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Monitoring of T-cell Acute Lymphoblastic Leukemia by Flow Cytometry

Research Article

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Abstract: Minimal residual disease (MRD) predicts the outcome of acute lymphoblastic leukemia (ALL). Flow cytometry (FC) is one of the most sensitive and most applicable methods for MRD diagnostics, but there is still no agreement on the "gold standard" of the method. We tried to optimize flow cytometric MRD detection in T-ALL. Fourteen adults and 11 children with T-ALL and 12 normal bone marrow (BM) donors were enrolled in the study. We found that the most common phenotypic aberrations in T-ALL were TdT and CD99 coexpression on T-cells in BM. Therefore for MRD detection we developed a limited four-color marker panel (TdT/CD7/cCD3/CD19 and CD99/CD7/cCD3/CD2) and a standard analysis strategy. This assay was evaluated on BM of healthy controls. Less than 0.01% TdT+ or CD99 bright T-cells were found in normal BM. MRD was detected in 9 adult patients and 1 child at different time-points of treatment. The average TdT and CD99 mean fluorescence intensity (MFI) value of residual blasts fluctuated during therapy, but it still remained higher than MFI of normal T-cells. Our established MRD detection method differentiated leukemic lymphoblasts with sensitivity in the range of 0.01% and did not give any false positive results in normal BM.

Keywords: *Minimal residual disease • flow cytometry • T-ALL • CD99 • TdT*

1. Introduction

Modern treatment protocols induce complete remission defined by clinical and morphological criteria in a high proportion of patients with T-cell acute lymphoblastic leukemia (T-ALL). Some of these patients eventually relapse as submicroscopic amounts of malignant cells (minimal residual disease, MRD) persist and subsequently grow. A number of studies showed that presence of detectable MRD at any stage of therapy can predict relapse in childhood and adult ALL [1-5]. Several methods are used for the detection of MRD, the most sensitive and most widely applicable being polymerase chain reaction (PCR) and multiparameter flow cytometry (FC) [6,7].

PCR assays for T-ALL MRD detection are usually based on identifying allele specific rearranged T-cell receptor (TCR) genes of leukemic subclones. The principle advantages of this method are its broad applicability and high sensitivity, but it might also have several disadvantages, such as high complexity, possible loss of rearrangements and the interference of

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normal cells. Leukemia specific gene rearrangements could provide even higher sensitivity by PCR, but they are found only in a minority of T-ALL patients [8,9]. Several studies showed that both flow cytometry and PCR are equally effective in the detection of minimal residual disease in ALL [6,10,11].

Detection of MRD by FC is based on the expression of aberrant leukemia associated phenotypes (LAP) that differentiate malignant cells from normal ones. In the case of T-ALL FC might seem to be an efficient and not very complicated tool for MRD diagnostics, since most often it simply consists of identification of T-cells with immature phenotype, e.g. CD3+TdT+, outside the thymus (ectopic phenotype) [12]. With this approach it is possible to detect one leukemic cell in 10 000 and about 90% of ALL cases could be followed [13].

Despite the apparent simplicity of an assay, several technical problems exist, such as nuclear detection of TdT, possible loss of aberrations, etc. Recently some surface markers such as CD99 have been shown to be of high value in T-ALL MRD detection [14]. Nevertheless, there is still no agreement on the "gold standard" of the method.

Our study presents an attempt to standardize flow cytometric detection of MRD in T-ALL: a flow cytometric MRD assay with a limited set of four-color markers, representing the most common leukemia associated phenotypes in T-ALL, and a standardized analysis technique.

2. Material and Methods

2.1. Patients

Twenty-five patients with T-ALL were enrolled in the study from 2006 to 2008: 14 adults (12 males, 2 females) and 11 children (8 males, 3 females). Mean age of adults was 28.2 years (17-50) and mean age of children was 8.0 years (17 months – 16 years). Twelve normal adult BM donors were included as a control group (10 males, 2 females, mean age 34.4 years). Patients were treated at the Vilnius University Hospital "Santariškių Klinikos" and Vilnius University Children Hospital according to hospital established standard treatment protocols corresponding to their risk groups. MRD assays were carried out at the time points consistent with treatment protocols; on average patients were followed for 18.2 months and 6.2 MRD tests were carried out per patient. The study was approved by the Lithuanian Bioethics Committee.

2.2. Flow cytometry

2.2.1. Specimen

All assays were performed on BM samples obtained by standard BM puncture procedure and anticoagulated with K_3 EDTA.

2.2.2. Sample preparation

All diagnostic samples, MRD samples and samples of healthy controls were prepared by lysed whole blood technique. In the dilution experiment both mononuclear density gradient separation and lysed whole blood techniques were used.

For whole blood sample preparation with cell membrane permeabilization and red cell lysis, the *Fix&Perm (Invitrogen)* cell fixation and permeabilization kit was used. To stain cell surface antigens 100 µl of anticoagulated bone marrow were incubated with 20 µl of pretitrated (1:4) antibodies for 20 minutes in the dark at room temperature. Then 100 µl of fixation medium were added and samples were incubated for 15 minutes in order to fix leukocytes and lyse red cells. Afterwards cells were washed in 3 ml of a wash buffer (*BD CellWash* with 0.5% fetal bovine serum) at 350 g for 5 minutes. 100 µl of permeabilization medium and 20 µl of pretitrated (1:10) antibodies for intracellular or intranuclear staining were added, samples were incubated for 20 minutes and then washed again under the same conditions. After aspirating the supernatant, cells were resuspended in 500 µl of prediluted (1X) paraformaldehyde-based *CellFix (BD)* solution. Samples were analyzed within 24 hours.

To separate mononuclear cells, the bone marrow sample was diluted 2:1 with PBS buffer. 4 ml of *Lymphoprep* (*Nycomed*) medium was placed in 15 ml conical tubes and 6 ml of diluted bone marrow was overlaid on the top. Tubes were centrifuged for 30 minutes at 400 g at room temperature. The mononuclear layer was aspirated and washed twice in *BD CellWash* for 10 minutes at 300 g. Cell count was measured with a hematological analyzer and a cell suspension containing approximately 10 000 cells per µl was prepared. Staining was performed the same way as with the whole blood procedure.

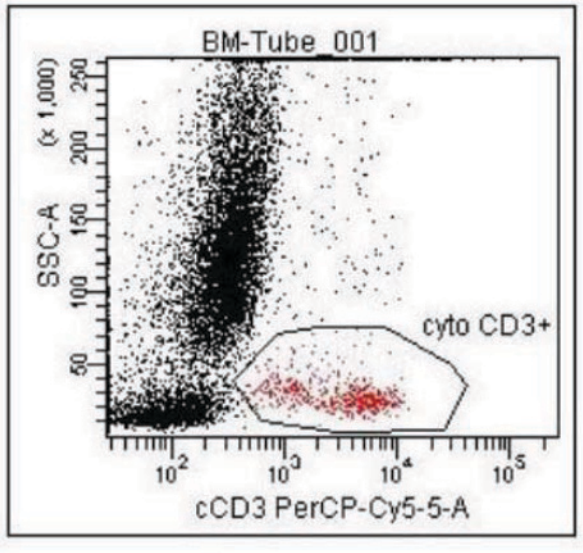
2.2.3. Monoclonal antibodies

Four-color markers labeled with FITC, PE, PerCP-Cy5.5 and APC were used. For diagnostics of T-ALL a broad marker panel was applied including precursor markers (CD34, TdT, CD99, CD117, CD10), T-cell markers (CD1a, CD2, CD3, CD4, CD5, CD7, CD8), B-cell markers (CD19, CD20, CD22, CD79a), myeloid markers (CD13, CD14, CD15, CD33, CD64) and isotype controls.

Table 1. Marker panel for T-ALL MRD detection.

FITC	PE	PerCP-Cy5.5	APC
TdT (E17-1519, BD Pharmingen)	CD7 (M-T701, BD)	Cytoplasmic CD3 (SK7, BD)	CD19 (SJ25C1, BD)
CD99 (TÜ12, BD Pharmingen)	CD7 (M-T701, BD)	Cytoplasmic CD3 (SK7, BD)	CD2 (S5.2, BD)

Figure 1. "Live-gate" for an acquisition of cCD3 positive events: a region is set on a cCD3 positive cells with a low-to-intermediate side scatter, cells in this region are saved for subsequent analysis while totally acquiring up to 1 million cells.



To detect MRD we built a limited panel based on the most common T-ALL phenotypic aberrations (Table 1).

2.2.4. Sample acquisition and analysis

Samples were analyzed on *FACSCalibur (BD)* and *FACSCanto (BD)* flow cytometers using *CellQuest* and *FACS Diva* software.

In diagnostic samples 10 000 cells were acquired from each tube. Blasts were identified as cells with low to intermediate side scatter properties and dim CD45 expression. Markers were considered to be positive when they were expressed on more than 20% of cells. T-ALL was defined by cytoplasmic or surface expression of CD3 in blast population.

For cell acquisition in MRD samples, a "live gate" was set on cytoplasmic CD3 (cCD3) positive cells and one million events were acquired or acquisition was stopped when the entire sample was aspirated (Figure 1).

Afterwards cells from tube 1 expressing CD19 were excluded from analysis (Figure 2) and all other cells were analyzed using "2x3 empty block" strategy.

CD7/TdT and CD7/CD99 2D dot plots were divided into 6 regions according to the antigen expression level: CD7 positive and CD7 negative, TdT and CD99 negative, dim and bright. Some of the blocks were filled with populations of normal T-cells, and the rest were ascribed to "empty blocks" (Figure 3).

Figure 2. Exclusion of CD19 positive events: a rectangular region is set on CD19- events, and only those are analyzed further.

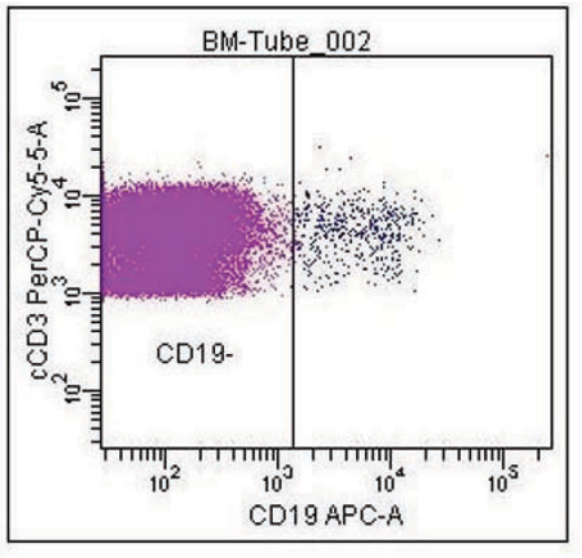


Figure 3. "2x3 empty block" analysis strategy: P4, P7 and P5 are blocks where normal T-cells can be found; P6 and P9 are blocks definitive for T-lymphoblasts; and P8 is an empty block, but T-lymphoblasts are not usually located here. In this case most T-lymphoblasts are located in block P6.

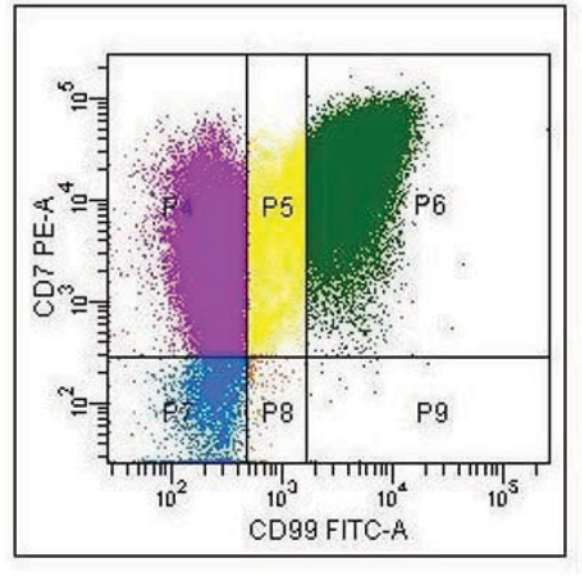


Figure 4. The most common phenotypic aberrations in T-ALL.

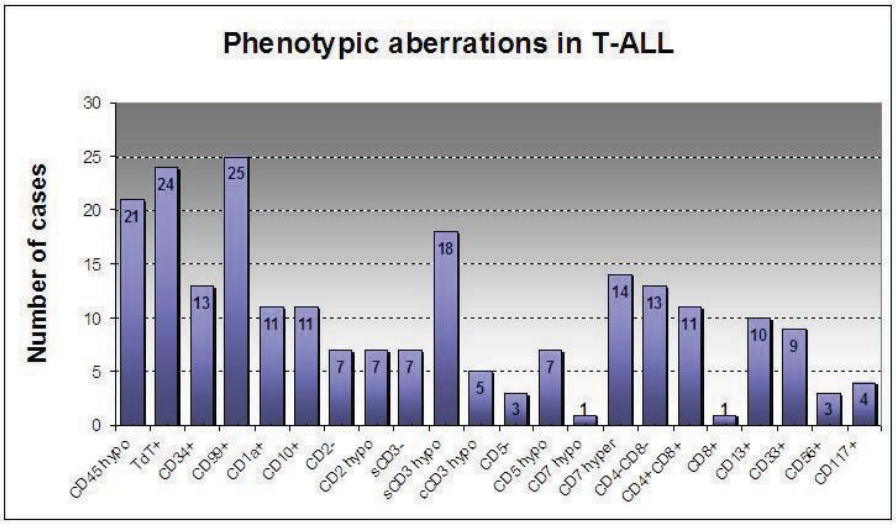


Table 2. Comparison of TdT and CD99 MFI between T-lymphoblasts and T-cells of BM donors using t value.

Parameter	T- lymphoblasts		T-cells of BM donors		t test result	
	Mean	Standard deviation	Mean	Standard deviation	t value	p value
MFI TdT	812,2143	729,35790	98,7500	18,16653	3,659	0,003
MFI CD99	7984,6667	4580,28503	614,4545	113,21781	5,572	0,000

For T-ALL MRD enumeration cell count in an empty block was divided by the total count of acquired cells; only cells with a proper side scatter and forward scatter features were acquired and described as viable nucleated cells. Cells within an empty block were defined as MRD only when they formed a cluster and there were more than 10 of them. Only in 2 cases did we have MRD as a 10-cell cluster; in all other cases there were more than 20 pathological cells.

2.2.5. The dilution experiment

BM sample from a patient with T-ALL was diluted in the peripheral blood of a healthy donor in titers of 1:100, 1:1000, 1:10 000, 1:100 000 and 1:1 000 000. Afterwards 2 sets of samples were prepared applying 2 different sample preparation techniques: lysed whole blood and mononuclear density gradient separation. Marker combination CD99/CD7/cCD3/CD2 was used. Data were analyzed the same way as detecting MRD.

2.2.6. Analysis of normal BM

In normal BM, TdT and CD99 mean fluorescence intensity (MFI) of the T-cell population was recorded. Then data were analyzed using "2x3 empty block" strategy. T-cells were classified by their phenotypic features into 3 groups and their quantity was calculated. Also "empty" blocks containing less than $1x10^{-4}$ cells were defined.

2.3. Statistical Analysis

Statistical analysis was carried out with SPSS software. To compare TdT and CD99 MFI of T-lymphoblasts and T-cells of BM donors, unpaired samples t-test was used. The average TdT and CD99 MFI values of residual T-lymphoblasts at different therapy time-points were compared to the MFI of T-lymphoblasts at the time of diagnosis using Friedman test (χ^2).

3. Results

At the time of diagnosis all T-ALL cases had at least one phenotypic aberration suitable for MRD follow-up. The most common aberration was TdT and CD99 expression on T-cells in BM (Figure 4). Another marker of early differentiation CD34 was found only in 52% of T-ALL cases.

The different pattern of TdT and CD99 expression between T-cells of healthy donors and T-lymphoblasts was shown to be statistically significant by comparing mean fluorescence intensities (MFI) and using t value (Table 2).

Serial dilutions of T-ALL BM sample in normal peripheral blood up to titer 1:100000 showed that sensitivity in the range of 10^{-4} could be achieved by both whole blood lysis and mononuclear density gradient

Figure 5a. T-ALL dilution 1:10 000, lysed whole blood. "2x3 empty block" analysis strategy: population of leukemic T-lymphoblasts located in an empty block P6 makes 0.04% of acquired nucleated cells.

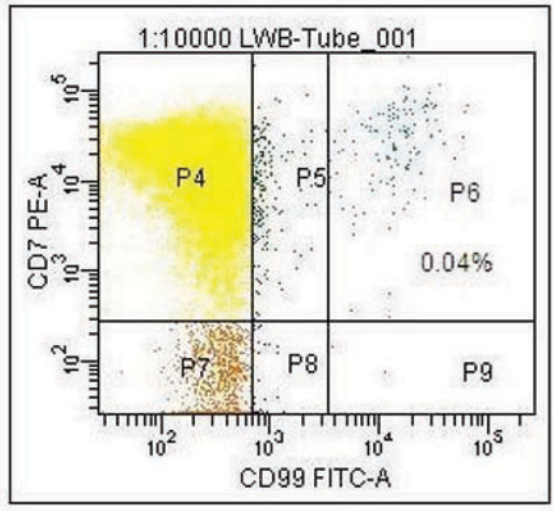


Figure 5b. T-ALL dilution 1:10 000, mononuclear density gradient separation. "2x3 empty block" analysis strategy: population of leukemic T-lymphoblasts located in an empty block P6 makes 0.03% of acquired mononuclear cells.

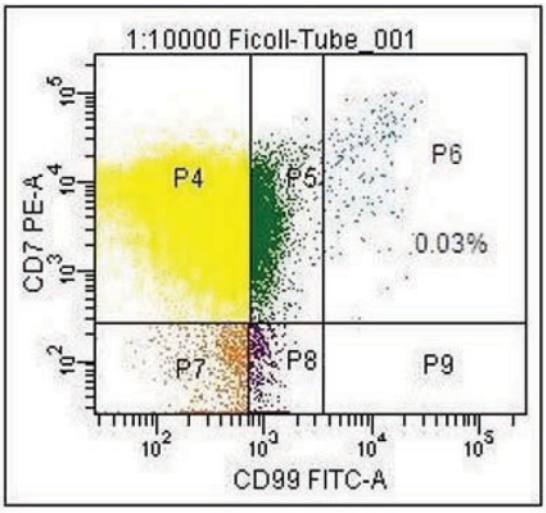
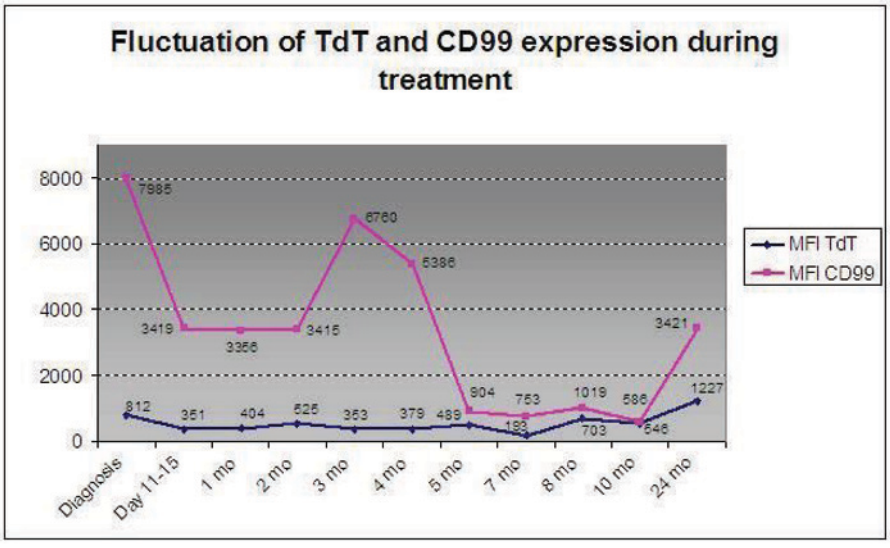


Figure 6. Variation of average TdT and CD99 MFI values of residual blasts during treatment.



separation techniques (Figure 5a, b).

The average amount of cCD3+ events in normal BM was 4.21% ± 1.95% of all BM cells.

In BM of healthy controls we identified 3 populations of normal T-cells:

- CytoCD3+CD7+TdT-CD99- T lymphocytes (3.81% ± 2.67% of all BM cells)
- CytoCD3+CD7+TdT-CD99dim T lymphocytes (0.18% ± 0.09% of all BM cells)
- CytoCD3+CD7-TdT-CD99- T lymphocytes (0.17% ±0.16% of all BM cells)

Cell amount in TdT dim ($0.9x10^{-4}$ ± $0.4x10^{-4}$) and bright ($0.0x10^{-4}$) and CD99 bright ($0.4x10^{-4}$± $0.4x10^{-4}$) blocks was lower than $1x10^{-4}$ thus making these regions applicable for MRD detection.

The amount of cCD3+CD19+ events in normal BM was $3.7x10^{-4}$ (±$3.4x10^{-4}$). This finding indicated that to achieve the sensitivity of an assay above this level, these events had to be excluded from the analysis in samples of patients.

Minimal residual disease was detected in nine adults and one child with T-ALL.

Average TdT MFI value of residual blasts fluctuated during treatment: starting from 812, it fell to 193 in the 7th month of treatment and increased again to the limit of 1227 in the 24th month. CD99 MFI altered even more: from the

initial value of 7985, it decreased to 586 in the 10th month and later increased again to 3421 in the 24th month (Figure 6). Nevertheless Friedman test did not reveal any trends: χ^2 was 1,600 in the case of MFI TdT (p=0,809), and it was 5,600 in the case of MFI CD99 (p=0,231).

4. Discussion

It is generally assumed that malignant blasts reflect phenotypes of normal cells in early stages of differentiation. T-cells co-expressing precursor markers (e.g., TdT) should normally be found in the thymus and their appearance in bone marrow is definitive of T-ALL [15,16].

Thus combination of an early marker (TdT, CD34, CD99) with a T-cell marker (CD2, CD3, CD5, CD7) is used for T-ALL MRD detection most widely, such as: TdT/CD5/cCD3 and CD34/CD5/cCD3 in Campana's study [17]; TdT/CD7/sCD3/cCD3, CD99/CD7/sCD3/cCD3 and CD99/CD7/CD5/CD3 in Dworzak's study [14]; or cCD3/TdT in Krampera's study [18].

However, it is obvious that T-ALL MRD detection by flow cytometry varies from laboratory to laboratory. One of the controversies in MRD detection by flow cytometry is selection of sample preparation technique. Density gradient separation yields more numerous cell populations and implies better sensitivity, but it has several disadvantages, such as increased amount of residual non-nucleated cells or selective loss of different cell populations. Anticoagulant imbalance or prolonged sample storage may influence the efficacy of this technique. Also the staining qualities of the cells can be affected. On the other hand, whole blood lysis allows us to examine intact cell composition of the sample and to characterize the population of interest as a percentage of all nucleated cells, not to mention it requires a smaller sample volume and a shorter and less complicated preparation procedure [19,20]. The dilution experiment we performed in our study showed that sensitivity in the range of 10^{-4} can be achieved with both techniques, so we selected whole blood lysis mainly to be able to explore all nucleated BM cells.

In addition, there are some other aspects that are not yet unified: different laboratories use different numbers of markers and their combinations, as well as different analysis strategies. Laboratories can choose a standardized way to test MRD or an individualized one depending on the patient's initial phenotype. In this situation a different sensitivity of assays might be achieved or results of different laboratories might not be comparable.

To improve and standardize the flow cytometric MRD detection, Biomed-1 Concerted Action was initiated [21]. Within this project normal bone marrow T-cells were investigated by flow cytometry to build a basis for the minimal residual disease detection. Five triple stainings were used – CD7/CD5/CD3, CD7/CD4/CD8, CD7/CD2/CD3, CD7/CD38/CD34 and TdT/CD7/CD3 – and the lysed whole blood sample preparation technique was applied.

We followed this model in our study, just with an approach towards a more limited antibody set. In our study we chose 2 four-color combinations: TdT/CD7/cCD3/CD19 and CD99/CD7/cCD3/CD2. We used CD19 to exclude TdT positive CD19 events admixed to cCD3+ gate. CD2 was included because it was hypoexpressed or negative in a considerable proportion of patients (56%). Our panel worked well for all our T-ALL patients and did not give any false positive results in normal BM. Our results were in line with those of the Biomed-1 group results. The most common aberrant phenotypic pattern in their study was found to be TdT+CD7+ (91%) and the CD34+CD7+ phenotype was less common (40%). In comparison, TdT was expressed in 96% and CD34 was expressed in 52% of our T-ALL cases. In our study 56% of cases had CD7 hyperexpressed, whereas single hyperexpression of CD7 was discovered in 14% of the Biomed-1 group patients (they identified CD2dim/negCD3-CD7+++ as a rare phenotype that we could not identify because of a different acquisition procedure with a live-gate on cCD3). CD2 was hypoexpressed in 56% of our T-ALL patients and 62% of the Biomed-1 group patients. In both studies no clearly defined precursor T-cell population (TdT+CD7+cCD3+) was found in normal bone marrow. Average CD7+ events in normal BM in the Biomed-1 group study made up 10% ± 3.6% of total BM cells. In our study we gated cCD3+ events and they composed 4.21 ± 1.95% of BM cells. The difference might be explained by the presence of CD7+CD3- population (e.g., NK cells).

Although TdT was proved to be a useful indicator of T-lymphoblasts, its detection is technically complicated (nuclear expression, sometimes dim on pathological cells). Attempts were made to find a marker that would be brightly expressed on the surface of T-lymphoblasts and differentiate them from normal T-cells. CD99 was shown to be such a marker. Dworzak and colleagues discovered that CD99 expression on T-lymphoblasts was in median 7.7 times higher than on normal T-lymphocytes and in 85% of their T-ALL cases leukemic MFI values were higher than MFI ± 2 SD of T-lymphocytes [14]. In our study all T-ALL cases were CD99 positive and we also statistically showed differences in CD99 expression between T-lymphoblasts and normal T-cells. So we

support the idea that CD99 should be included in a marker panel for T-ALL MRD detection.

It is well known that phenotypic shifts can occur during leukemia treatment, due to selective depletion of certain cell subpopulations or corticosteroid action on gene transcription [22,23]. These are better investigated in B-ALL, yet not so many studies focused on early phenotypic changes. Gaipa and colleagues investigated B-ALL follow-up samples from day 15 and found that in B-ALL the expression of CD10 and CD34 was downregulated while CD19, CD20, CD45RA and CD11a were upregulated, indicating a shift to a more mature population [22].

Even less research is done on early phenotypic shifts in T-ALL. Roshal et al. found that the median decline for CD99 positivity on the abnormal blasts was 24%, 26% and 62% at days 8, 15 and 29 of treatment respectively. The differences for TdT were 30%, 44% and 60% respectively. They recommended using a broader panel to avoid false negative MRD results in case of phenotypic shifts (M. Roshal et al., presented at the 50th annual ASH meeting, 6-9 December 2008, San Francisco, CA). In our study we checked how the MFI of the most commonly aberrant markers we chose for MRD panel (TdT, CD99) changed during treatment. Results showed that they did fluctuate, but they were always higher than MFI of TdT and CD99 in normal T-cells and no trends were revealed. So in our case a limited marker panel was justified.

Considering analysis of normal BM, our results were mostly concordant with a well proven approach that there should be no clearly observed T-cell precursor population in healthy adult bone marrow [21,24]. We did not find any T-cell population with a bright expression of CD99. However, we did detect some cytoplasmic CD3 and TdT positive events which also appeared to be CD19 positive. Most probably this happened due to unspecific staining of early B-cell precursors with anti-CD3 reagent. We added CD19 to our T-ALL MRD panel to exclude those events. Although the amount of these cells was not high, we will attempt to avoid this phenomenon by optimizing our sample preparation technique.

In summary, we consider flow cytometry as a sensitive, fast and affordable tool for monitoring T-ALL. In perspective of increasing interactions between different health care institutions, formation of laboratory networks and growing opportunities of patient mobility, we support the idea of standardization of MRD detection. Economic factors, especially in countries with more limited resources, play a remarkable role in the selection of a test method. We hope we have showed that a standard, sensitive and economically effective MRD test for T-ALL can exist. Yet we all face the continuous development of new technologies within flow cytometry, which may provide an opportunity to detect more cell parameters and lead to even better sensitivity of assays and more profound comprehension on a composition of cell populations. These technological advancements whenever it is possible should be exploited not only for research but also for diagnostic purposes.

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