Multicolor flow cytometric immunophenotyping for diagnosis of childhood precursor-B-ALL and monitoring of minimal residual disease

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Abstract

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most frequent cancer in childhood. Current treatment strategies induce complete remission in almost all patients. However, approximately one-third of patients suffer from relapse and most of these children die of this disease. Flow cytometric immunophenotyping is of high value both at diagnosis of BCP-ALL and for treatment monitoring – detection of minimal residual disease (MRD). In the current study, 70 bone marrow samples from childhood BCP-ALL patients were immunophenotyped with 3-color flow cytometry based on the BIOMED-I Concerted Action approach. Five triple monoclonal antibody combinations were used: TdT/C10/CD19 (I), CD10/CD20/CD19 (II), CD34/CD38/CD19 (III), CD34/CD22/CD19 (IV) and CD34/CD45/CD19 (V). Areas devoid of normal bone marrow cells (so called "empty spaces") were defined for each of the triple staining dot plots. In addition, further stainings were performed to investigate possible T-lineage (CD1a, CD2, CD5, CD7) and myeloid lineage (CD13, CD15, CD33) antigen co-expression. Every triple staining revealed different efficiency in detection of aberrant leukemic phenotypes ranging from about 44% for combination III and IV to as high as 96% for the combination II. The study confirmed relatively high incidence of myeloid marker coexpression - CD13 (24%) and CD33 (7%). The T-lineage markers (CD2, CD5 or CD7) were coexpressed rarely (6%). In conclusion, we could confirm the capability of the "empty spaces" method to detect abnormalities in antigen expression in BCP-ALL patients and to discriminate between normal and leukemic differentiation patterns in virtually all cases, which could be applied for MRD monitoring.

Key words: *acute lymphoblastic leukemia, immunophenotyping, flow cytometry, children, minimal residual disease.*

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Introduction

Detection of small numbers of persisting leukemic cells, i.e. monitoring of minimal residual disease (MRD) has strong prognostic value in childhood acute lymphoblastic leukemia (ALL) [1-3]. MRD status at the end of induction treatment is the most significant prognostic factor superior to previously identified risk factors such as age, blast count at diagnosis, immunophenotype at diagnosis, presence of chromosome aberrations, response to steroid prophase, and classical clinical risk group assignment. Moreover, children with high risk primary ALL and children with relapsed ALL subjected to allogeneic hematopoietic stem cell transplantation can profit from MRD monitoring [4, 5].

Currently three techniques can be successfully applied for specific and highly sensitive detection of MRD (sensitivity of at least one leukemic cell between 10000 of normal cells), namely multiparameter flow cytometric immunophenotyping, real-time quantitative polymerase chain reaction (RQ-PCR)-based detection of fusion gene transcripts or breakpoints, and

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RQ-PCR-based detection of clonal immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements [6-8].

Flow cytometry is particularly attractive technique of MRD monitoring because it is fast, cost-effective and measures single cells, which enables precise quantification [3]. Up till now, immunophenotypic MRD detection in ALL is mostly based on 3-4 color flow cytometry. This methodology relies on tracing the leukemia-specific immunophenotypes as the result of cross-lineage antigen expression, maturational asynchronous expression of antigens, antigen overexpression, absence of antigen expression, ectopic antigen expression (summarized in [6, 7]). BIOMED-1 consortium developed a new approach of multicolor flow cytometric MRD detection based on the observation that various combinations of aberrant features of leukemic cells bring the ALL blasts into the "empty spaces" between normal lymphoid differentiation [9-11]. In the current study we have evaluated BIOMED-I Concerted Action approach on a large series of consecutive childhood B-Cell Precursor (BCP) ALL patients.

Material and Methods

Patients

The study group consisted of 70 consecutively enrolled pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patients treated at the Departments of Pediatric Hematology and Oncology in Zabrze and Katowice of the Medical University of Silesia between 2002 and 2007. The age of the patients ranged from 1 month to 18 years. The male/female ratio was 1.5.

Sample collection and methods

The bone marrow samples were collected at diagnosis from all patients and processed within less than 10 hours after collection, generally within 1-2 hours. The samples were processed under standardized and optimized conditions. For surface antigen staining, 100 µl of sample was incubated for 20 min in room temperature with saturating amounts of relevant mouse anti-human monoclonal antibodies conjugated with fluorochromes. The monoclonal antibodies were conjugated with one of the following fluorochromes: FITC (fluorescein isothiocyanate), PE (phycoerythrin) or PE-Cy5 (phycoerythrin-cyanin 5). The full characteristic of used antibodies is listed in Table 1.

After the incubation step, erythrocyte lysis was performed by 10-minute incubation in lysing solution (BD FACSLysing Solution, Becton Dickinson, San Jose, CA, USA). Subsequently the sample was washed with modified PBS (CellWash, Becton Dickinson) and finally suspended in the volume of 0.5 ml. TdT as a nuclear marker was stained after surface staining and lysis steps, according to the manufacturer's instructions. Acquisition of data was performed in FACScan flow cytometer (Becton Dickinson) using the CellQuest software (Becton Dickinson) on Macintosh platform. The data analysis was performed in Paint-A-Gate software (Becton Dickinson).

Table 1. Characteristics of the fluorochrome-conjugated monoclonal antibodies used in the current study

Target	Clone	Fluorochrome	Manufacturer
CD1a	NA1/34	FITC	Dako[1]
CD2	S5.2	PE	BD[2]
CD5	L17F12	PE	BD[2]
CD7	M-T701	FITC	BD[2]
CD10	HI10a	PE	BD[2]
CD13	L138	PE	BD[2]
CD15	MMA	FITC	BD[2]
CD19	HIB19	PE-Cy5	BD Pharmingen[3]
CD20	L27	FITC	BD[2]
CD22	S-HCL-1	PE	BD[2]
CD34	8G12	FITC	BD[2]
CD38	HB-7	PE	BD[2]
CD45	T29/33	PE	Dako[1]
TdT	HT-6	FITC	Dako[1]

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Immunophenotypic data analysis

Diagnosis of BCP-ALL has been confirmed based on the standard morphological and immunophenotypic criteria [12]. We searched for aberrant immunophenotypes among BCP-ALL patients with 3-color flow cytometry and determined their relative frequencies. Because the pattern of antigen expression in normal bone marrow is highly reproducible, it was possible to create fixed images of normal B-cell differentiation profile by five triple monoclonal antibody combinations: TdT/C10/CD19 (I), CD10/CD20/CD19 (II), CD34/CD38/CD19 (III), CD34/CD22/CD19 (IV), CD34/CD45/CD19 (V) (Figure 1, Table 1) [10]. Areas devoid of normal bone marrow cells were defined on such designed dot plots (so called "empty spaces"), which served as the point of reference (coded as patterns A-F) [10]. Leukemic precursor B-cells localize in these areas due to aberrant antigenic expression (antigen over- or underexpression, and asynchronous or ectopic expression). All five triple stainings were applied to each patient. In addition to above-mentioned five triple stainings, further double and triple surface stainings were performed to investigate possible T-lineage (CD1a, CD2,

CD5, CD7) and myeloid lineage (CD13, CD15, CD33) antigen co-expression. The positivity of each cross-lineage antigen was assumed, if it was expressed on at least 20% of blasts.

Results

Aberrant BCP-ALL phenotypes

Particular monoclonal antibody combinations revealed different frequencies of aberrant leukemia-specific immunophenotypes, ranging from about 44% for combination III (CD34/CD38/CD19) and IV (CD34/CD22/CD19) to as high as 96% for the combination II (CD10/CD20/CD19). For the remaining triple stainings: I (TdT/CD10/CD19) and V (CD34/CD45/CD19), the percentages of disclosed aberrancies were 80 and 49%, respectively. The "empty spaces" within each combination were occupied with different frequencies. The summary of the immunophenotypic data is shown in Table 2.

In the (I) antibody combination, the most frequent aberrant immunophenotype observed (37.14%) was the one defined as "E" pattern representing $CD19^+$ cells with high-level expression of both CD10 and TdT ($TdT^{++}CD10^{++}CD19^+$) (see Figure 1). The immunophenotypes showing bright expression of one of the markers and dim of the other at the same time ($TdT^{dim/+}CD10^{dim/+/++}CD19^+$) occurred at lower frequencies (pattern C, D and F together – about 33%). The immunophenotype of dim expression of TdT and dim or lack of expression of CD10 was the least common ($TdT^{dim}CD10^{-/dim}CD19^+$, patterns A and B – 10%).

Table 2. The relative frequency of B-lineage aberrant phenotypes identified by five triple stainings. The values for each "empty space" are expressed as percentages of all cases and absolute counts (N). Total number of patients =70.

Empty space	I [TdT/CD10/ CD19]		II [CD10/CD20/ CD19]		III [CD34/CD38/ CD19]		IV [CD34/CD22/ CD19]		V [CD34/CD45/ CD19]	
	%	n	%	n	%	n	%	n	%	n
A	4.29	3	5.71	4	25.71	18	0.00	0	32.86	23
B	5.71	4	51.43	36	15.71	11	1.43	1	1.43	1
C	12.86	9	2.86	2	2.86	2	2.86	2	5.71	4
D	2.86	2	14.29	10	0.00	0	8.57	6	8.57	6
E	37.14	26	21.43	15			30.00	21		
F	17.14	12								
total	80.00	56	95.71	67	44.29	31	42.86	30	48.57	34

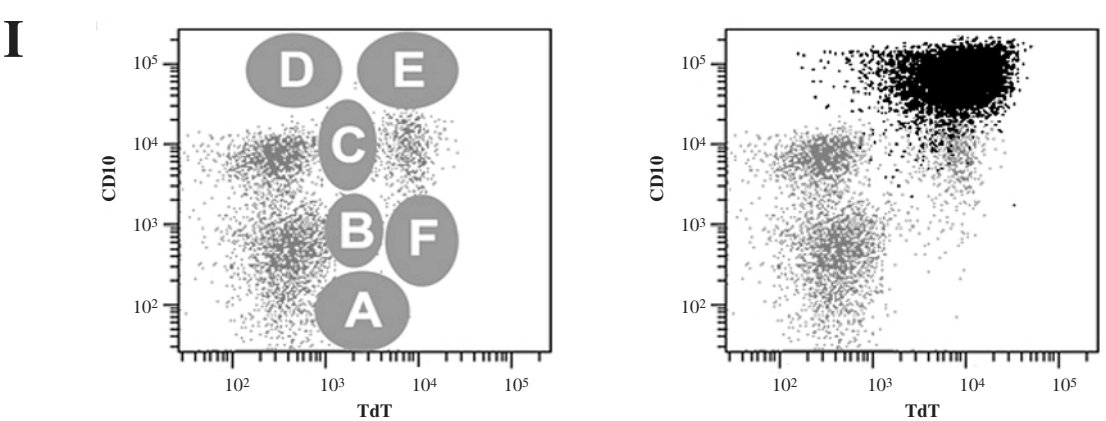
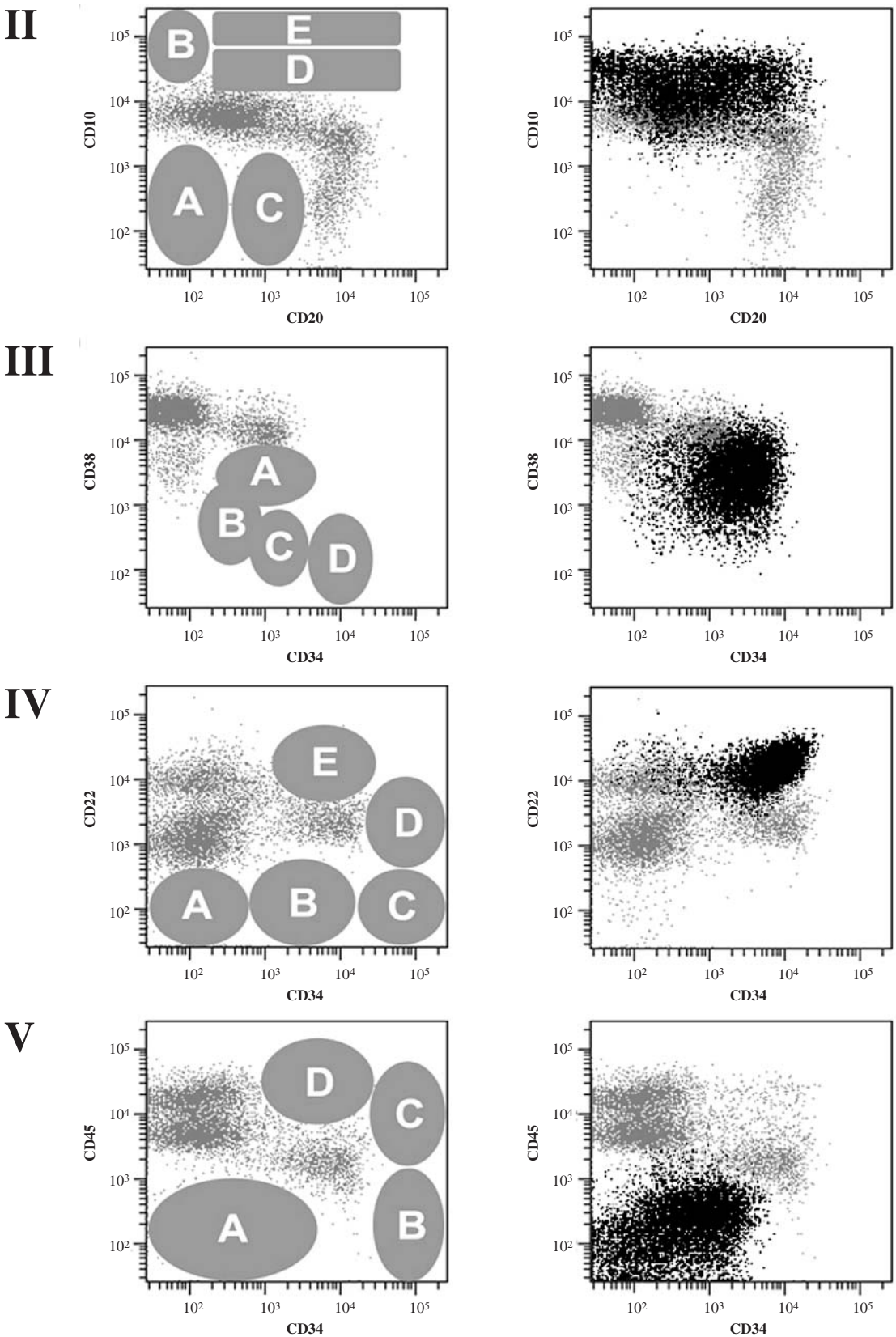


Fig. 1. Left column: localization of the "empty spaces" between normal bone marrow precursor-B-cells (depicted in grey). The "empty spaces" defined with letters A-F correspond to aberrant phenotypes in five triple stainings (I-V). Right column: examples of leukemic precursor-B-cells with aberrant phenotype distributed over one or more "empty space" (depicted in black). Normal cells are present in the background (grey dots)


II
III
IV
V
CD10
CD20
CD38
CD34
CD22
CD45
A
B
C
D
E
10^2
10^3
10^4
10^5

The (II) triple staining turned out to be the most informative for the discrimination between normal and leukemic cells. The most prevalent immunophenotype concerned CD10 overexpression by $CD20^-$ cells (pattern B – about 51%). Asynchronous expression of CD10 by CD20 dim to strong positive cells ($CD10^{+/++}CD20^{dim/+/++}CD19^+$) was also frequently observed (patterns D and E – about 36%). The least common was the immunophenotype of CD10 negativity with simultaneous underexpression or absence of CD20, which was detected in about 9% of cases (patterns A and C, $CD10^-CD20^{-/dim}CD19^+$).

The (III) antibody combination disclosed asynchronous dim or strong expression of CD38 by cells positive for CD34 in almost all detected aberrant cases ($CD34^+CD38^{dim/+}CD19^+$ – patterns A and B – about 41%). Only in 3% of cases dim expression of CD38 was accompanied by bright expression of CD34 (pattern C).

With the combination (IV) we observed mainly two different aberrant immunophenotypes. The most frequently expressed pattern E, occurred in 30% of cases and corresponded to the immunophenotype of bright CD22 expression on CD34 dim positive cells ($CD34^{dim}CD22^{++}CD19^+$). The other aberrant phenotype of bright positivity for CD34 and positivity for CD22 cells was revealed in about 9% of cases (pattern D). Aberrancies concerning negativity for CD22 with different level of CD34 expression were less frequently observed and together reached about 4% (patterns A, B and C, $CD34^{-/dim/+}CD22^-CD19^+$).

The last triple staining examined (V) revealed frequent occurrence of double negativity or double dim positivity for CD34 and CD45 cells (pattern A, $CD34^{-/dim}CD45^{-/dim}CD19^+$ – 33%). In addition, 14% cases showed bright expression of CD34 by CD45 positive cells (patterns C and D – $CD34^{+/++}CD45^+CD19^+$). There was only one case of CD45 negative cells overexpressing CD34 detected among $CD19^+$ cells (pattern B, $CD34^{++}CD45^-CD19^+$).

Aberrant cross-lineage expression in BCP-ALL cases

Using additional antibody combinations, coexpression of cross-lineage antigens was comprehensively studied. The highest coexpression incidence concerned myeloid markers – CD13 and CD33. Aberrant isolated expression of CD13 and CD33 was found in about 24 and 7% of cases, respectively. Simultaneous coexpression of these two markers was detected in 11% of cases. In contrast, expression of another myeloid marker CD15 was found in only 3 BCP-ALL cases (5%). The T-lineage markers were rarely coexpressed. In a total of 4 BCP-ALL cases isolated coexpression of CD2, CD5 or CD7 was demonstrated.

Discussion

In the present study we searched for aberrant immunophenotypes of blast cells among BCP-ALL patients, using five triple monoclonal antibody combinations and "empty spaces" approach as an analysis method [10]. The selected antibody combinations were applied to exhibit abnormal, i.e. asynchronous, ectopic, over-, or underexpression of antigens, hence, detection of any (as compared to the normal expression patterns – Figure 1) qualified the case to be pathological.

The method used, turned out to be fully appropriate to prove leukemia-specific aberrant immunophenotypes and to discriminate between normal and leukemic differentiation patterns in 100% of cases. The maximal percentage of cases in which pathological blasts were detectable by single staining was 96% (staining II, Table 2). Moreover, the use of five triple stainings revealed the existence of two different phenotypic aberrancies in over 94% of patients, three in about 64% and four in about 41%. The incidence of aberrant immunophenotypes in general was higher as compared to the reported by the other groups exploring the clinical value of flow cytometric investigation of MRD in ALL [2, 13-15]. For instance the single staining (with one monoclonal antibody combination) demonstrated an abnormal population in maximum 55% of cases in [10] and in 81% in [16]. In every triple staining there was one dominating "empty space" into which aberrant cells were falling. Some of the monoclonal antibodies, like CD10 were used in more than one staining (I and II) and in those cases the results correspondingly indicated high incidence of overexpression of CD10 (Table 2). Furthermore our results concerning the most frequent "empty space" within each antibody combination were concordant to as proved by BIOMED-I Concerted Action in all but staining I (TdT/CD10/CD19) [10].

In our study we also looked for cross lineage antigen expression on leukemic precursor-B-cells. It is commonly known that blasts in BCP-ALL sometimes coexpress antigens from other lineages, such as T-cell and myeloid lineages, which sometimes were assigned prognostic significance [17]. The frequency of such coexpressions reported in literature varies in a wide range, between 7 and 54% [2, 18] Our research revealed relatively high incidence of mostly dim coexpressions of myeloid markers CD13 and CD33 (total percent of cases with either of these markers – 42%), but not CD15. T-lineage markers were coexpressed at lower frequencies (about 5%). This observation is concordant with the previous notions that CD13 and CD33 are the most representative examples of cross-lineage antigen expression among BCP-ALL patients [10].

In conclusion, we can confirm the capability of the "empty spaces" method to detect abnormalities in antigen expression in leukemic patients. It can be noted that even the use of limited antibody panel can be of great diagnostic value, thus situating the method at the top of the simplest, fastest and most valuable diagnostic approaches [13]. However to gain even higher reliability of the technique, additional conditions must be fulfilled. One of the prospective solutions is to expand the antibody panels and enrich them in additional fluorochromes, preferably using simultaneously 6 to 8 colors [19, 20]. The possibility of

concurrent analysis of expression of antigens in new configurations would undoubtedly increase the sensitivity and specificity of MRD detection in ALL [21].

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