Optimizing detection of minimal residual disease in B-precursor acute lymphoblastic leukaemia by multiparameter flow cytometry

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INTRODUCTION

Flow cytometric (FC) detection of minimal residual disease (MRD) in acute lymphoblastic leukaemia (ALL) has generally been accepted as one of the most useful approaches, because of its speed, simplicity and sensitivity. From the clinical point of view immunological detection of MRD has been shown both to predict relapses and to discriminate high-risk patients (1–4).

Immunological detection of MRD is based on the expression of aberrant leukaemia associated phenotypes (LAP) that differentiate malignant cells from normal. The reported incidence of phenotypic aberrancies in B-precursor ALL ranges from 85 to 99% (5–9). First flow cytometric investigations of MRD were patient-specific assays depending on the blast immunophenotype detected at diagnosis. With constantly increasing application of flow cytometry for MRD detection in clinical laboratories this diagnostic strategy has become suboptimal because it is difficult to standardize between laboratories, it requires extensive specialist training and large antibody panels. Efforts were made to develop standardized, reproducible and cost effective FC techniques for MRD detection (8, 10, 11).

In our study we present flow cytometric MRD assay for B-precursor ALL developed at the Centre of Laboratory Diagnostics of Vilnius University Hospital Santariškių Klinikos, which is based on four-colour analysis of leukaemia-associated phenotypes and whole blood lysis sample preparation technique. Our purpose was to develop an optimal methodology for the investigation of MRD in B-precursor ALL that would be uniform, sensitive, and applicable for most patients and derived from the analysis of normal bone marrow.

MATERIALS AND METHODS

Between February 2004 and April 2006, 26 adult patients with newly diagnosed or relapsed B-precursor ALL were enrolled in this study: 19 males, 7 females, mean age 33.4 years. They were treated at the Centre of Haematology, Oncology and Transfusiology of Vilnius University Hospital Santariškių Klinikos according to the protocols corresponding to their risk groups.
Diagnosis of B-precursor ALL was based on the results of morphological examination of May-Grunwald-Giemsa stained smear of bone marrow aspirate, cytochemical reactions (myeloperoxidase and non-specific esterase), flow cytometry and cytogenetics (Ph chromosome).

Bone marrow (BM) aspirate taken into EDTA containing Vacutainer tube was used for the initial FC analysis of blast immunophenotype. Samples were prepared using standardized whole blood lysis technique proposed by NCCLS (12) and three-colour antibody panel (the list of monoclonal antibodies is given in Table 1).

Samples were analyzed by BD FACS Calibur and BD FACS Canto flow cytometers using Cell Quest and FACS Diva software. All the samples were processed within 24 hours.

10 000 events were collected for each tube. Blast population was defined in a SSC vs. CD45 dot plot. According to the European Group for the Immunological Characterization of Leukaemias (EGIL) recommendations positivity threshold for the surface markers was 20%, and for intracellular markers it was 10% (13).

B-precursor ALL diagnosis was established when leukaemic cells were positive for at least two B-lymphoid markers (CD19 and cytoplasmic CD79a), TdT and did not express surface immunoglobulin.

Before starting MRD assays we compared sensitivity of MRD detection using two sample preparation techniques: whole blood lysis and mononuclear density gradient separation. We diluted fresh B-precursor ALL bone marrow sample with a peripheral blood of a healthy donor making serial dilutions 1:10, 1:100, 1:1000, 1:10000 and 1:100000. Then we divided these samples into two aliquots. One was prepared using standard whole blood lysis technique, another was prepared using standard density gradient separation technique (13). TdT/CD19/CD34/CD45 staining was applied. Whole blood lysis technique was chosen for MRD assays.

For the detection of B-precursor ALL MRD we built a standardized four-colour antibody panel based on three-colour panel used in BIOMED-1 concerted action study (10) (Table 2).

Inclusion of at least one early marker is essential for the definition of blast population. As some cases of B-precursor ALL can be negative for CD34, the only early marker that is uniform for all B-precursor ALL cases is TdT (14). Besides, recent reports show blast shifts to the more mature phenotype during the treatment (15). So we included TdT into 4 of 5 tubes along with CD34 making the backup for an early marker. We included CD38 instead of TdT to the tube CD10/CD20/CD19 as the purpose of this tube in our study was not only to diagnose MRD, but also to check the regularity of regenerating B-cell population. This way any deviations from the haematogone maturation pattern in a CD20 vs. CD10 dot plot can be checked for the expression of CD38 which should be uniformly bright in regenerating B-cell population.

After developing marker panel and choosing sample preparation technique we examined bone marrow of healthy controls: 10 adult bone marrow donors (9 males, 1 female, mean age 32.9 years). Samples were analysed by BD FACS Calibur and FACS Canto flow cytometers using Cell Quest and FACS Diva software. For cell acquisition a "live" gate was set on cells that are CD19 positive and have low to intermediate side scatter (Fig. 1). One million events were acquired from each tube or acquisition was stopped when the tube ran empty, and the number of processed cells was recorded. Samples were analysed using standardized "3 × 3 empty block" strategy (Figs. 2a, 2b). The expression of each marker was divided into three levels: bright, dim and negative. So each two-parameter dot plot was divided into 9 "blocks". Some blocks were filled with regenerating B cells, and the rest were called "empty blocks".

Empty blocks according to the number of events within were divided into two levels:

– empty blocks with the highest sensitivity for MRD detection (events within those always compose less than $1 \times 10^{-5}$ of BM cells),

– empty blocks with sufficient sensitivity for MRD detection (events within those compose $1 \times 10^{-4} \sim 1 \times 10^{-5}$ of BM cells).

### Table 1. Monoclonal antibodies (BD, USA) used for the analysis of leukaemia immunophenotype

<table>
<thead>
<tr>
<th>Antibody group</th>
<th>CD classification</th>
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<tbody>
<tr>
<td>Non-specific markers</td>
<td>CD45, CD34, CD38, CD99, CD117, HLA-DR, TdT, CD10, CD56</td>
</tr>
<tr>
<td>Myeloid markers</td>
<td>CD13, CD14, CD15, CD33, CD11c, CD64, CD66c, MPO</td>
</tr>
<tr>
<td>T-lymphoid markers</td>
<td>CD1a, CD2, CD3, CD4, CD5, CD7, CD8</td>
</tr>
<tr>
<td>B-lymphoid markers</td>
<td>CD19, CD20, CD22, CD79a, IgM</td>
</tr>
<tr>
<td>Megakaryocytic markers</td>
<td>CD41a, CD61</td>
</tr>
</tbody>
</table>

### Table 2. Antibody panels for diagnostics of B-precursor ALL MRD used in BIOMED-1 study and in Vilnius University Hospital Santariskių Klinikos

<table>
<thead>
<tr>
<th>Antibody panel</th>
<th>CD classification</th>
</tr>
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<tbody>
<tr>
<td>TdT FITC/CD10 PE/CD19 PE-Cy5</td>
<td>TdT FITC/CD10 PE/CD34 PerCP-Cy5.5/CD19 APC</td>
</tr>
<tr>
<td>CD10 FITC/CD20 PE/CD19 PE-Cy5</td>
<td>CD38 FITC/CD10 PE/CD20 PerCP-Cy5.5/CD19 APC</td>
</tr>
<tr>
<td>CD34 FITC/CD38 PE/CD19 PE-Cy5</td>
<td>TdT FITC/CD19 PE/CD34 PerCP-Cy5.5/CD38 APC</td>
</tr>
<tr>
<td>CD34 FITC/CD22 PE/CD19 PE-Cy5</td>
<td>TdT FITC/CD22 PE/CD34 PerCP-Cy5.5/CD19 APC</td>
</tr>
<tr>
<td>CD19 FITC/CD34 PE/CD45 PerCP</td>
<td>TdT FITC/CD19 PE/CD34 PerCP-Cy5.5/CD45 APC</td>
</tr>
</tbody>
</table>
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BM samples of patients with B-precursor ALL for MRD detection were sent on days 11, 24, 44 and weeks 11, 16, 22, 30, 41 and 52 of treatment.

Since in case of B-precursor ALL only MRD in bone marrow but not in the peripheral blood has predictive value, the adequacy of sample is crucial, and dilution may have major interference to the results (16). Sample adequacy was checked by morphological examination of bone marrow smear. Sample was considered to be adequate for MRD testing when less than 5% of blasts were found and maturation of all cell lines was observed in BM. Morphologic complete remission was defined by the criteria proposed by Cheson et al: peripheral blood neutrophil count greater than 1.5 x 10^9/l, platelet count greater than 100 x 10^9/l, absence of leukaemic blasts in the peripheral blood, less than 5% blasts in BM, BM sample displays more than 20% cellularity with maturation of all cell lines and absence of extramedullary leukaemia (17).

Samples for MRD detection were prepared using whole blood lysis technique and a standardized four-colour B-lymphoid panel. For an acquisition CD19 positive "live" gate was applied. In all the samples it was possible to acquire at least 100 000 events, and this allowed to achieve a uniform sensitivity level of 1 x 10^-4. Samples were analyzed using standardized "3 x 3 empty block" strategy. At least 10 cells forming a cluster located in one or more empty blocks consistent with initial diagnosis were considered to be leukemic. Minimal residual disease size was calculated dividing the number of leukemic cells by total number of processed cells.

RESULTS

The dilution experiment demonstrated that the sensitivity of four-colour marker panel for B-ALL MRD detection was 1/10000 by both sample preparation techniques (Fig. 3).

In BM of healthy controls we identified 3 populations of normal B cells (Table 3):
- early haematogones that compose 0.08% of nucleated BM cells (TdT+, CD10+, CD19+, CD22dim, CD34+, CD38+, CD45dim, CD20-),
- late haematogones that compose 0.60% of nucleated BM cells (CD10+, CD19+, CD20dim, CD22dim, CD38+, CD45dim, TdT-, CD34-),
- mature B lymphocytes that compose 1.57% of nucleated BM cells (CD19+, CD20+, CD22+, CD34+, TdT-, CD10-, CD34-, CD38-).
Fig. 3. Dilution experiment showing detectable blast population in a titre 1:10 000 by whole blood lysis and mononuclear separation techniques. Blast cells (red) are located in an “empty block” P3 (23 and 22 events, respectively). Events in blocks P10 and P11 represent normal B cells.

Table 3. Antigen expression of normal B-cells in bone marrow (mean fluorescence intensity, ±SD)

<table>
<thead>
<tr>
<th>Fluorochrome labelled antibody</th>
<th>Early haematogones 0.08 ± 0.04%</th>
<th>Late haematogones 0.60 ± 0.03%</th>
<th>Mature B lymphocytes 1.57 ± 0.53%</th>
</tr>
</thead>
<tbody>
<tr>
<td>TdT FITC</td>
<td>212.0 ± 300.8</td>
<td>101.5 ± 34.7</td>
<td>80.8 ± 15.4</td>
</tr>
<tr>
<td>CD10 PE</td>
<td>1084.8 ± 2223.8</td>
<td>5405.5 ± 1667.2</td>
<td>109.0 ± 41.3</td>
</tr>
<tr>
<td>CD19 PE</td>
<td>4094.0 ± 1208.0</td>
<td>6235.5 ± 2224.2</td>
<td>7630.0 ± 2742.0</td>
</tr>
<tr>
<td>CD19 APC</td>
<td>5156.8 ± 854.4</td>
<td>6476.0 ± 754.2</td>
<td>8765.0 ± 2884.7</td>
</tr>
<tr>
<td>CD20 PerCP-Cy5.5</td>
<td>288.5 ± 79.4</td>
<td>9101.3 ± 1971.6</td>
<td>11291.3 ± 2666.3</td>
</tr>
<tr>
<td>CD22 PE</td>
<td>3824.3 ± 1182.5</td>
<td>4201.0 ± 1803.8</td>
<td>18712.3 ± 6449.7</td>
</tr>
<tr>
<td>CD34 PerCP-Cy5.5</td>
<td>1659.0 ± 640.6</td>
<td>82.3 ± 25.3</td>
<td>71.3 ± 34.9</td>
</tr>
<tr>
<td>CD38 FITC</td>
<td>14203.8 ± 2054.9</td>
<td>13144.5 ± 1401.2</td>
<td>1073.8 ± 275.8</td>
</tr>
<tr>
<td>CD38 APC</td>
<td>39783.3 ± 9075.6</td>
<td>39701.3 ± 9066.5</td>
<td>1848.8 ± 664.5</td>
</tr>
<tr>
<td>CD45 APC</td>
<td>3515.8 ± 1212.6</td>
<td>8322.5 ± 3183.6</td>
<td>25493.5 ± 9035.4</td>
</tr>
</tbody>
</table>

BM of healthy controls was also evaluated for the expression of myeloid antigens (CD13, CD33) on normal B cells. Dim partial expression of myeloid markers was found on more than 0.01% of normal bone marrow B cells (CD13 0.021%, CD33 0.019%, accordingly) while bright expression was detected on less than 0.01% B cells thus making bright expression of myeloid markers suitable for identification of leukaemic B lymphoblasts.

Phenotypic aberrations were found in all 26 B-precursor ALL patients, and their number ranged from 3 to 10 per case. We classified all phenotypic aberrations into four groups: asynchronous antigen expression, antigen hypoexpression, antigen hyperexpression and cross lineage antigen expression. The most common aberrations were hypoexpression of TdT (19 cases) and hyperexpression of CD34 (12 cases) (Fig. 4).

Analysis of healthy controls showed that cells with our identified aberrations were not found in normal BM (Fig. 5).

At least one myeloid antigen (CD13 or CD33) was coexpressed in 15 cases, but only in 3 cases they were homogeneous-ly present on the whole leukaemic population (CD33 in 3 cases and CD13 in 2 cases). The homogeneous expression of myeloid antigens remained stable also in cases with positive minimal residual disease (Fig. 6).

Other aberrations which in normal bone marrow were detected in percentage of 0.01–0.05% were considered not to be sensitive enough to define blasts as leukaemic (e.g. CD45bright CD34+, CD22bright CD34+, CD10+TdTdim).

MRD was detected in 20 patients out of 26. The most useful marker combination appeared to be TdT/CD19/CD34/CD45 and CD38/CD10/CD20/CD19. They revealed leukaemic population in 14 of 20 MRD positive cases (Fig. 7).

In 14 cases phenotype switches of residual leukaemic cells were found, but they still allowed differentiation from regenerating B cells. The most common phenotypic switch was down regulation of TdT (6 cases) (Fig. 8).
DISCUSSION

From the methodological point of view, standardized method for MRD assessment by flow cytometry does not exist. For sample preparation both density gradient separation and whole blood lysis techniques are used. Density gradient separation gives more numerous cell yields that result in a better sensitivity, but it has several disadvantages, e.g. increased amount of residual non nucleated cells, diminished efficacy of mononuclear separation by anticoagulant imbalance and prolonged storage. On the other hand, whole blood lysis technique allows us to have intact bone marrow cell composition and to calculate MRD as a percentage of all bone marrow nucleated cells the same way as it is done at the moment of diagnosis. This strategy gives more consistent evaluation of blast reduction process (18). The dilution experiment we made showed that whole blood lysis technique is sufficient to achieve the sensitivity of $1 \times 10^{-4}$ for B-ALL MRD detection.

Combinations of monoclonal antibodies used in BIOMED-1 concerted action "Investigation of minimal residual disease in acute leukaemia: international standardization and clinical evaluation" provided the identification of aberrant immunophenotypes in 98% of B-precursor ALL cases and the concordance between laboratories was >90%. They established immunophenotypes of normal regenerating B-cells and identified "empty spaces" of dot plots where minimal residual disease can be located. We adopted this standardized methodology modelling the

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**Fig. 4.** Most common phenotypic aberrations in B-precursor ALL patients

**Fig. 5.** Maturation pattern of B-lymphocytes in normal BM. Cells with aberrant phenotypes (blocks P4, P5, P7, P8, P10) compose less than 0.01% of BM cells
Fig. 6. Coexpression of CD33 on leukaemic B-lymphoblasts at different time points of therapy, gated on CD19+ cells

Fig. 7. Most useful marker combinations for the detection of MRD
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Fig. 8. Phenotypic switches of leukaemic B-lymphoblasts during treatment.

Panel according to the results of prevalence of immunophenotypic aberrations in our patients. As TdT was one of the most common abnormally expressed markers, we included it to the most tubes of the panel. TdT is an intranuclear enzyme that catalyses the addition of deoxyribonucleotides triphosphates to 3'-ends of DNA breaks without the need of a template strand (19). High frequency of TdT positive B-precursor ALL cases suggests that it might be a useful marker for the identification of abnormal B cell maturation. However, our later results showed that it is also one of the most frequently downregulated markers. Although TdT is an intranuclear marker, our dilution experiment proved that cell and nuclear membrane permeabilization does not result in a significant cell loss, and major changes of a light scatter and the sensitivity level of 1:10000 may still be achieved. So in spite of the fact that the applicability of TdT is not yet proven and needs further investigations we still suggest that it is useful as an indicator of the early stage of B-cell development.

Estimation of B-cell development by means of flow cytometry derived from studies of B- acute lymphoblastic leukaemia (20, 21). More recently studies have focused on investigation of normal bone marrow (22). Technical advances such as the development of multiparameter flow cytometry, the discovery of new fluorochromes with high sensitivity emission signals, optimization of intracellular staining protocols have contributed to better insight in B cell maturation stages. One of the most extensive studies that focused on B cell development was performed by Lucio et al. (23). Their aim was to investigate B cell differentiation in normal bone marrow by standardized triple staining. They divided B cell compartment into two parts: earliest CD19- progenitors and later CD19+ progenitors. Two subpopulations of CD19- progenitors were separated: CD34+/CD22+ and TdT+/CD10+ representing 0.11 ± 0.09% and 0.04 ± 0.05% of BM cells, respectively. In our study we concentrated only on CD19+ B cell development because our main purpose was to discover differences between normal and neoplastic B cells that are virtually always CD19+. In Lucio study CD19+ progenitor population was further subdivided into three maturation stages the same way as we did in our study. The earliest CD10+CD34+TdT+ subpopulation represented 0.44 ± 0.65% and 0.08 ± 0.04%, the intermediate CD10+CD34-TdT- subpopulation represented 3.75 ± 5.75% and 0.6 ± 0.03%, and the most mature CD10-CD34-TdT- subpopulation represented 2.58 ± 1.43% and 1.57 ± 0.53% of BM cells in Lucio and our studies, respectively. Lower values of all B cell populations in our study might have been the result of different donor age as children were not included in our group of healthy controls. Lucio study showed that during maturation TdT, CD34 and CD10 expression diminishes while CD20 and CD45 expression increases. This stands in line with our results. We found a clear distinction between the mean fluorescence intensity (MFI) of CD34 in early haematogone and late haematogone populations. CD10 was still positive on late haematogones, but it was considerably weaker. As in Lucio study, we found an increased CD20 and CD45 expression on late haematogone population. CD38 and CD22 expression was similar between two haematogone populations in both studies. The main differences between haematogones and mature B cells were the loss of CD10 and CD38 and increase in CD22 and CD45 expression.

Abnormalities of blast immunophenotype compared to normal bone marrow cells serve as a basis for the flow cytometric MRD detection. Leukaemic cells historically were considered to be “frozen” in a certain stage of maturation after loosing their ability to differentiate (14). However, more recent studies showed that in certain antigen expression leukaemic cells actually differ from normal cells that regenerate
CONCLUSIONS

Our study confirmed that four-colour flow cytometry can be considered as one of the most optimal tools for MRD detection in B-precursor ALL. In all B-precursor ALL patients we identified numerous phenotypic aberrations suitable for MRD detection, which were roughly subdivided into four groups: asynchronous antigen expression, antigen hypoexpression, antigen hyperexpression and cross lineage antigen expression. Standard marker panels we developed covered most of these aberrations, even when phenotypic switches occurred. In B-precursor ALL cases with prominent coexpression of myeloid markers we also suggest to use additional combinations to include them. This approach makes flow cytometry applicable for MRD investigation in most B-precursor ALL cases. Flow cytometry showed sufficient sensitivity of $10^{-4}$ by two sample preparation methods (whole blood lysis and density gradient separation). Our MRD assay was validated on normal bone marrow samples. It allowed us to establish three populations of B cells with distinct phenotypes: early B haematogones, late B haematogones and mature B cells. Phenotype of normal B cells served as a basis to define phenotypic aberrations.

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in bone marrow (24–26). These discrepancies were started to be used to monitor MRD for patients who achieved morphological complete remission (<5% of blasts in bone marrow assessed by morphological methods). Aberrations can manifest as asynchronous antigen expression, cross-lineage antigen expression, antigen hyperexpression, antigen hypoexpression or its absence. Such leukaemia-associated immunophenotypes can be identified in the vast majority of acute leukaemia patients. In our study we were able to identify immunophenotypic aberrancies in B-precursor ALL patients. This finding stands in line with the reported incidence of immunophenotypic aberrancies in B-precursor ALL that ranges from 85 to 99% (5–7). Considering individual markers CD10 is reported to be the most common abnormally expressed marker, followed by CD22, CD38 and TdT (11). In our hands CD10 expression was also the most frequently abnormal (hypoexpressed in 10 cases, hyperexpressed in 11 cases), followed by TdT which was hypoexpressed and CD34 which was hyperexpressed.

Cross-lineage antigen expression in our study was mostly represented by pan-myeloid markers CD13 and CD33. The reported incidence of these markers in B-ALL cases varies. In BIOMED-1 concerted action report overall incidence of CD13 and/or CD33 expression in B-precursor ALL was 39.6% while in our study it was 57.7% (15 cases of 26), thus making these markers suitable for MRD detection for a substantial fraction of patients. Besides, we found that myeloid markers were downregulated only in a small proportion of patients. Bright expression of myeloid markers was stable in all the cases.

Immunophenotypic switches that occur during the course of disease are considered to be the main disadvantage of flow cytometry and they may concern up to 90% patients (27–29). However, at least one aberrance is usually maintained in relapse in at least 80% of cases (30). This proves the importance to use at least two leukaemia-specific marker combinations for each patient to monitor MRD. Our results support these findings: immunophenotypic switches occurred in 14 of 20 (70%) of MRD positive cases but in all the cases it was still possible to differentiate between leukaemic and normal cells. Gaipa et al. (15) in the study of drug-induced phenotype changes discovered that the expression of early markers CD34 and CD10 tends to be downregulated, and the expression of more mature markers CD20, CD19, CD45RA, CD11a tends to be upregulated. The reason for this might be the administration of steroid hormones during the induction therapy that are potent regulators of gene transcription. Alternatively, it may be that more mature leukaemic cell subsets that express low levels of early markers and which are poorly represented at diagnosis could withstand the treatment and constitute the majority of MRD mimicking antigen modulation. Selective depletion of more immature cell subsets was demonstrated by Stahnke et al. (31) who proposed that they are more susceptible to drug-induced apoptosis. In our hands the most frequent immunophenotypic switch was downregulation of TdT which is also an early marker, so this is concordant with the results of the above studies.


Miglė Janeliūnienė, Rėda Matuzevičienė, Laimonas Griskiūnas, Zita Kučinskenė

ÜMINĖS B-PREKURSIORŲ LIMFOBLASTINĖS LEUKEMIJOS MINIMALIOS LIKTINĖS LIGOS TYRIMO DAUGIAPARAMETRĖS TĖKMĖS CITOMETRIJA OPTIMIZAVIMAS

Santrauka

Problemos aktualumas. Tėkmės citometrija yra įtariu ir praktiškas minimalios liktinės ligos tyrimo metodas ūmine limfoblaste ligui skirta siekiant mokslinių rezultatų. Tačiau nepaisyta, kaip metodas svarbus ir plačiai prieinamas, nera vieningosio metodologijos. Mūsų tikslas buvo optimizuoti minimalios liktinės ligos tyrimo tėkmės citometrijos metodą. Šiame straipsnyje pateikiami informacijos apie B-prekursorių limfoblastinės leukemijos atvejai.


Išvados. Keturspalvė tėkmės citometrija ūminės B-prekursorių limfoblastinės leukemijos atveju yra pakankamai efektyvus metodas minimaliai liktinei ligai tirti; jis gali būti taikomas beveik visiems pacientams, yra pakankamai jautrus ir leidžia patikimai atskirti leukemines B-limfoidines ląstes nuo regeneruojančių, net jei keičiasi leukeminių ląstelių fenotipas.

Raktažodžiai: minimali liktinė liga, tėkmės citometrija, ūminė B-prekursorių limfoblastinė leukemija