500	No	Frontline	No	—	—	CR1	33	NA
C3	ALL	10	Minor	ALL BFM 90	114 600	No	No	CR1	58	—	CR4	145	Concordant
C4	ALL	15	Minor	ALL BFM 90	8200	No	After relapse	CR2	116	—	CR2	204	Discordant
C198	ALL	10	Minor	ALL BFM 95	23 600	No	No	No	10	15	Exitus	—	Discordant
C212	ALL	4	Minor	ALL BFM 95	125 000	No	After relapse	CR2	112	—	CR2	214	Concordant
C375	ALL	6	Minor	ALL BFM 95	6800	No	Frontline	CR2	33	—	CR2	198	Concordant
C429	ALL	4	Minor	ALL BFM 95	100 900	No	After relapse	CR2	33	—	CR2	194	Discordant
C438	ALL	10	Minor	ALL BFM 95	32 000	No	Frontline	CR2	15	46	Exitus	—	Concordant
C533	ALL	9	Minor	ALL BFM 95	41 600	No	Frontline	CR1	27	28	Exitus	—	Concordant
C658	ALL	11	Minor	ALL IC-BFM 2002	40 000	No	After relapse	CR2	26	—	CR2	160	Discordant
C710	ALL	12	Major	EsPhALL	139 000	No	Frontline	CR1	—	—	CR1	153	Concordant
C718	ALL	4	Minor	EsPhALL	24 900	No	Frontline	CR1	—	—	CR1	152	Concordant
C769	ALL	14	Minor	EsPhALL	45 000	Yes	Frontline	CR1	17	19	Exitus	—	Concordant
C825	ALL	5	Minor	EsPhALL	145 000	No	Frontline	CR1	18	19	Exitus	—	Concordant
C861	ALL	17	Major	ALL IC-BFM 2002	190 800	Yes	Frontline	CR1	—	—	CR1	137	Concordant
C1029	ALL	13	Major	EsPhALL	135 000	No	Frontline	CR1	—	18	Exitus	—	Concordant
C1092	ALL	4	Minor	EsPhALL	184 700	No	Frontline	CR1	10	10	Exitus	—	Discordant
C1277	ALL	3	Minor	EsPhALL	154 000	No	Frontline	CR1	—	12	Exitus	—	Concordant
C1304	ALL	2	Minor	COG AALL0622	133 630	Yes	Frontline	CR1	31	—	CR3	87	Concordant
C1382	ALL	5	Minor	EsPhALL	9500	Yes	Frontline	No	—	3	Exitus	—	Discordant
C1519	ALL	4	Minor	EsPhALL	344 000	No	Frontline	CR2	34	42	Exitus	—	Concordant
C1822	ALL	11	Minor	EsPhALL	411 500	Yes	Frontline	CR2	28	—	CR2	45	Concordant
C1893	ALL	17	Minor	EsPhALL	6670	No	Frontline	No	—	—	CR1	40	Discordant
C1964	ALL	14	Minor	EsPhALL	5900	No	Frontline	CR1	—	—	CR1	36	NA*
C2248	ALL	10	Minor	EsPhALL	155 200	No	Frontline	No	—	—	CR1	20	Concordant
C2294	ALL	14	Major	EsPhALL	65 600	Yes	Frontline	No	—	—	CR1	20	Concordant
G001	ALL	7	Major	ALL BFM 2000	46 000	No	No	CR1	—	—	CR1	145	Discordant

A (Australian) and C (Czech) ALL patients (top 42 rows) represent unselected cohorts; G (German) ALL patients were selected based on discordance.

CNS, central nervous system; CR, complete remission; DG, diagnosis; mo, months from diagnosis; NA, not applicable (no Ig/TCR target); UPN, unique patient number; WBC, white blood cell count [$\times 10^9$/L].

*"Discordant" based on flow cytometric MRD vs genomic *BCR-ABL1* breakpoint quantification.

Table 1. (continued)

UPN	DG	Age (y)	BCR-ABL1 variant	Initial treatment protocol	Initial WBC	Initial CNS involvement	TKI	SCT	Relapse (mo)	Death (mo)	Status	Follow-up (mo)	BCR-ABL1 vs Ig/TCR MRD
G002	ALL	16	Major	ALL BFM 2000	135 000	Yes	Frontline	CR1	14	46	Exitus	—	Discordant
G003	ALL	3	Minor	ALL BFM 2000	1950	No	Frontline	CR2	33	—	CR2	140	Discordant
G004	ALL	14	Major	EsPhALL	49 600	No	Frontline	CR1	—	—	CR1	98	Discordant
G005	ALL	14	Major	EsPhALL	167 900	No	Frontline	CR1	—	—	CR1	67	Discordant
G006	ALL	18	Minor	EsPhALL	760	No	Frontline	CR2	84	—	CR2	92	Discordant
C543	CML	12	Major	ALL BFM 95	194 200	No	Frontline	CR1	9	12	Exitus	—	Discordant
C1123	CML	6	Major	CML Paedll-06	575 000	No	Frontline	CR2	2	—	CR2	108	Discordant
C1437	CML	14	Major	EsPhALL	477 000	No	Frontline	CR1	11	13	Exitus	—	Discordant

A (Australian) and C (Czech) ALL patients (top 42 rows) represent unselected cohorts; G (German) ALL patients were selected based on discordance.
CNS, central nervous system; CR, complete remission; DG, diagnosis; mo, months from diagnosis; NA, not applicable (no Ig/TCR target); UPN, unique patient number; WBC, white blood cell count [$\times 10^9$/L].
*"Discordant" based on flow cytometric MRD vs genomic *BCR-ABL1* breakpoint quantification.

the quantification of these 2 targets in childhood ALL,[15] demonstrating that ~20% of Ig/TCR-negative samples are positive (sometimes at high levels) using *BCR-ABL1* transcript, and we presented 1 patient in whom the discrepancy was caused by the presence of *BCR-ABL1* fusion outside the B-lymphoid blast population.[15] Recent data show that even among *BCR-ABL1*-positive childhood ALL patients with a very good treatment response assessed by Ig/TCR MRD monitoring, 25% to 35% of patients relapse.[16] However, *BCR-ABL1*-based MRD data and their concordance with the Ig/TCR levels in relapsing vs nonrelapsing patients were not analyzed.

Discrepancies in MRD levels assessed using these 2 techniques could be ascribed to the different targets (Ig/TCR vs *BCR-ABL1*) and/or the different methods for complementary DNA (cDNA)–based vs DNA-based detection. The RNA/cDNA-based transcript quantification depends on the expression levels of *BCR-ABL1*, which may be heterogeneous within the blast population and between cell types and may be influenced by treatment. Therefore, the number of transcripts per cell may vary significantly within the *BCR-ABL1*-positive cell population, while DNA-based MRD tests measure 1 target molecule per cell. One way to resolve this issue is to also measure the levels of the genomic *BCR-ABL1* fusion.

Several studies demonstrated the feasibility of characterizing the *BCR-ABL1* fusion at the genomic level.[17-33] The genomic fusion sequence was usually used for sensitive detection of rare (pre)leukemic cells or as an MRD target. In some studies, the DNA-*BCR-ABL1*-based MRD monitoring was compared with the transcript quantification, and the genomic approach was shown to be more sensitive.[17,18,20,23-25,29,31] However, with a single exception,[27] the published "genomic" MRD data are based on major-*BCR-ABL1* monitoring, which is probably because the characterization of minor-*BCR-ABL1* fusion is more demanding. The minor-*BCR* breakpoint region spans >70 kb, whereas the major-*BCR* breakpoint region is only ~3 kb long.

Here, we present both minor- and major-*BCR-ABL1* breakpoint identification in a large cohort of pediatric patients with *BCR-ABL1*-positive ALL. We used the patient-specific DNA breakpoint sequences for MRD quantification and compared the results with standard Ig/TCR MRD monitoring and *BCR-ABL1* transcript levels. Moreover, to complement these approaches, we used *IKZF1* deletions (present in two-thirds of childhood *BCR-ABL1*-positive ALL[34]) as an alternative MRD target. Finally, we investigated the cell lineages of *BCR-ABL1*-positive clones in several patients with large differences in the MRD results.

Methods

Patients and samples

This study included 67 patients diagnosed with either *BCR-ABL1*-positive childhood ALL (n = 64) or CML diagnosed in lymphoid blast crisis (LBC) (n = 3). Patients were treated according to various protocols (see Table 1 for details). Standard diagnostics were performed according to the practice of local diagnostic laboratories. Basic clinical/outcome data were collected from treating centers. Diagnostic and treatment procedures and protocols were approved by the local institutional review boards. Informed consent was obtained in accordance with the Declaration of Helsinki.

Quantification of the *ALB* gene[35] by quantitative PCR (qPCR) was performed to measure the DNA concentration. In some diagnostic samples, the whole genome amplification using REPLI-g Midi Kit (Qiagen, Hilden, Germany) was performed to obtain sufficient DNA for *BCR-ABL1* breakpoint characterization. The concentration of cDNA was measured using either *B2M*, *GUSB*, or *ABL1* as a housekeeping gene.[36,37]

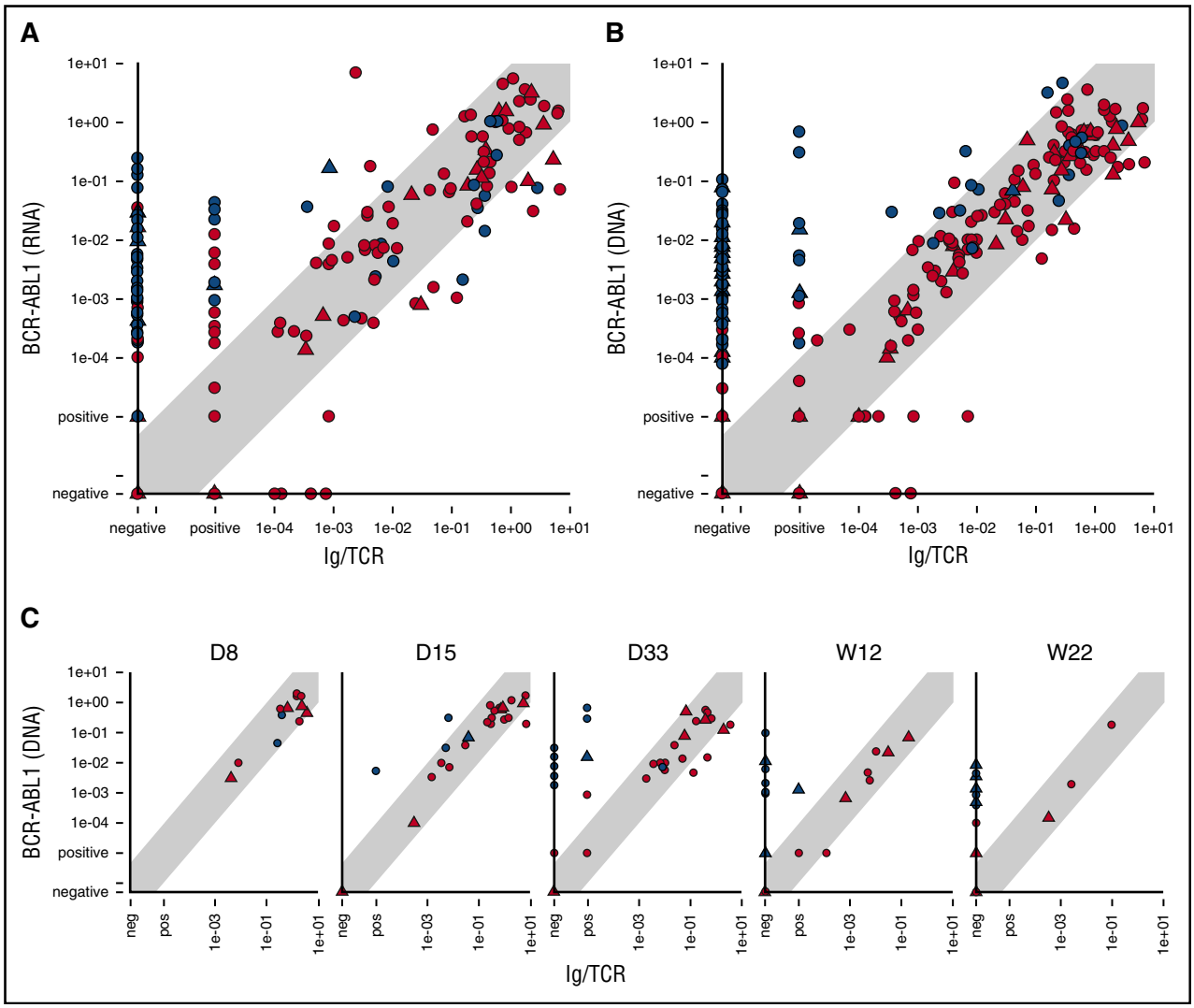

Figure 1. Comparison of the MRD levels in ALL patients. Comparison of the MRD levels in ALL patients measured by Ig/TCR vs *BCR-ABL1* transcript quantification (A) as well as vs *BCR-ABL1* genomic breakpoint quantification in all samples (B) and separately in selected time points during frontline treatment (C). Samples from patients with major-*BCR-ABL1* fusion variant are shown as triangles and minor-*BCR-ABL1* as circles. Samples from patients with concordant MRD course are in red, and samples from patients with discordant MRD are in blue. The light gray diagonal shape represents the area of concordance ±1 log. D, day; W, week (from the start of treatment).

Genomic *BCR-ABL1* breakpoint detection

Primers and their multiplexing in long-distance (LD) PCR for the *BCR-ABL1* genomic breakpoint detection were based on previously published data[27] with minor modifications and additional primers published elsewhere.[22,25] The complete list of primers is shown in supplemental Table 1 (available on the *Blood* Web site); for schematic representation of primer positions and PCR conditions, see supplemental Figure 1.

The specific product sequences were obtained by Sanger sequencing. In 7 patients with minor-*BCR-ABL1* fusion, the products were not successfully sequenced using the common Sanger approach. Here, we employed the GS Junior platform (454 next-generation sequencing technology; Roche Diagnostics, Rotkreuz, Switzerland)[38] to find the *BCR-ABL1* genomic breakpoint within the amplified PCR fragment. To annotate the sequencing results, BLAST,[39] ENSEMBL,[40] or University of California Santa Cruz BLAT[41] tools were used.

IKZF1-deletions screening and monitoring

Patients were screened for 4 intragenic *IKZF1* deletions (exons 2-7, 2-8, 4-7, and 4-8) using qPCR with KAPA Probe Fast mix (Kapa Biosystems, Wilmington, MA), as previously described,[42,43] and using additional primers and probe (supplemental Table 2). Patients with high *IKZF1* deletion levels at diagnosis were tested at later time points with the same assay and with their own standard curve to quantify MRD.

Ig/TCR and *BCR-ABL1* MRD quantification

In 49/51 patients, clonal Ig/TCR rearrangement was available for MRD monitoring. Quantification of patient-specific Ig/TCR rearrangements was performed and interpreted according to the standards of the EuroMRD international network.[44-49] MRD monitoring based on *BCR-ABL1* transcript quantification, including normalization to control gene expression (*B2M*, *GUSB*, or *ABL1*) and interpretation, was performed as described previously.[15,50]

For MRD quantification based on the *BCR-ABL1* genomic breakpoint, primers amplifying the fusion sequence were designed to produce the PCR product of 97 to 204 base pairs. First, the QuantiTect SYBR Green PCR (Qiagen) system was used. In 5 cases for which the optimization of SYBR Green system was not satisfactory, fluorescein/tetramethylrhodamine-labeled probe (preferentially covering the breakpoint sequence) was designed and TaqMan Universal Master Mix II (Thermo Fisher Scientific) was employed.

The MRD levels of all targets are measured relative to the reference diagnosis/relapse sample, which was set to 1 (100%). For statistical comparisons between MRD results, samples with low, nonquantifiable positivity (below

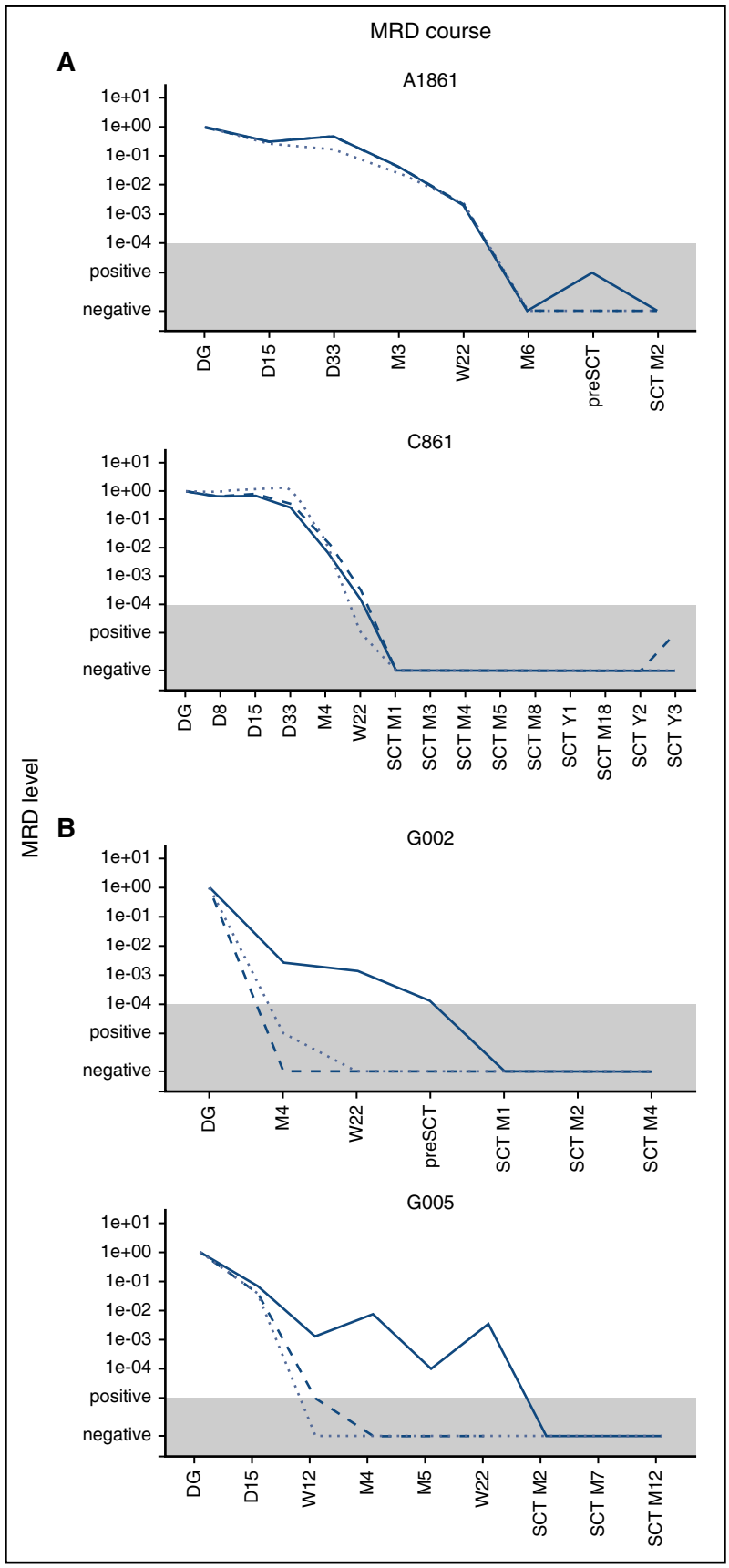

Figure 2. Examples of the MRD course. Examples of the MRD course measured by Ig/TCR (dashed line), *BCR-ABL1* genomic breakpoint (full line), and IKZF1 deletion (dotted line) quantification in 2 illustrative patients with concordant MRD (A) and 2 with discordant MRD (B). The gray area represents the level of sensitivity of the Ig/TCR quantification. M, month (from the start of treatment or from SCT).

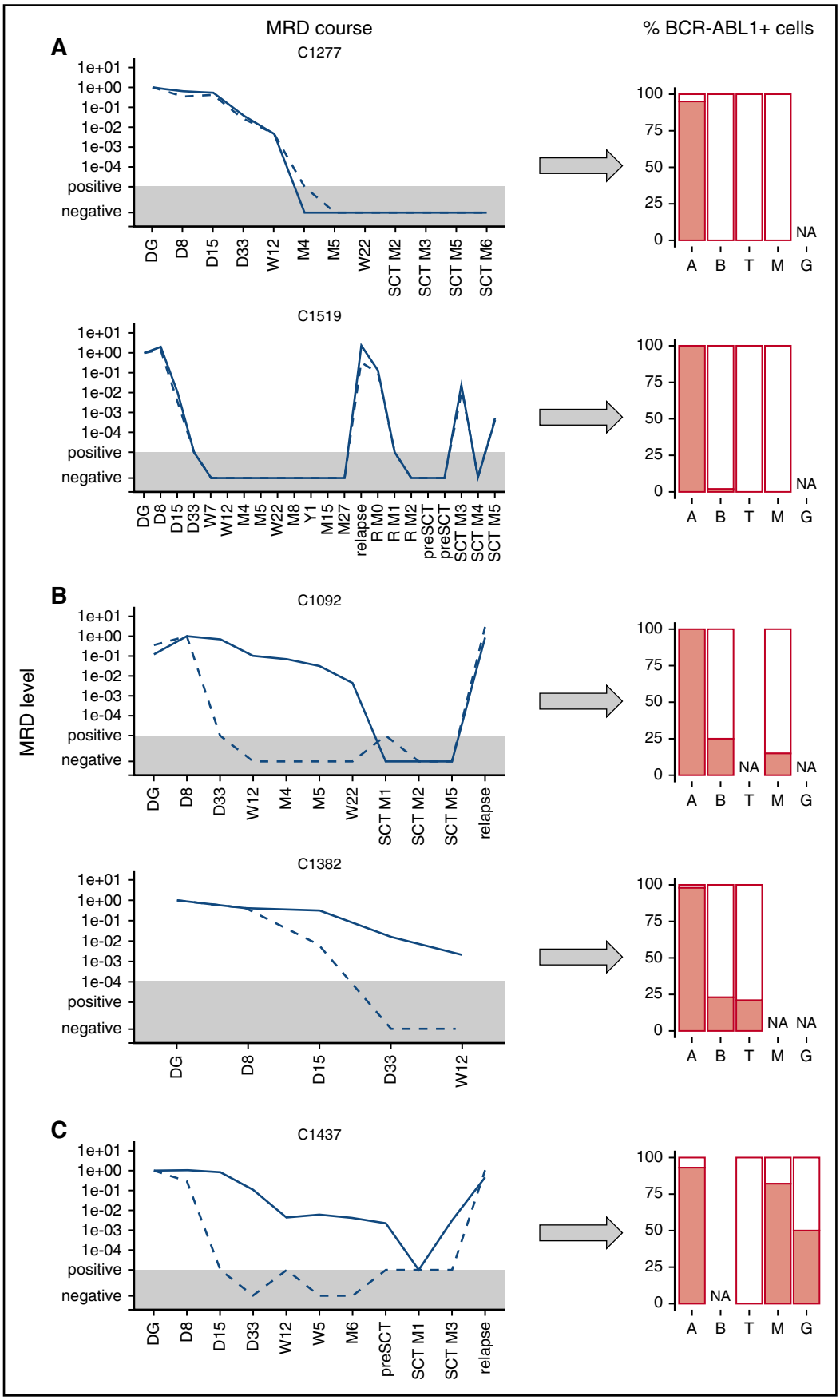
MRD course
% BCR-ABL1+ cells
A
C1277
1e+01
1e+00
1e-01
1e-02
1e-03
1e-04
positive
negative
DG D8 D15 D33 W12 M4 M5 W22 SCT M2 SCT M3 SCT M5 SCT M6
100
75
50
25
0
NA
A B T M G
C1519
DG D8 D15 D33 W7 W12 M4 M5 W22 M8 Y1 M15 M27 relapse R M0 R M1 R M2 preSCT preSCT SCT M3 SCT M4 SCT M5
B
C1092
MRD level
DG D8 D33 W12 M4 M5 W22 SCT M1 SCT M2 SCT M5 relapse
C1382
DG D8 D15 D33 W12
C
C1437
DG D8 D15 D33 W12 W5 M6 preSCT SCT M1 SCT M3 relapse

Figure 3.

quantitative range [QR]) were assigned an arbitrary level of 1×10^{-5}. Negative samples were assigned a level of 5×10^{-7}.

Definition of discordant MRD results

In this study, we sought to identify samples that gave clearly discordant MRD results using different tests. Normal variation is considered a <0.5 log difference. Consequently, when the variation between results was ≤1 log, the results were scored as concordant; discordant samples were defined as those for which the MRD levels differed by >1 log. Moreover, although this might artificially increase the number of concordant samples, with respect to QR and sensitivity, we considered 2 samples concordant if (1) 1 target was quantifiable at a level <1 log above QR/sensitivity of the other target and the other target was nonquantifiably positive/negative, respectively; or (2) 1 target was nonquantifiably positive, whereas the other target was negative, and sensitivity of the former was <1 log higher than the sensitivity of the latter.

Patients with more subsequent follow-up samples that were discordant for *BCR-ABL1* DNA vs Ig/TCR were considered as patients with discordant MRD.

Cell sorting and fluorescence in situ hybridization (FISH)

Frozen viable cells from bone marrow or peripheral blood samples were sorted on slides using a BD FACS Aria III (BD, Franklin Lakes, NJ). Following cell sorting into T cells ($CD3^+$), myeloid cells ($CD33^{+/dim}$), malignant B-cell precursors ($CD19^+CD10^+CD45^{dim}$), and nonmalignant B cells ($CD19^+CD10^-CD45^{++}$) (all supplemented in some cases with additional markers to minimize possible contamination of non-ALL subpopulations by leukemic blasts), FISH analysis was performed. The cells were fixed on slides using modified blastomere HCl/Tween20 fixation.[51] For *BCR-ABL1* chromosomal fusion visualization BCR-ABL Translocation, the Dual Fusion LPH 007 probe (Aquarius Haematology Probes, Cytocell, Cambridge, United Kingdom) or Vysis LSI BCR/ABL Dual Color Dual Fusion Translocation Probe (Abbott Molecular, Des Plaines, IL) was used. In selected cases, qPCR targeted to patient-specific genomic *BCR-ABL1* fusion and/or Ig/TCR rearrangements was performed using DNA isolated from the sorted subpopulations.

Twelve of the ALL patients with analyzed cell subpopulations represent unselected consecutive cases that were diagnosed/relapsed in the Czech Republic between June 2007 and December 2014 (only 1 patient from this period was not included because of a lack of material for cell sorting).

Statistical analysis

The correlation of individual methods was analyzed using the Spearman correlation rank. The double-negative samples were excluded from the analysis. For comparisons of groups, the Mann-Whitney *U* test and Kruskal-Wallis test were used. Initial WBC was compared by 2-way ANOVA to compensate for the effect of the *BCR-ABL1* variant (minor vs major).

Results

Breakpoint detection

We examined leukemic DNA for 67 childhood *BCR-ABL1*-positive ALL/CML cases to find the genomic *BCR-ABL1* breakpoint. We characterized the fusion sequence in 54 cases (81%), 16 with major and 38 with minor breakpoint variants. In 13 cases (4 major and 9 minor), we did not find the genomic breakpoint sequence; in 7 of these cases, the DNA quality was poor or suboptimal (control LD PCR amplified only products of <2 kb [3 cases] or <7 kb [4 cases]). In 1 patient, the DNA quality and integrity were not tested using control LD PCR because of a low amount of material for further analyses. In 3 cases, the LD PCR yielded a positive product; however, the sequencing (both Sanger and next-generation sequencing) was unsuccessful. In the remaining 2 patients, we did not obtain a specific LD PCR product despite the adequate DNA quality. The fusion gene sequences of the 54 successfully analyzed cases are listed in supplemental Table 3.

MRD samples and sensitivity

We analyzed MRD in 548 bone marrow samples from 48 ALL patients (minor-*BCR-ABL1*-positive ALL, 433 samples/36 patients; major-*BCR-ABL1*-positive ALL, 115 samples/12 patients) and 3 CML patients (34 samples) by quantifying the *BCR-ABL1* genomic breakpoint and at least one of the other targets (Ig/TCR, 560 samples; *IKZF1*del, 194 samples; and *BCR-ABL1* transcript, 410 samples). Moreover, in 143 samples, we assessed the MRD levels in the peripheral blood (supplemental Figure 2).

The sensitivity of the DNA approaches was generally comparable, with no statistically significant differences between Ig/TCR, *BCR-ABL1*, and *IKZF1* tests with mean sensitivities of 4.4 vs 4.5 vs 4.3 logs and median values of 4 vs 5 vs 4 logs, respectively. On the other hand, the QR (ie, the range in which MRD can be accurately quantified) was significantly lower for Ig/TCR compared with *BCR-ABL1* ($P = .0021$, mean 3.7 logs/median 4 logs vs mean 4.1 logs/median 4 logs). The QR for *IKZF1* assays was intermediate and not significantly different from either *BCR-ABL1* or Ig/TCR tests. The quantitative reverse transcription PCR for *BCR-ABL1* transcript detection reliably detected ≤10 copies of cDNA. As 1 standard quantitative reverse transcription PCR consisted of cDNA from ~100 000 cells, the sensitivity of this method is generally comparable to the DNA-based assays (4-5 logs).

Comparison of MRD by different targets

Comparison of *BCR-ABL1* RNA with Ig/TCR MRD levels confirmed our previous data[15] that showed a poor correlation (Spearman correlation coefficient, 0.63) and significant number of samples (23%) with quantifiable *BCR-ABL1* levels, while Ig/TCR MRD negative (Figure 1A).

The results of the genomic *BCR-ABL1* quantification also showed differences with the Ig/TCR levels (Figure 1B). Despite using the same DNA-based methodology, the Spearman correlation was low (0.62) for Ig/TCR vs *BCR-ABL1* DNA overall (with 0.61 for minor- and 0.65 for major-*BCR-ABL1*) and consistent with Ig/TCR vs *BCR-ABL1* RNA correlation. In contrast, the correlation coefficient was significantly better (0.85) between the *BCR-ABL1* DNA and RNA MRD levels. The comparison of 2 DNA targets also confirmed our observation that although the MRD results assessed by the different techniques correlated well in the majority of patients ("patients with concordant MRD"), there were some "patients with discordant MRD" and several consecutive samples with significantly higher (>1 log) *BCR-ABL1* levels compared with Ig/TCR. There was no significant difference in the frequency of patients with discordant MRD among minor- vs major-*BCR-ABL1* patients (10/34 vs 5/12 patients; $P = .49$).

In patients with concordant MRD, only rare individual samples (12/386 all samples, 3% or 12/255 non–double-negative samples,

Figure 3. Presence of BCR-ABL1-positive cells in hematopoietic lineages. Presence of *BCR-ABL1*-positive cells in hematopoietic lineages (A, ALL blasts; B, non-ALL B cells; G, granulocytes; M, myeloid cells/monocytes; T, T cells) at diagnosis in ALL patients with concordant (A) and discordant (B) MRD courses and in CML patient (C). The MRD levels are shown for Ig/TCR (dashed line) and *BCR-ABL1* genomic breakpoint (full line); the gray area represents the level of sensitivity of the Ig/TCR quantification. NA, not available.

4.7%) did not fit into the 1 log difference interval, and all divergences were only slightly higher, well within 2 logs. By contrast, in patients with discordant MRD, 50% of all and 60% of the non–double-negative samples (71/141 or 71/120, respectively) had *BCR-ABL1* levels higher than Ig/TCR by >1 log and 50% of those (n = 35) by >2 logs.

In patients with discordant MRD, the proportion of samples with significantly higher *BCR-ABL1* compared with Ig/TCR levels increased with the treatment time point from 0% (0/3 patients analyzed after 1 week of treatment) to 50% after 2 weeks (2/4 patients analyzed) and 80% to 90% after 1, 3, and 5 months of treatment (8/9, 8/10, and 8/10 patients, respectively) (Figure 1C).

We used *IKZF1* deletions as an alternative target for MRD monitoring. In patients with concordant MRD, the data from *IKZF1* quantification correlated well with the *BCR-ABL1* and Ig/TCR targets. In patients with discordant MRD, the levels of *IKZF1* deletion mimicked the Ig/TCR levels, which were significantly lower than *BCR-ABL1* MRD. The overall correlation is shown in supplemental Figure 2, and examples of the MRD course are shown in Figure 2.

BCR-ABL1 presence in hematopoietic lineages

To investigate the biological basis of discordant MRD, we analyzed hematopoietic cell subpopulations in selected patients and searched for the presence of *BCR-ABL1* fusion in distinct cell types. We analyzed sorted ALL B-cell precursors, non-ALL B cells, T cells, and myeloid cells from the diagnostic samples as well as performed FISH with *BCR-ABL1* probe in 12 patients (10 ALL with *BCR-ABL1* fusion and 2 CML) and qPCR BCR-ABL1 and Ig/TCR detection in 1 additional CML and 3 *BCR-ABL1*-positive ALL cases (1 was previously published[15]). In 2 ALL patients, one of the target sequences for MRD monitoring was not successfully identified; therefore, in this analysis, we used *BCR-ABL1* transcript quantification instead of the genomic *BCR-ABL1* fusion and flow-cytometric MRD assessment instead of Ig/TCR quantification, respectively, to assess the MRD concordance in these 2 patients.

In 7 patients with concordant MRD, the *BCR-ABL1* fusion was only found in the ALL B-cell precursors (93% to 100%), whereas non-ALL B cells, T cells, and myeloid cells were negative (<2%); 2 cases are shown in Figure 3A. Two patients with concordant MRD were diagnosed as ALL with significant aberrant expression of CD33 (40% of ALL blasts at diagnosis and 100% at subsequent relapse in 1 patient and bilineal B/Myelo-leukemia in the other). In addition to the ALL blasts, these 2 patients also had *BCR-ABL1*-positive cells in the $CD33^{pos}$-sorted myeloid subpopulation. Consequently, qPCR revealed that the sorted CD33-positive cells harbored both *BCR-ABL1* and Ig/TCR rearrangements, whereas non-ALL B cells and T lymphocytes were *BCR-ABL1*-negative.

In 4 patients with discordant MRD results, we detected *BCR-ABL1* fusion not only in the malignant B-cell precursors but also in non-ALL B cells (15% to 83%), T cells (12% to 21%), and myeloid cells (15% to 80%). Importantly, the populations other than ALL B cells were also tested by qPCR and all were positive for the *BCR-ABL1* genomic fusion, whereas they were low/negative ($<5 \times 10^{-2}$, representing maximal expected level of possible sort contamination by ALL blasts) for the patient-specific Ig/TCR rearrangements (5 populations tested).

In the control analysis of sorted populations from 3 patients with typical CML (1 in LBC, 2 in the chronic phase) tested for *BCR-ABL1* fusion, we found that the proportions of *BCR-ABL1*-positive cells were 93% and 100% of positive lymphoid blasts; 82%, 100%, and 34% of monocytes; 50%, 100%, and 34% of granulocytes; and 0%, 0%, and 0% of T cells, respectively, in the 3 CML cases (1 patient shown in Figure 3C).

Initial WBC count and diagnostic *BCR-ABL1* expression

A very high WBC count (usually $>100 \times 10^9$/L) is characteristic of CML LBC. Indeed, the 3 patients with CML LBC had higher WBC levels compared with ALL patients ($P = .015$), and ALL patients with the major-*BCR-ABL1* variant had a higher initial WBC count compared with patients with minor-*BCR-ABL1* ($P = .002$). With respect to the Ig/TCR vs *BCR-ABL1* correlation, the ALL patients with discordant MRD had a lower initial WBC count compared with patients with concordant MRD ($P = .058$).

The expression of the *BCR-ABL1* fusion transcript at diagnosis varied significantly between the patients. When normalized to the expression of a housekeeping gene (*B2M* or *GUSB*), the diagnostic levels differed by >2 logs for the major-*BCR-ABL1* variant and by >3 logs for the minor-*BCR-ABL1* variant. In all patients with major-*BCR-ABL1*, the minor-*BCR-ABL1* transcript was also detected at levels that were ~3 logs lower compared with the dominant major transcript. Patients with concordant MRD tended to have higher *BCR-ABL1* expression compared with patients with discordant results ($P = .098$).

Effect of discordant vs concordant MRD on outcomes

There was no significant difference in the outcome between the patients with concordant and discordant MRD in our cohort in which 80% of patients underwent SCT and all but 3 children received TKI therapy (albeit another 4 only after relapse). For patients with discordant MRD, 10 out of the 12 transplanted patients are alive with a median follow-up 10 years, whereas only 1 out of the 3 nontransplanted patients is alive (40 months from diagnosis; log-rank test for overall survival, $P = .019$). The outcome of the patients with concordant MRD who were transplanted did not differ significantly from those who received chemotherapy alone (10/25 and 1/6 died, respectively; log-rank test for overall survival, $P = .48$). For details on the treatment and outcome, see Table 1.

Discussion

Comparison of the MRD levels measured by quantification of DNA vs RNA can always be challenged; although the number of target DNA copies per cell is usually constant at 1 or 2 per genome, the expression levels of both the target and housekeeping gene used for normalization can vary significantly (eg, in different patients, among different cell types, and during treatment). Therefore, we aimed to investigate our previous data for *BCR-ABL1*-positive childhood ALL, which showed a poor correlation of 2 routine MRD approaches in some patients (DNA-based Ig/TCR quantification and RNA-based *BCR-ABL1* transcript quantification) by complementing it with MRD analysis using *BCR-ABL1* fusion at the DNA level.

We found the genomic breakpoint in most patients (>80%) with no prior selection in terms of the DNA quality. In the remaining cases, poor DNA quality for the LD PCR was usually the limiting factor; moreover, in rare cases, the fusion might be more complex at the genomic level, precluding successful analysis with this approach. In particular, we detected cases in which an inverted part of the ABL1 gene was inserted into the fusion and deduced that if the size of such insertion/inversion was longer (approximately >10 kb), we would probably be unsuccessful using our LD-PCR approach. Moreover, the affected introns harbor many repetitive sequences that hamper routine sequencing. We wanted to establish if our approach could be used for routine MRD monitoring; therefore, we did not perform any additional experiments

beyond standard testing to find the breakpoint in all samples subjected to analysis.

Our results confirmed and further extended our previous data from the analysis of *BCR-ABL1* transcript quantification; although in the majority of ALL patients the MRD levels correlated very well (within 1 log), other patients had several consecutive samples showing significantly higher *BCR-ABL1* levels compared with Ig/TCR. Our present study was artificially enriched for such samples, as we selected some cases based on already known discordance between the Ig/TCR and *BCR-ABL1* transcript levels. In the unselected consecutive cohorts that were analyzed within this study, the incidence of patients with discordant MRD was 22.5% (9/40 patients; 8/32 [25%] with the minor- and 1/8 [12.5%] with major-*BCR-ABL1* variant). These data also show that the poor correlation is not limited to the cases with major-*BCR-ABL1* fusion; in contrast, we detected more discordant cases among minor-*BCR-ABL1*-positive patients. Only ALL blasts were *BCR-ABL1*-positive in patients with concordant MRD, although 2 patients diagnosed with ALL with myeloid markers had *BCR-ABL1* positivity in the myeloid fraction. In all patients with discordant MRD in whom the sorted cell populations were analyzed (as well as in our control CML cases), we established that the source of the poor correlation was the presence of *BCR-ABL1* in cells that were not derived from ALL lymphoid blast clones.

These experiments provide evidence that a multipotent hematopoietic progenitor is affected by *BCR-ABL1* fusion in some cases and that the ALL patients with discordant MRD have a "CML-like" disease background. The absence of clonal Ig/TCR rearrangements in the *BCR-ABL1*-positive cells detected in sorted cell subpopulations other than ALL blasts and during the follow-up of patients with discordant MRD also rules out the possibility that these cells originate by dedifferentiation or transdifferentiation of the original ALL cell. *IKZF1* gene deletions, considered to be a subsequent hit cooperating with *BCR-ABL1* in the ALL pathogenesis[52] and known to emerge in LBC after being originally negative during the chronic CML phase,[53,54] correlated in ALL patients with the lymphoid Ig/TCR clone and were absent in Ig/TCR-negative *BCR-ABL1*-positive cells detected during treatment, which further supports the CML-like pathogenesis with *IKZF1* deletion acquired in progression to ALL. Moreover, the patients with discordant MRD, both ALL and CML patients, tend to have lower fusion transcript expression at diagnosis compared with patients with "typical" *BCR-ABL1*-positive ALL.

In contrast, the initial WBC level tended to be lower in the ALL cases with discordant MRD than both the concordant ALL patients and typical CML cases. Although we found *BCR-ABL1* in other cell types, including the myeloid lineage, in patients with discordant MRD, the proportion of positive cells was lower than in the classical CML-LBC patients. Moreover, unlike the CML patients, ALL cases with discordant MRD also harbored *BCR-ABL1* fusion in T cells, whereas putative stem cells ($CD34^+$ $CD38^-CD133^+$) were not conclusively *BCR-ABL1* positive (supplemental Figure 3). These data suggest that ALL cases with discordant MRD differ from both "typical ALL" and classical CML. In our experience, the multilineage involvement of the primary leukemogenic aberration is limited to *BCR-ABL1*-positive ALL and rare MLL-rearranged leukemia.[55] Bilineal leukemia cases have identical genetic aberrations, including the same Ig/TCR rearrangements present in both lymphoid and myeloid compartments[56]; therefore, they are similar to our 2 *BCR-ABL1*-positive ALL cases with myeloid markers and Ig/TCR detected in $CD33^{pos}$-sorted subpopulation, which we interpret as being the result of cell plasticity[57] instead of a "CML-like" pathogenesis.

The impact of the specific disease biology in patients with discordant MRD on the prognosis and optimal treatment needs to be systematically analyzed in contemporary protocols to draw definitive conclusions. Patients in our study were treated with several trials over an extended period. However, based on small numbers, our data suggest that patients with discordant MRD benefited from SCT in contrast to patients with concordant MRD who did well on chemotherapy alone. Considering that early and prolonged use of TKI is now often preferred to SCT in *BCR-ABL1*-positive ALL, whereas SCT is still the treatment of choice for advanced phases of childhood CML, this perspective should be considered carefully in future protocols for children diagnosed as *BCR-ABL1*-positive ALL, and the patients with discordant MRD should be identified.

In conclusion, *BCR-ABL1* monitoring at the genomic DNA level is a feasible approach that provides the most accurate and sensitive quantification of *BCR-ABL1*-positive cells during leukemia treatment. Moreover, used in combination with standard Ig/TCR monitoring, it can unmask cases that have discordant results associated with different disease biology, originating from a multipotent hematopoietic progenitor. A systematic, protocol-based study is needed to precisely define the prognosis and optimal treatment of MRD-discordant cases and the role of early SCT vs prolonged TKI therapy in these patients.

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Authorship

Contribution: L.H., M.Z., N.C.V., K.B., M.T., E.P., M.V., J.L., K.M.P., E.F., W.M., and J.E.G. performed research and analyzed and interpreted data; P.JS., G.C., R.S., J.S., and J.T. collected and interpreted data; J.Z. designed the research; L.H. and J.Z. wrote the manuscript; and all authors revised the manuscript and approved the final version.

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Monitoring of childhood ALL using *BCR-ABL1* genomic breakpoints identifies a subgroup with CML-like biology

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