Identification of residual leukemic cells by flow cytometry in childhood B-cell precursor acute lymphoblastic leukemia: verification of leukemic state by flow-sorting and molecular/cytogenetic methods

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ABSTRACT

Reduction in minimal residual disease, measured by real-time quantitative PCR or flow cytometry, predicts prognosis in childhood B-cell precursor acute lymphoblastic leukemia. We explored whether cells reported as minimal residual disease by flow cytometry represent the malignant clone harboring clone-specific genomic markers (53 follow-up bone marrow samples from 28 children with B-cell precursor acute lymphoblastic leukemia). Cell populations (presumed leukemic and non-leukemic) were flow-sorted during standard flow cytometry-based minimal residual disease monitoring and explored by PCR and/or fluorescence *in situ* hybridization. We found good concordance between flow cytometry and genomic analyses in the individual flow-sorted leukemic (93% true positive) and normal (93% true negative) cell populations. Four cases with discrepant results had plausible explanations (e.g. partly informative immunophenotype and antigen modulation) that highlight important methodological pitfalls. These findings demonstrate that with sufficient experience, flow cytometry is reliable for minimal residual disease monitoring in B-cell precursor acute lymphoblastic leukemia, although rare cases require supplementary PCR-based monitoring.

Key words: childhood B-cell precursor acute lymphoblastic leukemia, minimal residual disease, flow cytometry, real-time quantitative PCR, fluorescence *in situ* hybridization.

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Introduction

The level of minimal residual disease (MRD) in bone marrow (BM) during early phases of treatment is the most important prognostic factor in children with acute lymphoblastic leukemia (ALL).[1-3] Consequently, MRD monitoring is applied in the treatment stratification in most ALL protocols. One strategy for MRD detection is real-time quantitative PCR (PCR-MRD) analysis of immunoglobulin (Ig)/T-cell receptor (TCR) gene rearrangements.[4,5] Another strategy is flow cytometry-based immunophenotyping (FC-MRD), which differentiates leukemic cells from normal cells based on aberrant antigen expression (leukemia-associated immunophenotype, LAIP).[6,7] The longest clinical experience has been with PCR-MRD. This has the best standardization techniques and is the method used in most treatment protocols. However, at present neither method has 100% applicability, and so it can be difficult to provide sensitive MRD results for all patients if only a single method is used in a center.

A critical issue of MRD studies is the occasional discordance between PCR and FC results. In rare cases, one of the two methods fails to detect MRD, while more commonly minor quantitative differences occur. Both situations can lead to different treatment stratification depending on the MRD method used and the cut-off levels.[8-11] Despite this, the identification of malignant cells by FC has only been biologically verified in one study including 5 patients.[12]

To explore the background of such discrepancies, we investigated 53 follow-up BM samples from 28 children with B-cell precursor ALL (BCP-ALL) by flow-sorting of immunophenotype-defined residual leukemic cell populations, and subsequent analyses for leukemia-associated genomic markers by

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RQ-PCR and/or fluorescence *in situ* hybridization (FISH). Additionally, we explored to what extent cell populations scored as being non-malignant contained significant amounts of leukemic cells. Flow-sorting was carried out on fresh BM samples during the data acquisition for standard FC-MRD quantification. This procedure makes it possible to directly verify the FC-MRD analysis and exclude variation related to use of different cell material, and sequential cell acquisition and flow-sorting.

Design and Methods

Patient samples

We studied 53 follow-up BM samples obtained from 28 patients (diagnosed within the period June 2007 to January 2010) with childhood BCP-ALL. Patients without a useful genomic PCR/FISH marker for the leukemic clone when screened by the standard gene rearrangement and cytogenetic panels at diagnosis were excluded (*Online Supplementary Appendix*).[13] Patients' cytogenetic characteristics are listed in *Online Supplementary Table S1*. The ALL diagnosis was established according to conventional criteria.[14] The patients were enrolled in the Nordic Organisation for Paediatric Haematology and Oncology (NOPHO) ALL-2000, NOPHO ALL-2008 or Interfant-06 treatment protocols. The induction treatment included three drugs (Vincristin-Doxorubicin-steroids) with prednisolone 60 mg/m.sq. for BCP-ALL with white blood cell count less than 100K and dexamethasone 10 mg/m^2 for the remaining patients.[14] In NOPHO ALL-2000, BM samples were obtained from patients treated at the University Hospital Rigshospitalet (accounting for approximately half of Danish patients), while in NOPHO ALL-2008, BM samples were obtained from patients treated at all Danish hospitals. Protocol-defined standard MRD follow-up BM samples were obtained at day 15 (14 samples), day 29 (17 samples) or after day 29 (22 samples; six samples from approx. day 50, six samples from day approx. 79, three samples from day approx. 96, and seven samples from between days 106 and 213) after diagnosis. The study was approved by the Danish Ethics Committee (HC-2008-081 and 2001-10205), and all patients/parents gave informed consent to participate in the study.

Staining for flow cytometric immunophenotyping

BM samples were stained for flow cytometric immunophenotyping with standard procedures according to NOPHO guidelines (*Online Supplementary Appendix*).[1] As part of the standard FC-based MRD monitoring, patients were analyzed at diagnosis using a broad panel of antibody-combinations to identify leukemia-associated immunophenotypes (LAIPs). A minimum of 100,000 cells were analyzed to identify heterogeneity, i.e. bimodal and broad antigen expression patterns.[13] Patients were analyzed using protocol-defined four-color (NOPHO ALL-2000) or six-color (NOPHO ALL-2008) MRD panels (*Online Supplementary Appendix*). At follow up, patients were analyzed using the protocol-defined and additional patient-specific MRD antibody-combinations. MRD detection performed as part of the standard clinical MRD monitoring will be referred to as 'standard FC-MRD'.

Standard MRD data acquisition and simultaneous flow-sorting

Cell populations were isolated by flow-sorting on a FACSAria (BD) equipped with the FACS Diva 6 software (BD) during data acquisition (in 'real-time') for the standard 4-6 color FC-MRD monitoring, i.e. sorting and MRD analysis were carried out on the same tube of cells. This set-up is useful due to the limited amount of BM material obtained at these time points, and importantly the set-up allows for an exact verification of the MRD-FC analysis by excluding any variation related to use of different cell material, and sequential cell acquisition and sorting.

Initially, a small part of the sample was acquired and sort gates were defined. LAIP sort gates were defined based on: a) the LAIP

Table 1. Distribution of immunophenotype-defined leukemic subpopulations at diagnosis and follow up (day 15, 29 and >29), in cases for which the leukemic state of the cells was verified by PCR/FISH.

Patient number (bimodal expression pattern)	Subpopulation fractions (percentage of leukemic blast count)			
	Day 0	Day 15	Day 29	Day >29
#67/00 (CD34 +/++)	27/73	5/95	30/70	NT
#82/00 (CD34 -/++)	80/20	NT	70/30	NT
#8/08 (CD34 +/+++)	6/94	NT	NT	14/84*
#19/08 (CD34 -/+)	21/79	61/39	NT	NT
#20/08 (CD34 +/+++)	3/97	NT	60/40	NT
#63/08 (CD34 -/++)	80/20	55/45	NT	NT
#63/08 (CD10 -/++)	80/20	35/45	NT	NT

*#: patient number; -: negative; +: positive-dim; ++: positive-normal; +++: positive-bright; e.g. CD34+/++ means one CD34positive-dim and one CD34positive-normal leukemic cell population. NT, not tested. *BM sample from day 96 after relapse.*

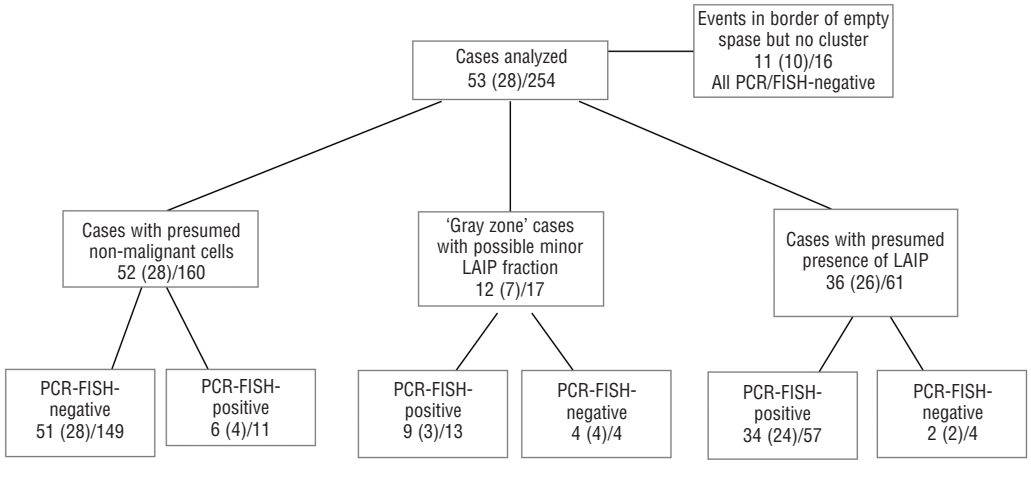

Figure 1. Schematic presentation of the BM samples and flow-sorted cell populations studied as well as the results from PCR/FISH-analyses in sorted cell populations. Two or more cell populations were sorted from each BM sample. Numbers in the boxes are: number of BM samples from which the flow-sorted cell populations derived (number of patients) / number of flow-sorted cell populations. E.g. in total 255 cell populations, deriving from 53 BM samples from 28 patients, were investigated.

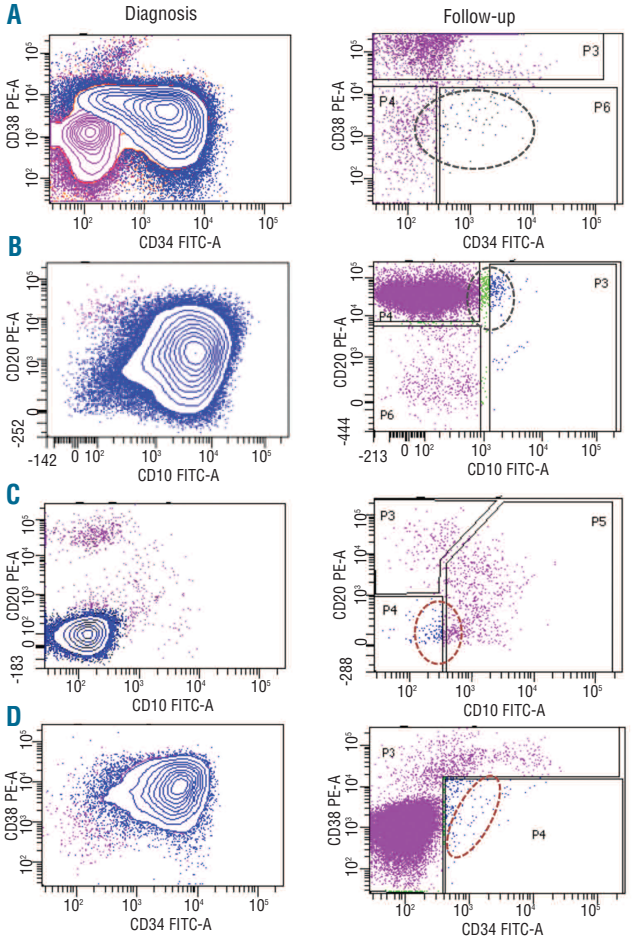

Figure 2. FC dot plots from discrepant cases with unexpected PCR/FISH-results in flow-sorted cell populations. Left hand plots are from diagnostic BM samples, while right hand plots are from follow-up BM samples with sort gates shown. All plots are gated on CD19^{pos} cells after excluding dead cells and debris based on FSC/SSC characteristics. Leukemic cells are shown in blue and non-leukemic B-cells in purple. The dashed circles indicate the plausible location of MRD cells (cases A and B), and areas with potential occurrence of MRD cells (cases C and D), respectively. (A) Risk of MRD underestimation due to partly informative LAIP at diagnosis and persistence of CD34^{neg} cells (or downmodulation of CD34) (Case #1). (B) Risk of MRD underestimation due to signification antigen modulation (CD10 downmodulation and CD20 upmodulation) (Case #2). (C) Risk of MRD overestimation in CD10^{neg}/CD20^{neg} BCP-ALL patient due to persistence of plasma cells (Case #3). (D) Risk of MRD overestimation due to dead cells in dot plot diagonal region (Case #4) (*Online Supplementary Appendix*).

identified at diagnosis; b) the marker expression in possible previous MRD samples; and c) knowledge of the typical modulations of antigen expression of the MRD blasts during early treatment related to the actual treatment protocol used.[15-17] Expected normal B-lineage cells were defined as either the normal mature B cells (CD19^{pos}/CD45^{bright}), normal regenerating precursor B cells (e.g. CD45^{pos}/CD19^{pos}/CD10^{pos}), or presumed plasma cells (CD45^{pos}/CD19^{dim}/CD10^{neg}/ CD20^{neg}).

After defining the sort gates, the maximum possible number of cells was acquired and sorted (a minimum of 300,000 events and preferably 1 million or more events per antibody-combination). When the amount of cells permitted, a part of the sorted populations were reanalyzed, showing a sorting purity of a minimum 98%. Cells for FISH and PCR analysis were flow-sorted as described previously.[18]

FC data analysis

Following FC data acquisition and flow-sorting, MRD was quantified according to the NOPHO guidelines (*Online Supplementary Appendix*).[1] Since flow-sorting was carried out simultaneously with the data acquisition for the standard FC-MRD, the *a priori* defined sort gates could not be identical to the *a posteriori* employed gating strategy for standard MRD quantification. By comparing the sort gates and the gates used for MRD quantification, we evaluated the individual isolated populations and categorized them as either: a) presumed residual malignant cell populations with high fraction of cells with LAIP; b) presumed normal cell populations with no detectable LAIP (CD19negative cells or normal B-lineage cells); or c) 'gray zone' cell populations with potential minor fraction of cells with LAIP (Figure 1).

Detection of cytogenetic markers in flow-sorted cells by FISH

As part of the diagnostics for childhood ALL, chromosome analyses (G-banding) and FISH were performed on diagnostic BM samples of all ALL patients (*Online Supplementary Appendix*).[13] The cell populations flow-sorted from the follow-up BM samples were analyzed for the clone-defining FISH marker using a selected probe in each patient (*Online Supplementary Table S1*). FISH was performed according to standard procedures. For evaluation of the FISH results, all nuclei possible (maximum 50) were analyzed. Results were expressed as percentage FISH-positive cells relative to number of cells analyzed.

Detection of clonotypic DNA-MRD markers in flow-sorted cells by RQ-PCR

As part of the standard PCR-MRD analyses at the Department of Clinical Immunology, patient-specific clonal Ig/TCR gene rearrangements were identified at diagnosis, and primers for RQ-PCR were designed and tested according to BIOMED-2 guidelines.[4,5] Clone-specific DNA markers were analyzed in flow-sorted cell populations by a semi-quantitative approach using the patient-specific Ig/TCR MRD primers (*Online Supplementary Table S1*), as previously described.[18] Percentage of PCR-positive cells in individual cell populations was calculated as follows: number of cells measured by *IG/TCR* RQ-PCR was divided by number of cells measured by *MBL2* RQ-PCR. This ratio was multiplied by a ratio calculated for one selected dilution of the standard curve, i.e. number of cells measured by *MBL2* RQ-PCR divided by number of cells measured by OD measurement (correlation for variance between *MBL2* RQ-PCR and the OD measurement on which the standard curve was based).

Definition of concordance between FC and PCR/FISH in individual cell populations

Populations were named PCR/FISH-positive when cells positive for the analyzed leukemia-associated marker by either method were detected, and PCR/FISH-negative when no cells were positive for the investigated marker(s). Concordance between FC and PCR/FISH-based detection of residual malignant cells in individual populations was defined to occur in cases with: a) presence of LAIP by FC and PCR/FISH-positive results; and b) non-malignant populations with no LAIP by FC and PCR/FISH-negative results.

Results and Discussion

PCR/FISH analyses in flow-sorted cell populations

In 53 follow-up BM samples from 28 BCP-ALL patients, FC data was acquired from 106 antibody-combinations and 330 immunophenotype-defined cell populations were iso-

lated for subsequent genomic analyses using *a priori* defined sort gates. In 75 sorted cell populations, PCR/FISH results could not be obtained due to too few cells and cell loss during preparation or FISH/PCR failure with no FISH/*MBL2*-PCR signals. Thus, in total 254 cell populations (isolated from 94 antibody-combinations) were analyzed by PCR and/or FISH depending on available markers and number of sorted cells (173 by PCR, 53 by FISH, 28 by both PCR and FISH) (Figure 1). Standard FC-MRD results for the investigated BM samples are shown in *Online Supplementary Figure S1*.

Overall evaluation of concordance

Overall, we found good concordance between FC and PCR/FISH in flow-sorted cell populations from the BM follow-up samples. Cell populations with presumed presence of malignant cells were PCR/FISH-positive in 34 out of 36 BM samples analyzed (57 of 61 sorted cell populations analyzed, i.e. 93%), and the majority of sorted presumed normal cell populations (149 of 160 cell populations analyzed, i.e. 93%) derived from 51 of 52 BM samples were PCR/FISH-negative (Figure 1). This proves that MRD monitoring methods do indeed evaluate the same cells.

'Gray zone' cell populations with potential minor LAIP fractions were studied in 12 of the BM samples. These cell populations were positive in 9 of the 12 BM samples (13 of 17 cell populations analyzed), while such populations with PCR/FISH-negative results were found in 4 BM samples (from 4 patients) (Figure 1). The latter was possibly due to similarity between low levels of MRD and regenerating precursor B cells, the occurrence of which is a well-known pitfall of FC-MRD at late follow-up time points (*Online Supplementary Appendix*).[19,20] Thus, in such cases FC might provide slightly overestimated MRD levels, however with no clinical importance here.

In 11 BM samples from 10 patients, 16 cell fractions were initially flow-sorted on the basis of predefined empty spaces. However, the events did not form a cluster in all relevant plots, and back-gating on FSC/SSC dot plots showed a location outside the blast region. None of these were classified as MRD-cells in the standard MRD analysis. All these sorted populations were PCR/FISH-negative (Figure 1).

Four BM samples (from 4 patients) showed PCR-positive signals among sorted non-malignant cell populations, explained by relative high standard FC-MRD-values (MRD=1.3E-2, 4.3E-2, 7.8E-2 and 1.5E-1, respectively) and MRD cell populations that clearly overlapped with the applied 'normal-cell sort gate' (*Online Supplementary Appendix and Table S2*).

Discrepant cases

In a few cases, unexpected PCR/FISH-results in sorted cell populations were found; in 2 BM samples (from 2 patients) sorted presumed malignant cells showed discrepant PCR/FISH-negative results, and in 2 BM samples (from 2 patients) PCR-positive cells were detected among sorted presumed normal cells. These 4 cases were carefully reviewed in order to search for explanations and to evaluate the impact on the MRD estimate (Figure 2, *Online Supplementary Appendix and Table S2*). Possible explanations for the observed discrepancies were: a) only partly informative immunophenotypes at diagnosis resulting in MRD underestimation; b) antigen modulation; c) high number of plasma cells in patients with $CD10^{neg}/CD20^{neg}$ ALL; and d) dead cells in diagonal regions.

A discrepant case with heterogeneous LAIP ($CD34^{broad}$) at diagnosis emphasizes the importance of defining whether the FC-markers are fully informative (Figure 2A). Another discrepant case was possible due to significant CD10 downmodulation and CD20 upmodulation resulting in overlap between the MRD population and the mature B-cell population (Figure 2B). Patients with $CD10^{neg}/CD20^{neg}$ BCP-ALL are a special case. In such a patient, we found discrepant results probably due to the lack of distinction between MRD and plasma cells (Figure 2C). The appearance of plasma cells in higher fractions during ALL follow up has been described previously.[21] This highlights the importance of including antibody-markers allowing such a distinction by including CD38 (as in the current NOPHO ALL-2008 FC set-up) or the plasma cell-specific marker CD138. Some of the cell populations with discrepant results were derived from either BM samples with unavoidable presence in FSC/SSC gating of dead cells (leading to fluorescence background) reducing the discriminatory power by FC, or from BM samples with very few cells in total reducing the overall MRD-FC sensitivity (e.g. to only 1.0E-3) (Figure 2D). This illustrates the importance of using only BM samples of good quality for credible FC-MRD results. Addition of dyes to exclude dead cells in each tube is recommended.

Overall, in retrospect, we found that the samples with 'discrepant' PCR/FISH-results in flow-sorted cell populations were from patients in whom the suitability of FC-MRD had already been questioned at diagnosis or where the sample was of poor quality at the time of the standard MRD evaluation.

Immunophenotypic modulation of LAIP markers

We evaluated LAIP modulations in samples in which clearly detectable MRD levels (1.4E-3 to 2.1E-1) were present and in which the malignant state of the populations with modulated antigen expression was verified by flow-sorting and PCR/FISH. The LAIP modulations included CD10 downregulation (8 out of 9 patients at day 15, and 6 out of 7 patients at day 29) and upregulation of CD20 (5 out of 7 patients at day 15, and one out of 3 patients at day 29). Other changes observed were downregulation of CD34 and upregulation of CD45 and CD22 at early time points (*data not shown*). These modulation patterns, here verified by flow-sorting experiments, are in line with previous studies[15,16] and support the view that FC-MRD should not target only narrowly defined LAIPs but be adjusted according to the marker modulation induced by the actual treatment protocol.

Persisting bimodality

We previously found that bimodal expression of antigen markers, most often CD34 and CD10, is common at diagnosis in BCP-ALL.[13] In this study, 6 patients were characterized by: a) bimodal expression of CD34 (and CD10 in one patient) at diagnosis; b) inclusion of CD34 (or CD10, respectively) as informative marker for MRD detection; as well as, c) relative high MRD levels. In 7 follow-up BM samples from these patients (3 from day 15, 3 from day 29, one from day 96), the leukemic state of both immunophenotype-defined subpopulations was verified by PCR/FISH-positive results in flow-sorted cells (Table 1). In one sample, a small subpopulation at diagnosis became the dominating cell population at day 29, suggesting differential therapy sensitivity in $CD34^{neg}/CD34^{pos}$ subpopulations, as previous-

ly observed.[22,23] This persistence of subpopulations, at least at early follow-up time points, indicates that FC should target all subpopulations to avoid MRD underestimation. In complex cases with bimodal expression of two (or several) markers, disease monitoring might be supplemented with PCR-MRD.

In conclusion, this study confirms that the cells identified as MRD in BCP-ALL based on their LAIP are indeed the leukemic cells harboring the clone-specific genomic markers, and that the cell populations scored as non-malignant in general do not contain significant amount of leukemic cells. However, it is important to define up-front whether the FC-markers are fully informative at diagnosis by analyzing a higher number of cells (e.g. 100,000), and it must be recommended in the few BCP-ALL cases with non-optimal LAIPs to also support this with PCR-based MRD monitoring.

Authorship and Disclosures

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