Brief Report

Hematopoietic stem cell involvement in *BCR-ABL1*-positive ALL as potential mechanism of resistance to blinatumomab therapy

Short title: Mechanism of blinatumomab resistance in ALL

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Key Points

-   *BCR-ABL1*-positive cells outside the B-lineage compartment are found in around 40% of *BCR-ABL1*-positive adult BCP-ALL
-   Selection of preexistent CD19-negative subclones is a potential source of tumor escape after CD19-targeted therapies in adult *BCR-ABL1*-positive BCP-ALL

Abstract

The bispecific T-cell engager blinatumomab targeting CD19 can induce complete remission in relapsed or refractory B-cell precursor acute lymphoblastic leukemia (BCP-ALL). However, some patients ultimately relapse with loss of CD19-antigen on leukemic cells which has been established as a novel escape mechanism to CD19-specific immunotherapies. Here, we provide evidence that CD19-negative relapse after CD19-directed therapy in BCP-ALL may be due to selection of preexisting CD19-negative malignant progenitor cells. We present two *BCR-ABL1*-fusion-positive BCP-ALL patients with CD19-negative myeloid lineage relapse after blinatumomab therapy and show *BCR-ABL1*-positivity in their hematopoietic stem cell (HSC)/progenitor/myeloid compartments at initial diagnosis by fluorescence in situ hybridization after cell sorting. Using the same approach in 25 additional diagnostic samples of patients with *BCR-ABL1*-positive BCP-ALL, HSC involvement was identified in 40% of the patients. Patients with major-*BCR-ABL1* transcript encoding P210[BCR-ABL1] mainly showed HSC involvement (6/8), whereas in most of the patients with minor-*BCR-ABL1* transcript encoding P190[BCR-ABL1] only the CD19-positive leukemia compartments were *BCR-ABL1*-positive (9/12) (p=0.02). Our data are of clinical importance, because they indicate that not only CD19-positive cells, but also CD19 negative precursors should be targeted to avoid CD19-negative relapses in patients with *BCR-ABL1*-positive ALL.

Introduction

B-cell directed therapies have shown promising results in the treatment of chemotherapy resistant or relapsed B-cell-precursor acute lymphoblastic leukemia (BCP-ALL). Blinatumomab, a bispecific T-cell engager (BITE®) antibody that links CD3-positive T-cells to CD19-positive B-cells and thereby triggers serial lysis of B-cells, results in up to 69% complete response rate in relapsed/refractory BCP-ALL.[1-4] Similarly, CD19-specific chimeric antigen receptor modified T-cell (CART-19) therapy induces complete response in up to 90% of patients with BCP-ALL.[5,6] However, a considerable proportion of patients relapse after an initial molecular response while others remain in a lasting remission after CD19-directed treatment. For both therapy settings CD19-negative relapses have been described.[1,5,7-9] Epitope loss due to mutations, alternative splicing or disrupted CD19 membrane trafficking pretending a CD19-negativity may contribute to resistance against CART-19 therapy.[9,10] Furthermore, a few patients with CD19-negative relapse have been described which appear as acute myeloid leukemia (AML) indicating a previous lineage switch and a real CD19-negativity.[8,11] The mechanism behind, such as the cell of origin or clonal evolution in those patients, has not been described until now. By applying fluorescence in situ hybridization (FISH) after fluorescence activated cell sorting (FACS) in two *BCR-ABL1*-positive BCP-ALL with relapse after blinatumomab and in 25 additional diagnostic samples of *BCR-ABL1*-positive BCP-ALL patients, we here provide evidence that CD19-negative, myeloid lineage relapses in adult *BCR-ABL1*-positive BCP-ALL occur in association with hematopoietic stem cell (HSC) involvement.

Study Design

Cryoconserved bone marrow and peripheral blood samples from adult *BCR-ABL1*-positive BCP-ALL patients treated within the GMALL elderly, 07/2003, MT103-206 and Alcantara trials (trials NCT00198978, NCT00198991, NCT01209286, and NCT02000427) were FACS sorted onto slides and analyzed by FISH for *BCR-ABL1*-fusion (LSI BCR/ABL Dual Color, Dual Fusion Translocation probe, Abbott, Illinois, USA; cut-off was set up to 3%) (Patient characteristics: supplemental Table 1). In patient 21 a centromere 7 probe (CEP7, Abbott; cut-off was defined as 3%) was added to the *BCR-ABL1*-fusion probe. The following populations were investigated based on the approach described by Castor et al.[12]: CD34$^+$38$^-$19$^-$3$^-$ (HSC and multipotential progenitor cells (MPP)), CD34$^+$38$^+$19$^-$3$^-$ (myeloid and lymphoid progenitors), CD34$^+$19$^+$20$^-$3$^-$ (leukemia cells without CD20 coexpression; LAIP 20$^-$), CD34$^+$19$^+$20$^+$3$^-$ (leukemia cells with CD20 coexpression; LAIP 20$^+$), CD34$^-$19$^+$20$^+$ (mature B-cells), CD34$^-$19$^-$20$^-$3$^+$ (mature T-cells), CD34$^+$19$^-$13/33$^+$10$^-$16$^-$ (early myeloid compartment), CD34$^-$19$^-$13/33$^+$10$^-$16$^-$ (late myeloid compartment), CD34$^-$19$^-$13/33$^+$10$^-$16$^+$ (mature myeloid compartment) (supplemental Figure 1; supplemental Table 2-4, and supplemental methods). Cell sorting, FISH, array comparative genomic hybridization (array CGH), molecular analysis of immunoglobulin (IG) and T-cell receptor (TR) gene rearrangements and *BCR-ABL1* real-time quantitative PCR were performed as described in Supplemental Methods. The clinical study was approved by the local ethics committee (D448/14). Informed consent was obtained in accordance with the Declaration of Helsinki.

Results and Discussion

Patient 21 presented with a relapse of a *BCR-ABL1*-positive BCP-ALL after standard induction/consolidation treatment. She received blinatumomab monotherapy and achieved rapid complete molecular response. However, within the third application cycle of blinatumomab *BCR-ABL1*-positive blasts reoccurred exhibiting a CD19-negative myeloid phenotype (Figure 1A). Molecular analysis did not show clonal IG/TR gene rearrangements in the relapse bulk suggesting that leukemic relapse derived from cells of a CD19-negative progenitor compartment (Figure 1B). Array CGH revealed a monosomy 7 at initial diagnosis but not at relapse (supplemental Figure 2). To elucidate the clonal evolution of malignancy, at initial diagnosis bone marrow cells were FACS sorted for distinct cellular compartments and subsequently analyzed by FISH with a three-color *BCR-ABL1*/CEP7 probe (Figure 1C). Indeed, *BCR-ABL1*-fusion/monosomy 7 positive cells were not only found in the CD19-positive leukemia compartments (CD34$^-$19$^+$20$^-$ and CD34$^+$19$^+$20$^-$) but also within the HSC and progenitor (CD34$^+$38$^-$19$^-$ and CD34$^+$38$^+$19$^-$) as well as within the myeloid compartments (CD34$^+$19$^-$13/33$^+$10$^-$16$^-$, CD34$^-$19$^-$13/33$^+$10$^-$16$^-$ and CD34$^-$19$^-$13/33$^+$10$^-$16$^+$). *BCR-ABL1*-fusion-positive cells without monosomy 7 were also present at initial diagnosis within the myeloid, but not within the CD19-positive leukemia compartments. In the HSC/progenitor cells, they remained with 4% and 3% respectively in the range of FISH- and sorting purity-cut-off. The same analysis was performed in a second *BCR-ABL1*-positive BCP-ALL patient (patient 29) who suffered a CD19-negative myeloid relapse after blinatumomab therapy. Also in this patient the progenitor and subsequent myeloid compartments showed *BCR-ABL1*-positivity at initial diagnosis.

To investigate the clonal architecture of *BCR-ABL1* ALL systematically, pretherapeutic samples from additional 25 patients with *BCR-ABL1*-positive BCP-ALL were analyzed accordingly (Figure 2). Ten of these patients (40%) also revealed HSC involvement. Nine of them showed the same pattern of *BCR-ABL1*-positivity (HSC/progenitor, myeloid and leukemia involvement) as patients 21 and 29 which we now termed "MPP-pattern". In 13/25 patients (52%), a second predominant pattern, referred to as "B-lineage-pattern", was identified, in which *BCR-ABL1*-positivity was restricted to the B-lineage-determined CD34$^+$19$^+$ cells. In 23/25 evaluable patients, independent of the pattern of *BCR-ABL1*-positivity, the mature B-cell compartment was *BCR-ABL1*-negative indicating a lymphatic maturation stop of *BCR-ABL1*-positive cells. Patients with major-*BCR-ABL1* transcript mainly showed the MPP-pattern (6/8) and much less frequently the B-lineage-pattern (1/8). In contrast, patients with minor-*BCR-ABL1* transcript predominantly exhibited the B-lineage-pattern (9/12) and much less frequently the MPP-pattern (3/12) (p=0.02; Fisher exact test).

Both patients with *BCR-ABL1*-positive BCP-ALL developing a CD19-negative myeloid lineage relapse after blinatumomab therapy, showed *BCR-ABL1*-positive cells in the HSC/progenitor and myeloid compartments at initial diagnosis. These results as well as the fact that the IG genes of the CD19-negative relapses showed germline configuration and not the clonal rearrangements present at initial diagnosis suggest that the CD19-negative relapses in these patients evolved from CD19-negative *BCR-ABL1*-positive progenitor cells. It also indicates that the CD19-negative myeloid lineage relapses are not

a consequence of dedifferentiation or reprogramming. Jacoby et al.[13] did not identify myeloid leukemic clones prior to therapy when they induced CD19-negative myeloid lineage relapses in a murine BCP-ALL model by long-term CART-19 exposure. However, this does not disprove the presence of uncommitted leukemic progenitors at low frequencies.[13]

Recently, Gardner et al. described two patients of mixed lineage leukemia (*MLL*) gene rearranged BCP-ALL which relapsed after CART-19 therapy presenting a myeloid, CD19-negative phenotype. One of the patients, an infant *MLL*-positive BCP-ALL, did not show a clonal IG rearrangement in the myeloid blasts analogous to our index patient whereas the other patient showed the same clonal IG rearrangement already existing at initial diagnosis obviously representing a different mechanism.[8]

Similar to two pediatric BCP-ALL cohorts we demonstrated multilineage involvement of the *BCR-ABL1*-positive clone.[12,14] In the study of Castor et al.[12] this finding was restricted to P210$^{BCR-ABL1}$ positive ALL whereas we also demonstrated clonal involvement of the HSC/MPP compartment in patients with to P190$^{BCR-ABL1}$ positive ALL. Thus, characterization of fusion transcripts does not allow a clear assignment to the cell of origin of the *BCR-ABL1*-fusion even though mainly patients with major-*BCR-ABL1* transcript showed MPP-pattern.

In the two index patients reported herein, blinatumomab was able to eliminate the aggressive B-determined clone but not the ancestral CD19-negative *BCR-ABL1*-positive precursor that gave rise to the CD19-negative relapse. It appears that the *BCR-ABL1*-fusion frequently represents an antecedent event in adult BCP-ALL leukemogenesis which may occur early in the hierarchy of hematopoiesis prior to B-lineage determination. More frequently than assumed BCR-ABL1 positive ALL resembles a chronic myeloid leukemia-like disease in lymphoid blast crisis.[14] Therefore, novel therapeutic strategies should target CD19-negative malignant precursor cells in addition to the B-cell leukemic bulk, especially in patients with MPP-pattern. As an example, Ruella and colleagues described a dual CD19- and CD123-CART approach that prevented antigen-loss relapses in xenograft models.[15,16] Our findings also favor a combination with tyrosine kinase inhibitors to target BCR-ABL1 positive cells without CD19 expression. Prospectively, it needs to be verified whether patients with an MPP-pattern of their *BCR-ABL1*-positive ALL show a higher frequency of CD19 escape after application of CD19-directed treatment compared to those with a B-lineage pattern.

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Authorship

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Figure Legends

Figure 1. Leukemic involvement and evolution of *BCR-ABL1*-positive blasts in patient 21 (A) Blast morphology and flow cytometric dot plots of pre-blinatumomab blasts (first row) and post-blinatumomab blasts (second row). Blasts at initial diagnosis were positive for CD19, CD10, cyCD22, cyCD79a, CD34, TdT, HLA-DR (not shown), cyIgM (not shown), showed aberrant expression of CD13, and were negative for CD33, CD117 and MPO (not shown). Expression profile did not fulfil WHO criteria for classification as mixed phenotype leukemia. At relapse after blinatumomab therapy, blasts were negative for CD19, CD10, cyCD79a, CD34, TdT, HLA-DR (not shown) and MPO (not shown), and expressed their myeloid antigens CD13, CD33 and CD117. **(B)** Hypothetical model of clonal evolution and selection of different subclones based on *BCR-ABL1* and immunoglobulin heavy chain (IGH) and T-cell receptor beta (TRB) gene rearrangement patterns. Leukemia was screened at initial diagnosis for clonal IG and TR gene rearrangements. Two clonal IGH gene rearrangements (VH3-23-DH2-2-JH6 and VH6-1-DH3-22-JH4) and one clonal cross-lineage TRB gene rearrangement (DB2-JB2.7) were detected, and clone specific real-time quantitative (RQ)-PCR assays were established based on sequence information. RQ-PCR and *BCR-ABL1* FISH showed dominance of the IGH rearranged (R)/R, TRB R/germline (G) and *BCR-ABL1* rearranged clone, whereas at first relapse IGH R/G TRB G/G clone was dominant, whereas the second IGH rearrangement was only detected at a level of 0.1%, the TRB only at a level below quantitative range of 0.1%. At second relapse the leukemic bulk did not show an IG/TR gene rearrangement but only the *BCR-ABL1* translocation, RQ-PCR revealed a subclonal IGH gene rearrangement (0.3%). A clonal evolution of the leukemic bulk with occurrence of a new dominant IGH/TRB gene rearrangement was excluded by IGH/TRB multiplex PCR, which has a sensitivity of about 1-5%. **(C)** Subclonal architecture of *BCR-ABL1*-fusion and monosomy 7 in immunophenotypic compartments of patient 21 at initial diagnosis analyzed by FISH after FACS sorting. Left: FISH results of each compartment. Orange circle, aberrant signal constellation; green circle normal signal constellation; *, in the range of the FISH- and sorting purity-cut-off. Right: Representative interphase nuclei showing the two different aberrant signal constellations in a false color display using MetaSystems software. The meaning of signals is as follows: isolated red, *ABL1*; isolated green, *BCR*; red green fusion signal. *BCR-ABL1*-fusion; blue, centromere 7.

B, mature B cells; CEP7, centromere 7 signal; F, *BCR-ABL1*-signal fusion; LAIP 20[-], leukemia cells without CD20 coexpression; LAIP 20[+], leukemia cells with CD20 coexpression; M1, early myeloid compartment; M2, late myeloid compartment; M3, mature myeloid compartment; MPP, multipotent progenitor cells; n.d., not determined ; neg., negative (not detected); Pro, myeloid and lymphoid progenitors; SCT, stem cell transplantation; T, mature T-cells.

Figure 2. Analysis of relevant immunophenotypic compartments in 27 adult patients with *BCR-ABL1*-positive BCP-ALL using FISH after FACS sorting. (A) *BCR-ABL1*-positivity of immunophenotypic compartments in the two predominant patterns of *BCR-ABL1*-occurence. The green

and orange color content of the boxes represents the ratios of *BCR-ABL1*-fusion positive and negative signal constellations observed in the 27 patients. For details of sorting strategies see supplement.

(B) Detailed results of the analysis for each patient and compartment. Green, *BCR-ABL1*-fusion negative; orange, *BCR-ABL1*-positive; grey, not analyzed. Overall, 11/27 patients showed an MPP pattern, 13/27 showed a B-lineage pattern, and in 3/27 patients pattern was in between (n. as., not assignable).

B, mature B cells; LAIP 20$^-$, leukemia cells without CD20 coexpression; LAIP 20$^+$, leukemia cells with CD20 coexpression; M, major-*BCR-ABL1* transcript; m, minor-*BCR-ABL1* transcript; mM, minor- and major-*BCR-ABL1* transcripts identified; M1, early myeloid compartment; M2, late myeloid compartment; M3, mature myeloid compartment; MPP, multipotential progenitor cells; n.a., not analyzable; n.as., not assignable; Pro, myeloid and lymphoid progenitors; T, mature T-cells
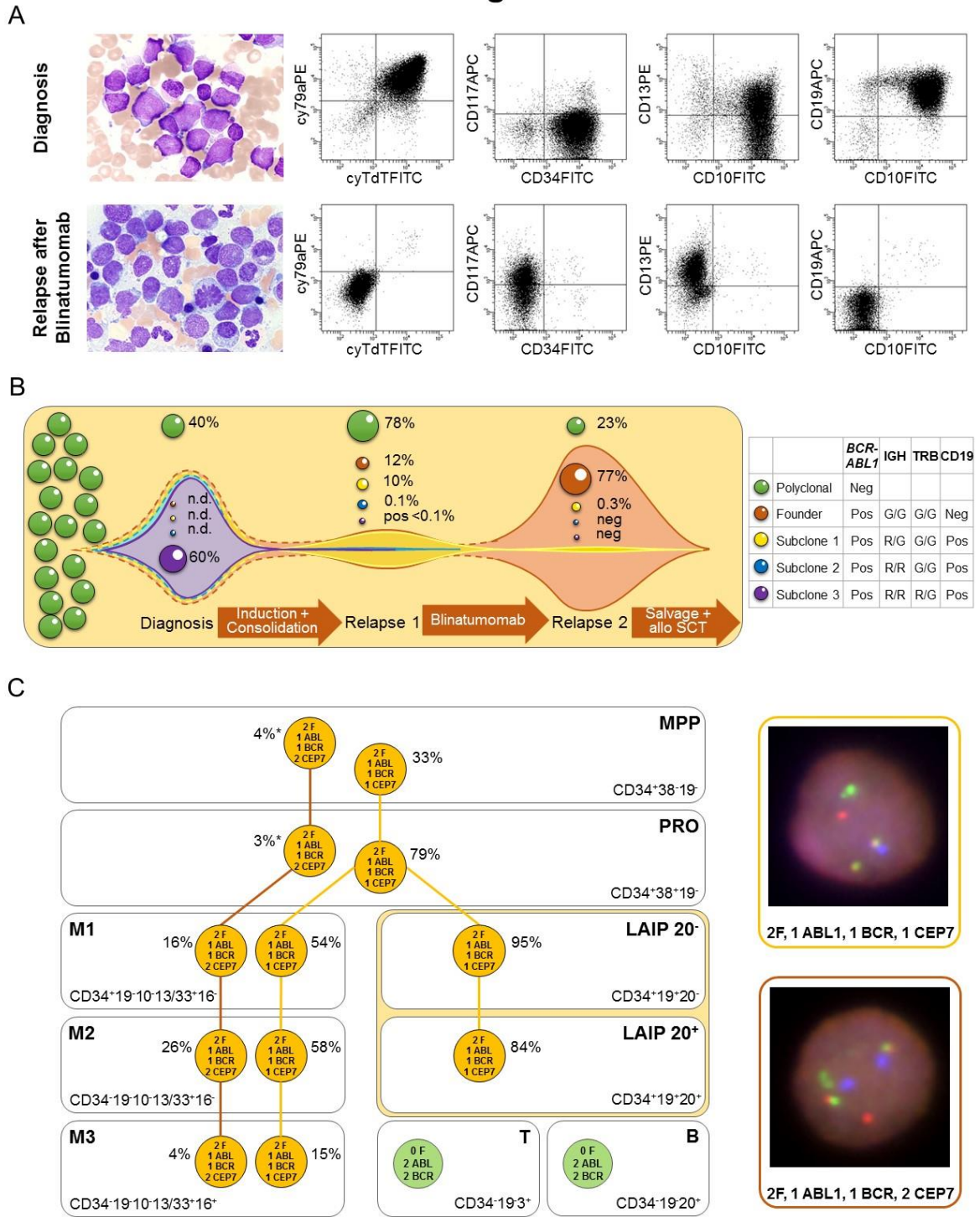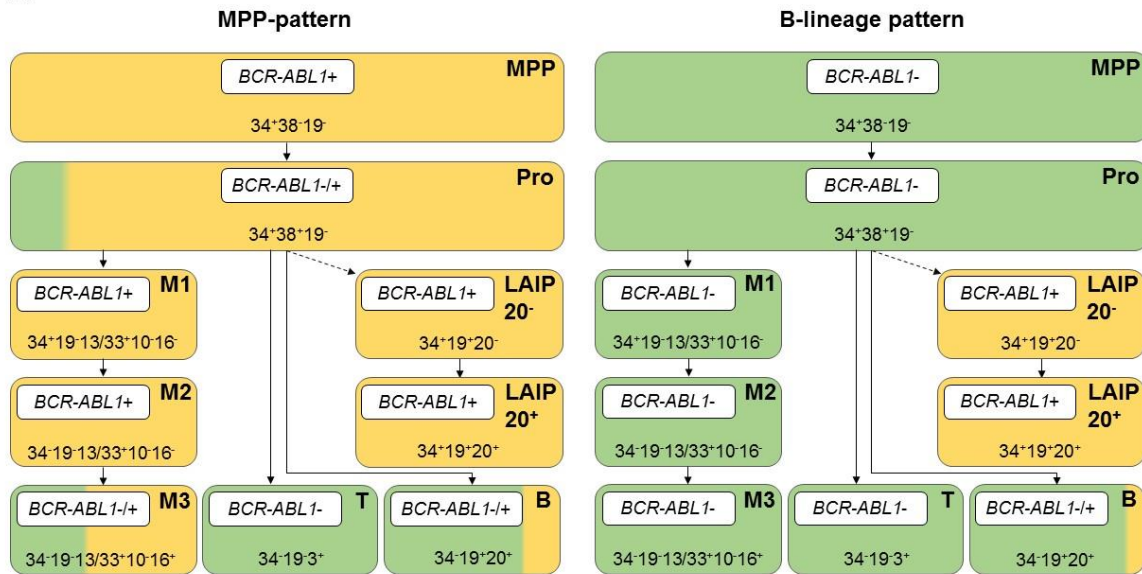
Figure 1

Figure 2

A

MPP-pattern

| BCR-ABL1+ | **MPP** |
| 34⁺38⁻19⁻ | |

$BCR\text{-}ABL1+$ — **MPP** — $34^{+}38^{-}19^{-}$

$BCR\text{-}ABL1\text{-}/+$ — **Pro** — $34^{+}38^{+}19^{-}$

$BCR\text{-}ABL1+$ — **M1** — $34^{+}19^{-}13/33^{+}10^{-}16^{-}$
$BCR\text{-}ABL1+$ — **LAIP 20⁻** — $34^{+}19^{+}20^{-}$

$BCR\text{-}ABL1+$ — **M2** — $34^{-}19^{-}13/33^{+}10^{-}16^{-}$
$BCR\text{-}ABL1+$ — **LAIP 20⁺** — $34^{+}19^{+}20^{+}$

$BCR\text{-}ABL1\text{-}/+$ — **M3** — $34^{-}19^{-}13/33^{+}10^{-}16^{+}$
$BCR\text{-}ABL1\text{-}$ — **T** — $34^{-}19^{-}3^{+}$
$BCR\text{-}ABL1\text{-}/+$ — **B** — $34^{-}19^{+}20^{+}$

B-lineage pattern

$BCR\text{-}ABL1\text{-}$ — **MPP** — $34^{+}38^{-}19^{-}$

$BCR\text{-}ABL1\text{-}$ — **Pro** — $34^{+}38^{+}19^{-}$

$BCR\text{-}ABL1\text{-}$ — **M1** — $34^{+}19^{-}13/33^{+}10^{-}16^{-}$
$BCR\text{-}ABL1+$ — **LAIP 20⁻** — $34^{+}19^{+}20^{-}$

$BCR\text{-}ABL1\text{-}$ — **M2** — $34^{-}19^{-}13/33^{+}10^{-}16^{-}$
$BCR\text{-}ABL1+$ — **LAIP 20⁺** — $34^{+}19^{+}20^{+}$

$BCR\text{-}ABL1\text{-}$ — **M3** — $34^{-}19^{-}13/33^{+}10^{-}16^{+}$
$BCR\text{-}ABL1\text{-}$ — **T** — $34^{-}19^{-}3^{+}$
$BCR\text{-}ABL1\text{-}/+$ — **B** — $34^{-}19^{+}20^{+}$

B

	LAIP 20⁺	LAIP 20⁻	B	T	MPP	Pro	M1	M2	M3	Pattern
Index patients (Patient 21 M-*BCR-ABL1*-positive; Patient 29 m-*BCR-ABL1*-positive)										
Patient 21	98/100	97/100	3/100	2/100	41/100	91/100	81/100	95/100	19/100	MPP
Patient 29	100/100	99/100	0/100	0/100	n.a.	10/100	9/100	6/100	0/100	MPP
M-*BCR-ABL1*-positive patients										
Patient 1	94/100	99/100	n.a.	2/100	89/100	98/100	95/100	96/100	n.a.	MPP
Patient 2	90/100	95/100	n.a.	2/100	63/100	92/100	96/100	98/100	n.a.	MPP
Patient 3	86/100	84/100	0/100	0/100	20/100	70/100	63/100	55/100	n.a.	MPP
Patient 4	91/100	98/100	0/100	0/100	1/100	0/100	0/100	0/12	0/100	B-lineage
Patient 8	99/100	90/100	0/100	0/100	42/100	90/100	88/100	69/100	0/100	MPP
Patient 11	89/100	81/100	0/100	0/100	55/100	0/100	0/100	0/18	0/68	i.d.
Patient 14	99/100	97/100	26/100	0/100	59/100	93/100	28/100	14/100	27/100	MPP
m-*BCR-ABL1*-positive patients										
Patient 6	92/100	97/100	1/100	0/100	0/100	1/100	1/100	2/100	n.a.	B-lineage
Patient 7	n.a.	92/100	0/100	0/100	0/100	0/100	0/100	0/19	n.a.	B-lineage
Patient 9	62/100	84/100	1/100	0/100	0/100	0/100	0/100	2/100	n.a.	B-lineage
Patient 10	94/100	93/100	2/100	0/100	0/100	0/100	0/100	0/45	0/100	B-lineage
Patient 12	n.a.	100/100	44/100	0/100	67/100	97/100	83/100	92/100	n.a.	MPP
Patient 13	91/100	95/100	n.a.	0/100	0/54	1/39	0/100	n.a.	n.a.	B-lineage
Patient 17	100/100	100/100	0/100	0/100	0/100	0/100	0/100	0/100	0/100	B-lineage
Patient 18	99/100	99/100	0/100	0/100	0/100	0/100	0/100	0/100	0/42	B-lineage
Patient 23	85/100	88/100	0/100	0/100	95/100	97/100	96/100	93/100	n.a.	MPP
Patient 24	97/100	92/100	0/100	0/100	2/67	1/100	0/100	n.a.	0/38	B-lineage
Patient 26	96/100	97/100	0/100	0/100	0/46	2/100	0/100	0/100	0/100	B-lineage
Mm-*BCR-ABL1*-positive patients										
Patient 5	82/100	94/100	2/100	0/100	80/100	2/100	29/66	78/100	n.a.	MPP
Patient 15	99/100	100/100	0/100	0/100	1/100	0/100	6/100	5/100	0/17	i.d.
Patient 19	95/100	97/100	0/100	0/100	0/100	0/100	0/100	0/100	n.a.	B-lineage
Patients with unknown type of *BCR-ABL1* fusion transcript										
Patient 16	94/100	95/100	0/100	0/100	1/100	4/100	3/88	n.a.	0/100	B-lineage
Patient 25	100/100	100/100	12/100	0/100	0/100	0/100	0/100	2/100	2/100	B-lineage
Patient 27	100/100	91/100	0/100	0/100	0/100	1/100	100/100	n.a.	67/100	i.d.
Patient 28	54/100	88/100	0/100	0/100	19/100	92/100	n.a.	91/100	62/69	MPP

Hematopoietic stem cell involvement in *BCR-ABL1*-positive ALL: a potential mechanism of resistance to blinatumomab therapy

I.   Supplemental Methods
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I.   Supplemental Methods

Flow cytometry and cell sorting

After thawing of cryopreserved mononuclear cells, each vial was suspended in PBS containing 2 % FBS and washed twice before antibody labeling. Using the fluorochrome-antibody conjugate combinations which are listed in the supplemental Table 2 the following populations were sorted for each patient: CD34$^+$38$^-$19$^-$3$^-$ (hematopoietic stem cells and multipotential progenitor cells (MPP)), CD34$^+$38$^+$19$^-$3$^-$ (myeloid and lymphoid progenitors, Pro), CD34$^+$19$^+$20$^-$3$^-$ (leukemia cells without CD20 coexpression; LAIP 20$^-$), CD34$^+$19$^+$20$^+$3$^-$ (leukemia cells with CD20 coexpression; LAIP 20$^+$), CD34$^-$19$^+$20$^+$ (mature B-cells), CD34$^-$19$^-$20$^-$3$^+$ (mature T-cells), CD34$^+$19$^-$13/33$^+$10$^-$16$^-$ (early myeloid compartment, M1), CD34$^-$19$^-$13/33$^+$10$^-$16$^-$ (late myeloid compartment, M2), CD34$^-$19$^-$13/33$^+$10$^-$16$^+$ (mature myeloid compartment, M3) (supplemental Figure 1). CD3 and CD16 were combined in a single fluorescence channel. Therefore, CD16 positivity in the M3 compartment was assumed for CD3/CD16 positive events due to concomitant CD13/CD33 positivity of the cells. Cells were sorted onto slides and fixed as previously described.[1] Reanalysis (supplemental Figure 1B) of all flow sorted subpopulations was performed whenever there was a sufficient number of mononuclear cells available after the initial thawing and sorting procedure. Consequently, the sort purity for each population was determined in three patients (supplemental Table 4).

Fluorescence in situ hybridization

For FISH analysis, the commercially available LSI BCR/ABL Dual Color, Dual Fusion Translocation probe and centromere 7 probe (CEP7), both obtained from Abbott (Illinois, USA), were used. Pre-treatment of cells in Carnoy's fixative, hybridizations, washings and evaluation was performed in accordance with previously described protocols. [2] The hybridization time was 48 hours. Slides were evaluated by two observers using Zeiss fluorescence microscopes equipped with appropriate filter sets and documented using the ISIS digital image analysis system version 5.0 (MetaSystems, Altlusheim). In patient of sufficient cells 100 nuclei per hybridization were evaluated and counted.

Array comparative genomic hybridization analysis

Array CGH on peripheral blood from initial diagnosis and relapse of patient 21 was performed using the SurePrint G3 Human CGH Microarray Kit, 4x180K (Agilent Technologies, Santa Clara, CA, USA). Experimental procedures were performed according to the manufacturer's instructions. One µg of DNA from each of both patient probes and, respectively, one µg of reference DNA from a pool of 10 healthy donors with a normal female karyotype were hybridized. The array was scanned with the G2565CA Microarray Scanner (Agilent Technologies) at a scan resolution of 5 µm/pixel. Signal intensities from the generated images were measured and evaluated with the Feature Extraction 10.10.11 and Agilent Genomic Standard Workbench Edition 6.5.0.58 (AGW6.5) software (Agilent Technologies) applying the Aberration Detection Method-2 (ADM-2) algorithm with a threshold of 6.0.

Array comparative genomic hybridization analysis

Multiplex ligation-dependent probe amplification (MLPA) was performed on peripheral blood from initial diagnosis and relapse of patient 21 to screen for IKZF1, PAX5, ETV6, RB1, BTG1, EBF1, CDKN2A, CDKN2B, SHOX-AREA, CRLF2, CSF2RA, IL3RA, P2RY8 and JAK2 copy number alterations according to the manufacturer´s instructions using the SALSA MLPA P335 ALL-IKZF1 kit (MRD-Holland).

Molecular IG /TR analysis

Molecular analysis of clonal immunoglobulin (IG) and T-cell receptor (TR) gene rearrangements was performed as described previously.(1) Clonal IG and TR gene rearrangements were sequenced, and primers were designed annealing to the hypervariable region of the respective IG or TR gene rearrangement and put in a real-time RQ-PCR together with a consensus primer and a TaqMan probe according to published protocols to quantify the respective target rearrangement.(2, 3) Data interpretation followed the EuroMRD guidelines.(4)

Molecular *BCR-ABL1* analysis

BCR-ABL1 RQ-PCR was performed following standard procedures.(5) Briefly, total RNA was extracted from bone marrow using Trizol based extraction and was reverse transcribed from 1-5 µg RNA using random oligonucleotide primers. Real-time PCR was performed using 5 µl cDNA, with primers and hydrolysis probes specific for *BCR-ABL1* mRNA and for the *BCR* control gene.

II.   Supplemental Tables

Supplemental Table 1. Patient characteristics

Patient no.	Age at initial diagnosis	Sex	*BCR-ABL1-* fusion transcript	Material
Patient 1	63	Male	M	Peripheral blood
Patient 2	35	Female	M	Bone marrow
Patient 3	67	Male	M	Peripheral blood
Patient 4	62	Female	M	Peripheral blood
Patient 5	53	Male	Mm	Peripheral blood
Patient 6	47	Male	m	Peripheral blood
Patient 7	44	Female	m	Bone marrow
Patient 8	22	Female	M	Bone marrow
Patient 9	45	Female	m	Bone marrow
Patient 10	45	Female	m	Bone marrow
Patient 11	77	Female	M	Bone marrow
Patient 12	71	Female	m	Peripheral blood
Patient 13	45	Male	m	Bone marrow
Patient 14	32	Female	M	Bone marrow
Patient 15	59	Male	M	Bone marrow
Patient 16	69	Female	unknown	Peripheral blood
Patient 17	50	Female	m	Peripheral blood
Patient 18	40	Female	m	Bone marrow
Patient 19	60	Male	Mm	Bone marrow
Patient 20*	52	Male	m	Bone marrow
Patient 21	66	Female	M	Bone marrow
Patient 22*	21	Male	M	Peripheral blood
Patient 23	44	Male	m	Peripheral blood
Patient 24	35	Male	m	Peripheral blood
Patient 25	36	Male	unknown	Bone marrow
Patient 26	31	Female	m	Peripheral blood
Patient 27	49	Male	unknown	Peripheral blood
Patient 28	60	Male	unknown	Peripheral blood
Patient 29	73	Female	m	Bone marrow

M, major-*BCR-ABL1* transcript; m, minor-*BCR-ABL1* transcript; mM, major- and minor-*BCR-ABL1* transcripts identified
* Patients were excluded because FISH was not evaluable after cell sorting

Supplemental Table 2. Sorting Panels with conjugates

Panel 1		FITC	PE		PE-Cy7	APC	PB	
			Fluorochrome					
Tube 1	Antigen	CD34	CD3		CD19	CD38	CD20	
	Volume	30µl	30µl		10µl	10µl	3µl	
	Clone Name	8G12	SK7		J3-119	HB7	2H7	
	Company	BD	BD		BD	BD	BL	
Tube 2	Antigen	CD34	CD13	CD33	CD19	CD38	CD3	CD16
	Volume	30µl	15µl	25µl	10µl	10µl	2 µl	7µl
	Clone Name	8G12	SJ1D1	P67.6	J3-119	HB7	UCHT1	3G8
	Company	BD	BC	BD	BD	BD	BD	BL

Panel 2		FITC	PE		PE-Cy7	APC	PB	
			Fluorochrome					
Tube 1	Antigen	CD34	CD3		CD19	CD38	CD20	
	Volume	30µl	30µl		10µl	10µl	3µl	
	Clone Name	8G12	SK7		J3-119	HB7	2H7	
	Company	BD	BD		BD	BD	BL	
Tube 2	Antigen	CD34	CD13	CD33	CD19	CD10	CD3	CD16
	Volume	30µl	15µl	25µl	10µl	10µl	2 µl	7µl
	Clone Name	8G12	SJ1D1	P67.6	J3-119	HB7	UCHT1	3G8
	Company	BD	BC	BD	BD	BD	BD	BL

BD, Becton Dickinson; BC, Beckman Coulter

Supplemental Table 3. Sort-Panel and leukemia associated immunophenotype of each patient

| Patient no.	Sort Panel	LAIP
Patient 1	2	$CD34^+19^+38^+10^+13/33^+20^-$
Patient 2	2	$CD34^+19^+38^-10^+13/33^-20^+$
Patient 3	1	$CD34^+19^+38^+13/33^+20^-$
Patient 4	2	$CD34^+19^+38^+10^+13/33^-20^-$
Patient 5	1	$CD34^+19^+38^-13/33^-20^-$
Patient 6	1	$CD34^+19^+38^+13/33^-20^-$
Patient 7	1	$CD34^+19^+38^+13/33^+20^-$
Patient 8	1	$CD34^+19^+38^+13/33^+20^-$
Patient 9	1	$CD34^+19^+38^+13/33^+20^-$
Patient 10	2	$CD34^+19^+38^+10^+13/33^+20^+$
Patient 11	2	$CD34^+19^+38^+10^+13/33^+20^-$
Patient 12	2	$CD34^+19^+38^+10^+13/33^+20^-$
Patient 13	2	$CD34^+19^+38^+10^+13/33^+20^-$
Patient 14	2	$CD34^+19^+38^+10^+13/33^+20^+$
Patient 15	2*	$CD34^+19^+38^+10^+13/33^+$
Patient 16	1	$CD34^+19^+38^+13/33^+20^-$
Patient 17	2	$CD34^+19^+38^-10^+13/33^+20^+$
Patient 18	2	$CD34^+19^+38^+10^+13/33^+20^-$
Patient 19	2	$CD34^+19^+38^+10^+13/33^+20^-$
Patient 21	1	$CD34^+19^+38^+13/33^+20^-$
Patient 22	1	$CD34^+19^+38^-13/33^-20^+$
Patient 23	1	$CD34^+19^+38^+10^+13/33^-$
Patient 24	2	$CD34^+19^+38^+10^+13/33^+20^-$
Patient 25	2	$CD34^+19^+38^+10^+13/33^+20^-$
Patient 26	2	$CD34^+19^+38^-10^+13/33^-20^-$
Patient 27	2	$CD34^+19^+38^+10^+13/33^+20^+$
Patient 28	2	$CD34^+19^+38^-10^+13/33^-20^-$

LAIP, Leukemia associated immunophenotype.

*In patient 15, markers of sorting panel 2 were used, but partly
in other fluorescence channels.

Supplemental Table 4. Sorting purity as determined by post-sort analysis in three patients.
Percentage of events with immunophenotype of sorted population is stated in relation to all measured events. Sort Purity ranged between 94% and 99%.

Pat. #	Immunephenotypic compartment								
	34+19+20+3-	34+19+20-3-	34-19+20+3-	34-19-20-3+	34+38-19-3-	34+38+19-3-	34+19-10-13/33+3/16-	34-19-10-13/33+3/16-	34-19-10-13/33+3/16+
	LAIP 20+	*LAIP 20-*	*B*	*T*	*MPP*	*Pro*	*M1*	*M2*	*M3*
12	n.a.	99%	99%	95%	94%	95%	95%	97%	n.a.
17	98%	98%	99%	99%	98%	96%	99%	96%	95%
27	99%	99%	99%	97%	99%	98%	99%	96%	97%

III. Supplemental Figures

Supplemental Figure 1. (A) Gating strategy for sorting of the following subpopulations: CD34$^+$38$^-$19$^-$3$^-$ (hematopoietic stem cells and multipotential progenitor cells (HSC/MPP)), CD34$^+$38$^+$19$^-$3$^-$ (myeloid and lymphoid progenitors), CD34$^+$19$^+$20$^-$3$^-$ (leukemia cells without CD20 coexpression; LAIP 20$^-$), CD34$^+$19$^+$20$^+$3$^-$ (leukemia cells with CD20 coexpression; LAIP 20$^+$), CD34$^-$19$^+$20$^+$ (mature B-cells), CD34$^-$19$^-$20$^-$3$^+$ (mature T-cells), CD34$^+$19$^-$13/33$^+$10$^-$16$^-$ (early myeloid compartment), CD34$^-$19$^-$13/33$^+$10$^-$16$^-$ (late myeloid compartment), CD34$^-$19$^-$13/33$^+$10$^-$16$^+$ (mature myeloid compartment). **(B)** Strategy to evaluate the sorting purity. Sorted populations were reanalyzed by flow cytometry to determine sorting purity (in this figure purity of the CD34+CD19+CD20-CD3- population). The purity of sorted cell populations ranged between 94% and 99%.
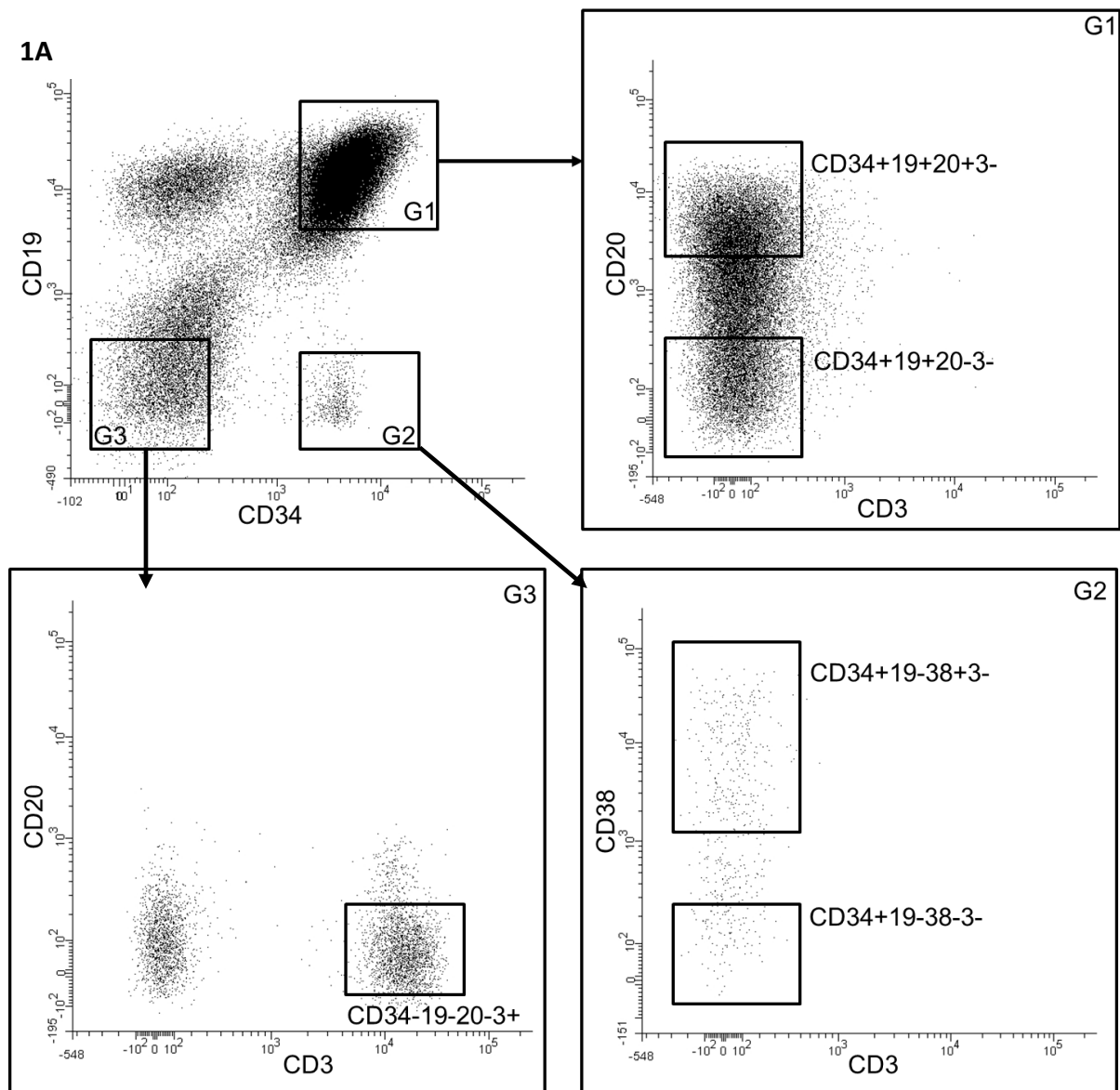
1B
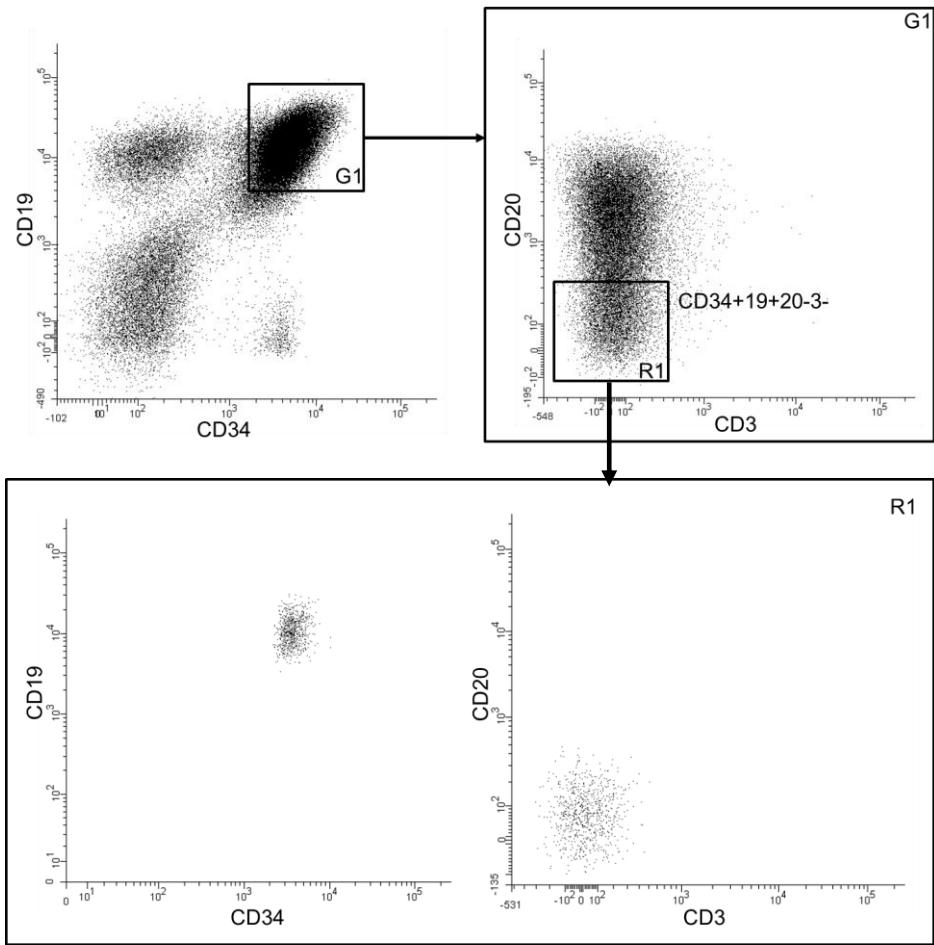
Supplemental Figure 2. Array CGH on patient 21. Genomic profile showing the monosomy 7 of patient 21 at initial diagnosis. In concordance to this finding multiplex ligation-dependent probe amplification detected an exon 1-8 IKZF1 deletion in the diagnostic but not in the relapse sample. Red: Initial diagnosis; Blue: Relapse.

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