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Letters to the Editor

comparison had a lower *P*-value and had a higher degree of deregulation than the differentially expressed genes from the comparison *TET2* mutated versus wild type (Figure 1 b; Supplementary Figure 1 and Supplementary Tables 2 and 3). These results indicate that 5hmC levels are most likely a more relevant measurement to define biologically distinct secondary leukemia subtypes than the *TET2* (or *IDH1/2*) mutational status. The fact that in some patient samples with low 5hmC levels neither *TET2* nor *IDH1/2* mutations could be identified suggests that additional genes might be directly or indirectly involved in the regulation of 5hmC levels. To further elucidate the regulation of 5hmC levels and their role in leukemogenesis, larger groups of sAML as well as *de novo* AML patients need to be studied.

Conflict of interest

The authors declare no conflict of interest.

Author Contributions

NK and FS designed and performed the mutation screening with the help of AD and BK, and wrote the manuscript. SB, AS and HL designed and performed the 5hmC measurements and wrote the manuscript. HL supervised the project. PMK and SS performed cytogenetics and fluorescence in situ hybridization analysis. TH and MM analyzed the GEPs. KS designed experiments and wrote the manuscript. SKB designed experiments, analyzed the data, supervised the project and wrote the manuscript.

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Immunophenotype-defined sub-populations are common at diagnosis in childhood B-cell precursor acute lymphoblastic leukemia

Neoplasms often display significant heterogeneity in morphology, gene expression (including cell surface markers), genetic aberrations, cell proliferation kinetics and response to therapy.[1] Heterogeneity in antigen marker expression is well known in acute myeloid leukemia (AML);[2] however, has rarely been studied in acute lymphoblastic leukemia (ALL). In ALL, shifts in immunophenotypic and genetic profiles can occur between

diagnosis and relapse.[3] Such changes of immune gene-rearrangement profiles can reflect the presence at diagnosis of minor, but genomically distinct sub-populations.[4] Likewise, changes in immunophenotype might reflect phenotypically distinct sub-populations at diagnosis that potentially could be detected in a more sensitive flow-cytometric analysis than used routinely. If such sub-populations are present and different immunophenotypes are related to differences in gene rearrangement profiles, this could cause discordances between the methods for minimal residual disease (MRD) monitoring, that is, flow-cytometric immunophenotyping and PCR-based detection of clonotypic immunoglobulin (Ig)/T-cell receptor (TCR) gene rearrangements. Importantly, sub-populations are also relevant for the understanding of the ontogenesis of the malignant cells and might give clues for understanding the biological mechanisms of therapy resistance and relapse.

In this study, we explored the presence at diagnosis of immunophenotypically heterogeneous leukemic cell populations, that is, distinct sub-populations with bimodal marker expression or populations with broad marker expression, in childhood B-cell precursor (BCP) ALL. Immunophenotypically heterogeneous cells were isolated by flow sorting and investigated for the clone-defining cytogenetic marker and a broad spectrum of clonal Ig/TCR gene rearrangements.

We studied 40 bone marrow (BM) samples and one peripheral blood sample obtained at diagnosis from 41 consecutive patients, with childhood (below 18 years of age) BCP-ALL diagnosed from March 2008 to July 2009. Three patients diagnosed during this time period could not be included due to lack of diagnostic samples (two patients) or lack of flow data (one patient). One additional patient diagnosed in October 2009, who had immunophenotypically distinct sub-populations, was included only in the cytogenetic/molecular analysis. The diagnosis was established according to the conventional criteria. In all, 39 of the patients were enrolled in the Nordic Organization for Pediatric Hematology and Oncology (NOPHO) ALL-2000 or NOPHO ALL-2008 treatment protocols. Two infant patients were enrolled in the Interfant-06 protocol. In ALL-2000, BM samples were obtained from patients admitted to The University Hospital Rigshospitalet, Copenhagen (accounting for approximately half of Danish patients), whereas in ALL-2008, samples were obtained from all Danish patients. Samples were collected in heparin-RPMI1640 and processed within 24 h of collection. All patients/parents gave informed consent to participate in this study according to the guidelines of the Danish Ethics Committee (HC-2008-081 and 2001-10205).

Samples for FC analysis were subjected to Lymphoprep separation of mononuclear cells. Staining for flow-cytometric immunophenotyping was performed with standard procedures according to the NOPHO guidelines. Data acquisition was performed on FACSAria cytometer equipped with the FACS Diva 6 software (Becton Dickinson, San Jose, CA, USA). A minimum of 100 000 events was acquired. Dead cells and debris were excluded in forward scatter (FSC)/side scatter (SSC) dot plots based on propidium-iodide staining. Aggregated cells were excluded based on FSC height versus FSC area dot plots. BCP blast populations were identified using CD45/CD19 dot plots. By using backgating into FSC/SSC plots, we verified that minor sub-populations were always located in the region where viable blasts are normally found.

Immunophenotypic marker expression on blast populations was given a score as negative (−), positive dim (+), positive

normal (+ +) or positive bright (+ + +), as compared with the expression on normal precursor B cells. Normal BM samples were analyzed in parallel using the same flow-cytometry settings. Antigens not expressed by normal precursors were only scored as negative or positive. Only antigen markers normally expressed by precursors were scored referring to the normal expression. Special attention was given to dim populations, which were defined as positive dim when more than 20% of the population was brighter than the corresponding isotype control. Distinct sub-populations were defined as separate populations, each having their own peak in contour plots (In FACSDiva 6; resolution: 128, percentage: 10) and histograms (described as bimodal expression). The size limit to what was named sub-populations was set to >2% of total blast count, as populations smaller than this were difficult to classify by our FC setup (4- or 6-color and 100 000 cells analyzed). We defined broad expression of a marker to occur when a population that only has one peak—using the outer line of the 10% contour plot as boundary—extends from one score into the middle of the neighboring score.

Patients from the ALL-2000 protocol were analyzed using the following protocol-defined four-color combinations: CD45/CD34/CD19/CD10; CD10/CD20/CD19/CD45; CD34/CD22/CD19/CD45; CD34/CD38/CD19/CD45; TdT/CD10/CD19/CD45. Additional antibody combinations were used: CD10/CD13 + 33/CD19/CD34; kappa/lambda/CD19/CD45; CD7/CD2/HLA-DR/CD45; cyMPO/cyCD79α/cyIgM/cyCD22; CD66c/CD135/CD34/CD133.

Fluorochromes in the four channels were: FITC/PE/PC5/APC, respectively.

Patients from ALL-2008 protocol were analyzed with the protocol-defined six-color combinations:CD10/CD20/CD34/CD19/CD38/CD45; CD66c/CD123/CD34/CD19/CD22/CD45; TdT/CD58/CD34/CD19/CD10/CD45. Additional tubes used were: CD10/CD58/CD34/CD19/CD133/CD45; cyMPO/cyCD79α/cyIgM/CD19/cyCD22/CD45; kappa/lambda/CD33/CD19/CD13/CD45; HLA-DR/CD7/CD34/CD2/CD1a/CD45.

Fluorochromes in the channels were: FITC/PE/PerCP-Cy5.5/PE-Cy7/APC/APC-Cy7, respectively. The following abbreviations are used: cy, cytoplasmic; kappa, Ig kappa light chain; lambda, Ig lambda light chain; TdT, terminal deoxynucleotidyl transferase; HLA, human leukocyte antigen.

Flow sorting was performed on FACSAria (Becton Dickinson). Sorting was carried out in 'high speed' with a 70-μm nozzle, sheath pressure of 70 PSI and sort precision mode set to 'Purity'. When feasible with respect to sample size and cell number, a part of the sorted populations were reanalyzed, showing a sorting purity of minimum 98%. Cells for fluorescence in situ hybridization (FISH) analysis were sorted onto polylysine-coated glass slides into a droplet (50–100 μl) of phosphate-buffered saline or CytoFix (Becton Dickinson) placed on the slide. When cells were not fixated before sorting and sorted into a phosphate-buffered saline droplet, the cells on the slides were fixated in methanol/glacial acid (3:1). Fixated slides were dehydrated and then kept at –20 °C for later FISH analysis. Cells to be analyzed by PCR were sorted into eppendorf tubes containing 500 μl RPMI medium, and cell pellets were kept at −80 °C for later DNA purification.

As part of the diagnostics for childhood ALL, chromosome analyses (G-banding) and FISH were carried out on diagnostic BM samples of all ALL patients. The applied FISH probes were MLL dual color, break apart rearrangement probe, TCF3/PBX1 dual color, dual fusion probe, BCR/ABL dual color, dual fusion probe, EVT6/RUNX1(TEL/AML1) ES dual color

probe (all probes from Vysis, Abbott, Weisbaden, Germany). Patients with a normal or failed karyotype were in addition screened with FISH probes for chromosomes 4, 10 and 17 (CEP4, CEP10, CEP17, Vysis, Abbott). The flow-sorted cell populations were analyzed for the clone-defining FISH marker using a selected FISH probe in each patient (for details, see Supplementary Table). FISH was performed according to standard procedures. At least 50 nuclei were analyzed when possible.

DNA from the sorted-cell populations was purified by the SpinTissue XS kit (Macherey-Nagel, Düren, Germany) and eluted in 30 μl H$_2$O. As the PCR clonality assay requires larger amount of DNA, we used whole-genome amplified DNA as template. Whole-genome amplification (WGA) was performed using REPLI-g Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with a minimum of $\sim$10-ng input DNA to minimize amplification bias. WGA was performed on DNA from both unsorted and sorted specimens, and amplified DNA (WGA-DNA) was purified using the QIAamp DNA Mini Kit (Qiagen). WGA-DNA from sorted-cell populations was analyzed for the presence of a broad spectrum of clonal Ig and TCR gene rearrangements (*IGH V$_H$-J$_H$, IGH D$_H$-J$_H$, IGK V-Kde/intronRSS-Kde, TCRB V$_\beta$-J$_\beta$, TCRB D$_\beta$-J$_\beta$, TCD* and *TCRG*) using the IdentiClone Clonality Assays (InVivoScribe Technologies, San Diego, CA, USA). The gene loci studied covers the majority of all possible gene rearrangements. All the Ig/TCR gene loci were analyzed in each unsorted BM samples and in flow-sorted each cell population. For each patient, the gene rearrangement profiles were compared between: genomic DNA from unsorted sample, WGA-DNA from unsorted sample and WGA-DNA from sorted subfractions. GeneScanning of PCR products were performed by capillary electrophoresis (3130 Genetic Analyzer, Carlsbad, CA, USA). In cases where unexpected clonal products were found, the WGA and PCR were repeated for verification of the findings.[5]

Analyzing the expression of the 14 antigen markers (CD10, CD34, CD38, CD45, TdT, HLA-DR, CD19, CD20, CD22, cyCD22, cyCD79α, lambda, kappa and cyIgM), we observed immunophenotypically distinct sub-populations defined by their bimodal antigen expression in more than half (27/41) of the patients studied (Table 1 and Figure 1). The sub-populations were most commonly characterized by a bimodal expression of CD34 (18/41 patients) and of TdT (11/41 patients), but five patients showed bimodal expression of both CD34 and TdT. Few examples of bimodal expression of other markers were also observed: for example, CD10 (two patients), CD45 (two patients) and lambda (two patients). The sub-populations often comprised more than 10% of the total blast count, seen in 15 of the 18 patients with bimodal CD34 expression and in 6 of the 11 patients with bimodal TdT expression. In the cases with bimodal CD34 expression, it was random whether the major population had the highest or lowest expression. In contrast, in almost all of the cases with bimodal TdT expression (10 cases out of 11), the major population had the highest TdT expression, being positive normal in all cases.

Broad expression, as defined above, of one or more markers was observed in almost all patients, that is, 38/41 of the patients studied. The markers involved were most commonly CD20 (23 patients), CD45 (14 patients) and TdT (13 patients) (Table 1). The broad expression patterns were verified by single-color FC in seven representative cases (three CD20broad, two TdTbroad and two CD34broad) from five BM samples. There was no tendency that broad expression of one marker was specifically correlated to broad expression of another specific marker.

Table 1 Number of patients showing bimodal and/or broad expression of the analyzed markers

Marker	Patients with bimodal expression/Patients analyzed[a]	Patients with broad expression/Patients analyzed[a]
CD34	18/41 (7)	8/41 (4)
TdT	11/41 (1)	13/41
CD10	2/41 (2)	10/41
CD45	2/41 (1)	14/41
Lambda	2/41 (2)	0/41
CD22	1/41	4/41
CD66c	2/25	3/25
CD20	0/41	23/41 (3)
CD38	0/41	6/41
cyCD79α	0/41	5/41
CD123	0/25	4/25

The following markers were also analyzed: CD19 (41 patients), cyCD22 (41 patients), HLA-DR (41 patients), CD33 (41 patients), kappa (41 patients), cyIgM (41 patients), CD13 (41 patients), CD2 (31 patients), CD7 (39 patients) and CD133 (10 patients), but no cases of bimodal expression of these markers were observed, and broad expression was observed in none or less than 10% of patients analyzed.
[a]Numbers in parentheses show number of patients in whom the immunophenotypically heterogeneous cell populations were isolated by flow sorting.

In 15 of the patients in whom distinct sub-populations or broad expression emerged, the immunophenotypically heterogeneous populations were isolated by flow sorting for subsequent genomic analyses (Figure 2). As the cytogenetic abnormalities are considered a primary event in leukemogenesis, we analyzed the flow-sorted populations by FISH to verify the malignant state of the cells. We found that all flow-sorted presumed leukemic cell populations showed similar dominance of cytogenetic aberrant cells by having 60–100% FISH-positive cells (29 populations from 10 patients), irrespective of bimodal or broad expression of the defining marker. In the remaining five patients, we did not obtain FISH results due to bad quality of the cells or lack of a FISH marker. Expected normal cell populations with normal immunophenotype (six populations from five patients) had normal karyotype (92–100% FISH-negative cells).

Screening each flow-sorted cell population (37 suspected leukemic and 12 expected normal cell populations from 15 patients) for a broad spectrum of Ig/TCR targets, we found that in 14 out of the 15 patients analyzed, the unsorted sample and all sorted expected leukemic subfractions (36) shared identical patterns of clonal gene rearrangements (Figure 2): Clonal Ig/TCR gene rearrangements detected in the unsorted specimen were also detected in all malignant subfractions at similar signal intensities when analyzing equal amounts of DNA. In unsorted specimens with polyclonal normal rearrangements, no clear clonal products were subsequently detected in sorted leukemic subfractions. One patient, however, had a distinct Ig/TCR gene rearrangement profile in one sub-population. This patient showed two clonal *IGH-VJ* rearrangements in a population of CD34dim cells, but only one clonal product in the CD34normal cells. Both of these populations had an immuno-phenotype consistent with common BCP-ALL. Loss of one marker in one sub-population may be due to secondary rearrangements or deletion of a chromosomal segment (genomic instability affecting this gene locus), as has been suggested in cases of loss of *IG/TCR* markers at relapse.
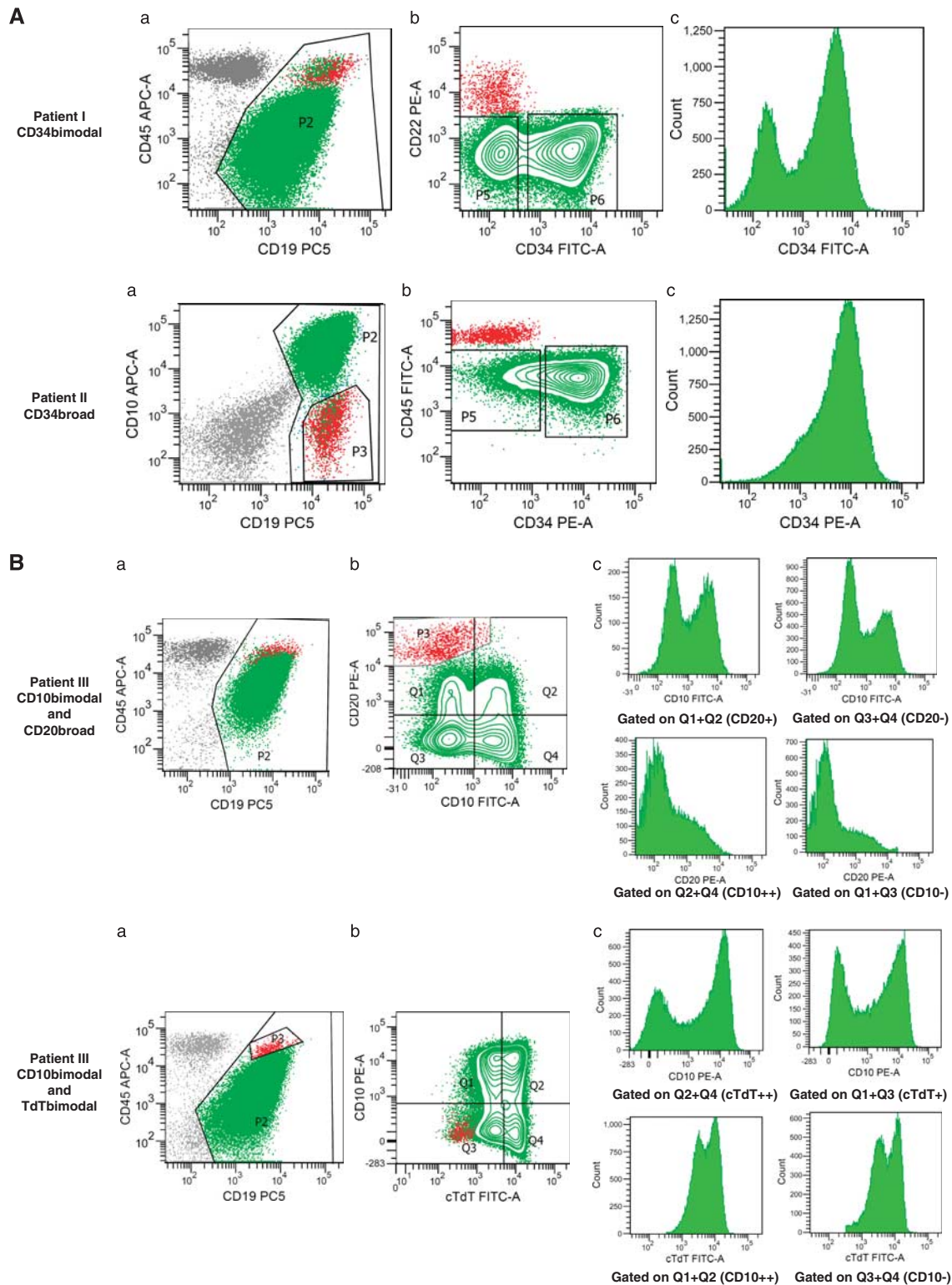
Figure 1 Examples of heterogeneous antigen expression at diagnosis in BCP-ALL. The left hand dot plots (**a**) are all gated on cells with blast FSC/SSC characteristics, while the right hand plots (**b**) are further gated on CD19-positive cells. Green color denotes leukemic cells; and red color denotes normal mature B cells. The histograms (**c**) are gated on the CD19-positive leukemic cells. (**A**) Examples of bimodal and broad expression of CD34. (**B**) Examples of simultaneous bimodal/broad expression of two markers in one antibody combination. Histograms (**c**) are gated on selected fractions of leukemic cells as described below each plot.

Cell populations regarded to be normal were analyzed in nine patients. In six of these patients with clear separation between immunophenotype-defined normal and malignant cells, the flow-sorted normal cells had normal *IG/TCR* polyclonal profiles. In two patients, normal cells were incompletely separated from the malignant cells, resulting in presence among the flow-sorted
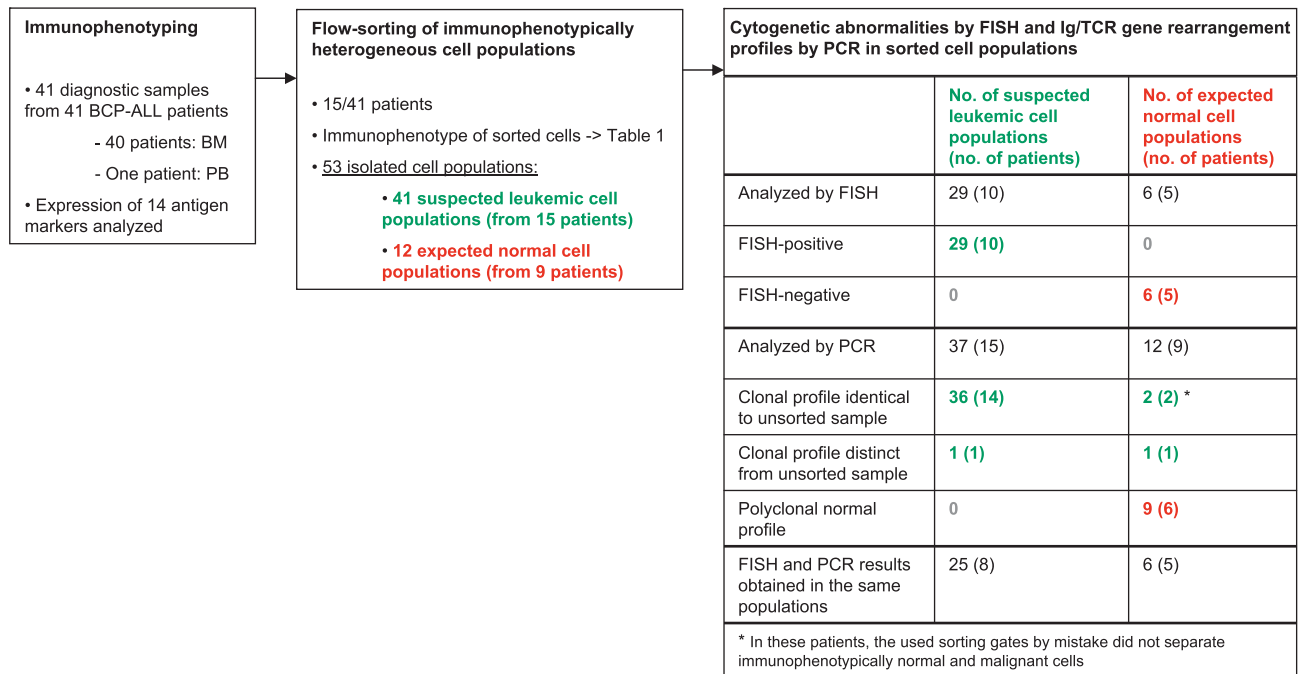
Immunophenotyping	Flow-sorting of immunophenotypically heterogeneous cell populations

• 41 diagnostic samples from 41 BCP-ALL patients
  - 40 patients: BM
  - One patient: PB
• Expression of 14 antigen markers analyzed
	• 15/41 patients
	• Immunophenotype of sorted cells -> Table 1
	• 53 isolated cell populations:
	  • **41 suspected leukemic cell populations (from 15 patients)**
	  • **12 expected normal cell populations (from 9 patients)**

Cytogenetic abnormalities by FISH and Ig/TCR gene rearrangement profiles by PCR in sorted cell populations

	No. of suspected leukemic cell populations (no. of patients)	No. of expected normal cell populations (no. of patients)
Analyzed by FISH	29 (10)	6 (5)
FISH-positive	**29 (10)**	0
FISH-negative	0	**6 (5)**
Analyzed by PCR	37 (15)	12 (9)
Clonal profile identical to unsorted sample	**36 (14)**	**2 (2)** *
Clonal profile distinct from unsorted sample	**1 (1)**	**1 (1)**
Polyclonal normal profile	0	**9 (6)**
FISH and PCR results obtained in the same populations	25 (8)	6 (5)

* In these patients, the used sorting gates by mistake did not separate immunophenotypically normal and malignant cells

Figure 2 Diagram of the experimental workflow and the results from FISH and Ig/TCR gene rearrangement analyses in flow-sorted cell populations. A total of 41 leukemic-cell populations were sorted. Of these, 37 were analyzed by PCR, 29 were analyzed by FISH and 25 were analyzed by both PCR and FISH. In all, 12 normal cell populations were sorted. Of these, 12 were analyzed by PCR, 6 were analyzed by FISH and 6 were analyzed by both PCR and FISH.

cells classified as normal of the same clonal immune-gene markers as identified in the leukemic cells. In one patient, we found a distinct clonal profile in the presumed normal mature B cells. This patient (classified as infant on the basis of the age at diagnoses, but with typical BCP-ALL phenotype and without MLL rearrangement) who presented with two distinct CD34negative/CD34normal sub-populations, had a clonal *IGH V-J* product in the expected normal cells that was not present in any of the leukemic sub-populations. We cannot determine whether this reflects a normal lymphocyte population undergoing an immune response with oligoclonal proliferation or a leukemic subclone. Another clonal *IGH V-J* product was identified in the leukemic cells and this was not present in the presumed normal mature B cells.

Our finding of one case with absence of one of the dominating clonal PCR markers suggests that this could lead to misjudgment of MRD, and the use of at least two independent markers in real-time quantitative PCR-based MRD analyses may reduce the risk of such false conclusions.

In ALL, several studies have focused on genomic heterogeneity and the genetic basis of relapse by comparing genomic profiles in paired diagnosis and relapse samples. It is hypothesized that outgrowth of therapy-resistant subclones, genomically distinct from the predominant clone and present already at diagnosis, is the mechanism of therapy resistance and relapse in the majority of ALL relapse cases. This hypothesis is supported by the finding that in some relapsed patients new Ig/TCR gene rearrangements were detected at relapse and that these 'relapse clones' could be detected at diagnosis using more sensitive methods than those used routinely.[4] Although restricted to sub-populations accounting for at least 2% of the total number of leukemic cells, our findings show that the immunophenotypically distinct sub-populations in general do not define clones with distinct rearrangements. Backtracking of relapse clones by sensitive

PCR analyses in flow-sorted cell populations in stored diagnostic samples could specify in which immunophenotypically defined population(s) these cells would be found. Another important question is whether the immunophenotypically distinct sub-populations have different *in vivo* leukemia-initiating capacity or different sensitivity toward therapy, as indicated by studies of sub-populations differing in CD34 expression.[6]

The observed bimodal expression and broad expression of antigen markers in BCP-ALL could reflect two different biological phenomena, respectively. The observed broad expression patterns of some markers, for example CD20, resemble the maturation spectrum seen in normal precursor B cells.[7] Thus, it is likely that it reflects partial maturation of the blasts—resembling the phenotypic heterogeneity seen in AML. In AML, there is increasing evidence that immunophenotypically distinct leukemic cells are also functionally heterogeneous and that rare sub-populations of leukemia-initiating cells exists. It is thought that AML, like the normal hematopoietic system, is organized as a hierarchy of distinct cell classes, sustained by a subset of leukemic stem cells that alone have long-term repopulating potential and give rise to progeny that lack this potential.[8]

It is recognized that immunophenotypic modulations of leukemic cells frequently occur during early treatment, possibly induced by the steroids used in induction therapy.[9] We observed a marked overlap between markers with broad expression at diagnosis and markers typically modulated during treatment. Thus, the broad expression at diagnosis might be related to physiological stress factors released during overt disease.

Bimodal expression in ALL might reflect the co-existence of genetically distinct sub-populations, generated early during oncogenesis possibly due to genomic instability. However, in the patients analyzed here, the immunophenotypic differences

were, in most cases, not related to differences in the immune gene-rearrangement profiles. Thus, these populations must have evolved from a common clonal origin, and the rearrangement process must have been completed early in the common progenitor cell. Consequently, possible genomic differences must be at loci other than the Ig/TCR genes, or might involve epigenetic differences, for example, DNA methylation and chromatin remodeling.

In conclusion, immunophenotypically distinct sub-populations are common at diagnosis in BCP-ALL. Occasionally, leukemic sub-populations may lack the dominating PCR MRD marker, which could result in underestimation of MRD, the risk of which may be reduced by the use of several independent PCR markers. Furthermore, awareness of the sub-populations is important in flow-cytometric MRD monitoring to avoid underestimation of residual disease.

Conflict of interest

The authors declare no conflict of interest.

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Donor KIR haplotype B improves progression-free and overall survival after allogeneic hematopoietic stem cell transplantation for multiple myeloma

Allogeneic hematopoietic stem cell transplantation from related or unrelated donor has become a standard treatment for many patients with hematological malignancies, but the role of this treatment approach in the treatment of multiple myeloma remains controversial, as high risk of relapse remains a major concern.[1] In acute leukemias, especially acute myeloid leukemias, alloreactive donor-derived natural killer cells (NK cells) have been correlated with an improved survival, especially after T-cell-depleted transplant procedures such as haploidentical stem cell transplantation.[2] Small reports after allogeneic stem cell transplantation for multiple myeloma as well as pre-clinical models showed that alloreactive NK cells might have a role regarding cytotoxicity and relapse prevention in multiple myeloma.[3] The NK cell function is determined by several receptor families including activating and inhibitory killer cell immunoglobulin-like receptors (KIRs). For the majority, especially for the active KIRs, the correspondent ligands are