ORIGINAL ARTICLE, MEDICINE

Immunophenotypic Modulation of the Blast Cells in Childhood Acute Lymphoblastic Leukemia Minimal Residual Disease Detection

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Received: 17 Feb 2016

Accepted: 11 April 2016

Published: 30 April 2016

Key words: MRD, flow cytometry, immunophenotypic modulation, childhood ALL

Citation: Burnusuzov HA, Spasova MI, Murdjeva MA, Stoyanova AA. Immunophenotypic modulation of the blast cells in childhood acute lymphoblastic leukemia minimal residual disease detection.

Folia Medica 2016;58(1):28-35, doi: 10.1515/folmed-2016-0004

Early clearance of leukemic cells during induction therapy of childhood acute lymphoblastic leukemia (ALL) is a basis for treatment optimization. Currently, the most widely used methods for the detection of minute residual malignant cells in the bone marrow and/or peripheral blood, minimal residual disease (MRD), are PCR and flow cytometry (FCM). Immunophenotypic modulation (IM) is a well known factor that can hamper the accurate FCM analysis.

Aim: To report the IM detected by 8-color FCM during the BFM-type remission induction in 24 consecutive MRD-positive samples of children with B-cell precursor ALL and the possible implications for MRD detection.

Patients and methods: Between 2010 and 2012 we prospectively followed up the MRD on days 15 and 33 of induction treatment in bone marrow (BM) samples and on day 8 in peripheral blood (PB). The IM was assessed by comparative analyses of the changes in the mean fluorescence intensity of 7 highly relevant antigens expressed by the leukemic cells and normal B-lymphocytes.

Results: IM occurred, to different extents, in all analyzed day 15 BM and in most day 33 BM samples. Statistically significant changes in the MFI-levels of four CDs expressed by the leukemic blasts were observed: downmodulation of CD10, CD19 and CD34 and upmodulation of CD20. No changes in the expression of CD38, CD58 and CD45 were noticed.

Conclusions: Measuring the MRD by standardized 8-color flow cytometry helps improve the monitoring of the disease, leading to better therapeutic results. However, the IM of the different antigens expressed by the leukemic blasts should be taken into consideration and cautiously analyzed.

INTRODUCTION

Recent studies demonstrating the prognostic significance of minimal residual disease (MRD) detection in childhood acute lymphoblastic leukemia (ALL) during remission induction have yielded a strong clinical implication for the management of these patients.¹⁻⁵ Currently, the most widely used, highly sensitive (1×10^{-4} – 1×10^{-5}) and specific methods, are immunophenotyping by multi parameter flow cytometry (FCM) and real-time quantitative PCR (RQ-PCR).⁶⁻⁹ The time point-dependent concordance of FCM and RQ-PCR has also been shown.^{10,11} Briefly, FCM utilizes the aberrant phenotypic expression of leukemic cells to discriminate them

from their normal counterparts.¹²⁻¹⁴ The stability of the leukemia-associated immunophenotype (LAIP) during therapy influences the FCM sensitivity and specificity. Such changes in the LAIP (also known as therapeutic immunophenotypic modulation, TIM) have continuously been described and include changes of the level of expression, acquisition or loss of lineage-associated antigens, or lineage switch.¹⁵ These could lead to false negative FCM results.¹⁶ Modern flow cytometers give the advantages of simultaneous staining with multiple immunophenotypic markers (> 6 colors) which could help prevent compromising the analysis.¹⁷⁻¹⁹

AIM

In this first Bulgarian childhood precursor B-cell ALL MRD study we report the immunophenotypic modulation detected by 8-color FCM during the BFM-type remission induction in 24 consecutive children with B-cell precursor ALL and the possible implications for MRD detection.

PATIENTS AND METHODS

PATIENTS

From December 2010 to December 2012, a series of 43 children with ALL were consecutively diagnosed and treated in the pediatric oncohematology departments of Plovdiv and Varna University Hospitals, Bulgaria. Leukemia associated phenotypes (B- and TCP) were detected in 40 and a total of 31 patients were systematically followed up. Of these, 24 subjects (13 girls, 11 boys, median age 7 yrs, range 17 mos – 17 yrs), were included in this study according to the following criteria: 1. B-cell precursor phenotype; 2. LAIP, suitable for MRD analysis; 3. Flow cytometric MRD-positivity (a cluster of ≥ 30 cells with related characteristics) in the peripheral blood (PB) and/or in bone marrow (BM) follow-up samples. The study was approved by the institutional ethics committee and individual written informed consents were obtained by the patients' guardians. Flow cytometry was performed in the immunological research facility of the Plovdiv Medical University.

All these 24 patients received a BFM-type induction therapy. Later on the treatment continued according to the assigned risk group. Twenty-three patients were prednisone good responders (PGR) according to the protocol criteria (less than 1000 blasts/ μ l PB on day 8, as assessed by morphological evaluation). The prednisone poor responding (PPR) subject (morphologically more than 1000 blasts/ μ l PB on day 8) was also the only Pro-B ALL case, the rest being classified as "common" or Pre-B ALL. Six patients were MRD negative ($< 0.01\%$) on day 33. Diagnostic non-malignant BM samples ($n = 8$) and BM from pediatric T-ALL patients ($n = 7$) undergoing the same treatment were used for comparisons. Twenty patients had a normal karyotype, translocation t(9;22) was found in two cases and translocation t(12;21) was also found in two out of the 24 analyzed patients as assessed by the initial molecular and cytogenetic screening.

FLOW CYTOMETRY

Both PB and BM samples were collected at diagnosis. Follow-up samples for MRD studies were collected from PB at day 8 and from BM at days 15 and 33. Leukemia-associated immunophenotypes were assessed by 8-color multiparametric FCM. The BCP-panel consisted of the following fluorochrome-conjugated antibodies: SYTO41 Pacific Blue/ CD45 Pacific Orange/ CD58 FITC/ CD10 PE/ CD34 PerCP-Cy5.5/ CD38 PE-Cy7/ CD19 APC/ CD20 APC-Cy7. Antibodies to CD45 and SYTO41 were provided by the local representative of Molecular Probes, Invitrogen, and the rest - by the local representative of Becton Dickinson (BD, USA).

Stain-lyse procedure was used. Briefly, BM or PB sample was incubated with the antibodies for 15 min at room temperature. For red blood cell lysing, 2 ml of 1:10 demineralized water-diluted commercially available solution (BD Lysing solution, 10x concentrate) was added for 10 minutes. Specimens were centrifuged at 400 G, 4°C (1400 rpm) for 10 min. Then the pellet was washed in phosphate-buffered saline (PBS), again centrifuged and finally the pellet was resuspended in 0.5 ml of PBS. The cell-permeant, live-cell nucleic acid fluorochrome SYTO 41 was added prior to measurement, in order to exclude residual non-nucleated erythroid cells, thrombocytes, or debris. All samples were processed within 24 to 48 h after collection. At diagnosis and on day 8 a total of 100 000 SYTO41+ cells were collected. In order to achieve a sensitivity of at least 1×10^{-4} on day 15 the cell input was increased to 300 000 SYTO41+ events, and on day 33- one million SYTO41+ events were collected. Cell acquisition and the following analyses were performed using a BD FACS Canto II flow cytometer (BD, USA), in 4-2-2 configuration (405, 488 and 635 nm) with BD FACS Diva v. 6.1.3 software. Due to the low number of cases instrument setup was carried out at the beginning of each acquisition day (no less than once weekly) using the BD Cytometer Setup and Tracking (CST) Beads and the cytometer's CST module.

GATING STRATEGY

Combined immunological gate: CD19 expression-based and by a physical parameter (90° side scatter) was used for the initial B-lineage restriction (**Fig. 1**). Then leukemic cells were discriminated from the normal B lymphocytes on the basis of

Figure 1. Gating strategy. Sequence of the analysis (A to D): A - general presentation of the populations in FSC/SSC –dot plot. B - SSC/Syto41®- dot plot. Only Syto41®-positive events are acquired; C - in SSC /CD19-dot plot, the analysis is restricted to CD19+ events; D - determining the MRD in the field CD10/CD45; additional secondary dot plots are used to determine the expression of other CD-markers (not shown). All Syto41®-positive events are colored gray; CD19+ events are in blue; green represents the CD19-negative events; MRD-leukemic cells are stained in red.

leukemia-associated immunophenotypes (i.e. over-expression of antigens such as CD10, CD34, CD58, CD38 and/or underexpression of CD20 and CD45). Antigen expression was quantified using the mean fluorescence intensity (MFI) values, measured by the cytometer.

STATISTICAL ANALYSIS

The Wilcoxon signed ranks test was used to compare the median MFI values at diagnosis and at follow-up time points. The results of the quantitative variables are indicated as an arithmetic mean or a median. Comparison of quantitative variables between two groups was performed by independent T-test or Mann-Whitney U test and for more than two groups - using One-Way ANOVA. The adopted significance level was $p < 0.05$. Box- and whisker plots and run charts are used for descriptive purposes.

RESULTS

MODULATION IN LEUKEMIC CELLS

We examined the median MFI values of seven highly relevant antigens - CD10, CD20, CD34, CD38, CD58, CD19 and CD45, expressed by the leukemic blasts in the MRD positive samples. As an internal control we analyzed the changes of the forward scatter (FSC) and side scatter (SSC) properties of the leukemic cells. Moreover, we also checked the expression of CD19, CD20, CD45 and CD58, FSC and SSC of the residual normal CD19+ cells in each sample. Paired samples from diagnosis and early follow-up from 24 consecutive B-cell precursor ALL cases (PB: day 0 – day 8; BM: day 0 – day 15, day 15 – day 33 and day 0 – day 33) were investigated. Immunophenotypic

modulation occurred, to different extents, in all analyzed day 15 BM samples and in most day 33 BM samples compared to diagnosis. We observed statistically significant changes in the MFI levels of four CDs expressed by the leukemic blasts: CD10, CD20, CD34 and CD19 (**Fig. 2**). Downmodulation of CD10 was noticed in the interval day 0 – day 33, predominantly in the second half- after day 15 ($Z: -3.007$; $P = 0.003$). Significant downmodulation was also seen for CD34 on day 15 ($Z: -3.425$; $P = 0.001$) and on day 33 ($Z: -2.213$; $P = 0.027$) in BM. By contrast, small but stable upmodulation was detected for CD20 both on day 15 ($Z: 1.964$; $P = 0.05$) and on day 33 ($Z: 1.891$; $P = 0.05$). Curiously, CD19 showed significant upmodulation on day 15 ($Z: -2.16$; $P < 0.001$), but later on day 33 we noted a steep downmodulation ($Z: -4.02$; $P < 0.0014$). On the other hand, expression of CD58, CD45 and CD38 was not significantly changed at all time points. Analyses for the same antigens by comparing PB samples at diagnosis and at day 8 yielded very similar results: CD34 downmodulation ($Z: -2.89$; $P = 0.004$), CD19 ($Z: -2.44$; $P = 0.015$) and CD20 ($Z: -2.84$; $P = 0.005$) upmodulation; no changes for CD38, CD45 and CD58. Of note, there was also no modulation detected for CD10 ($Z: -0.23$; $P = 0.82$). Scattergram (FSC vs. SSC) analyses of the leukemic cells confirmed that these blasts were always situated in the viable cells' regions. Furthermore, no significant changes in their FSC and SSC linear channel values, measured both at diagnosis and on follow-up, were seen. An example of IM as seen on the dot plots is given in **Fig. 4**.

MODULATION IN NORMAL RESIDUAL CELLS

There was a statistically significant reduction in the

Figure 2. Modulation of leukemic cells. Changes in the MFI of CD10, CD19, CD20, CD38, CD34 and CD58 during induction therapy. D0, D15 and D33 mark the day of diagnosis, day 15 and day 33 of therapy. The levels of statistical significance are given in each box above the respective interval investigated.

Figure 3. Modulation of B-lymphocytes. Changes in the MFI of CD19, CD20, CD45 and CD58, expressed by the leukemic cells (in red dashed line) compared to that of normal mature B-lymphocytes (in blue solid line).

proportion of leukemic cells among total CD19+ population (day 0 – day 33; Z :-3.82; $P < 0.001$), the greatest being during the first two weeks of induction treatment (Z :-4.11; $P < 0.001$): day 0: 86.49%; day 15: 19.55%; day 33: 4.93%. Therefore sufficient counts of normal CD19+, CD10 low/–, CD20++, CD38–, CD34–, CD45++ B-lymphocytes for analysis were

present at any time point of the study. Accordingly, we examined whether only the leukemic population is subjected to IM, by analyzing the variations in the expression of CD19, CD20, CD58 and CD45 by the normal residual B-cells. As shown in Fig. 3, the antigen expression patterns of normal mature B cells frequently appeared to undergo modulation.

Figure 4. Visual comparison of representative dot plots from the diagnosis (D0) in the upper row and follow-up (D15) in the lower row of a child with precursor B-cell ALL. All Syto41®-positive events are colored gray; CD19+ events are in blue; green represents the CD20+CD10- “mature” compartment; MRD-leukemic cells are stained in red. Shifts of the leukemic cells are clearly seen, aided by the superimposed referent lines.

Statistically significant downmodulation of CD19 was found between day 15 and day 33 (Z : -2.66; P = 0.008) (**Fig. 3**). In terms of CD20 and CD45, significant upmodulation was noted both on day 15 (CD20 Z : -3.59; P < 0.001 and CD45 Z : -3.23; P = 0.001) and on day 33 (CD20 Z : -3.81; P < 0.001 and CD45 Z : -2.98; P = 0.003). The expression of CD58 by the normal residual B-lymphocytes showed substantial downmodulation in both time points: day 15 (Z : -3.46; P = 0.001) and day 33 (Z : -2.21; P = 0.03). Similarly, day 8 PB results were compared to those from diagnosis and modulation of the same pattern was noted.

DISCUSSION

Immunophenotypic modulation of the leukemic cells during chemotherapy is a well-known phenomenon which could hamper the MRD analysis. This first Bulgarian pediatric ALL MRD prospective study reports changes in the median MFI-values of four of the analyzed CD-markers during the induction, although some data vary in a time-point aspect as compared to data published so far.²⁰⁻²² Differences concern the downmodulation of CD10 that has been detected mostly after day 15 and persists in all day 33 MRD-positive samples. The upmodulation of CD19 is verified on day 15, followed by

a significant downmodulation on day 33. Changes in the modulation of CD34 and CD20 are similar to those already reported in other studies, as well as the stable expression of CD58 detected on day 15. Additionally, no statistically significant modulation was also found for CD38 and CD45. Taken together, knowing the usual pattern of the different CDs' MFI changes during the induction therapy is of crucial importance and could help facilitate the differentiation of the MRD-population in cases with concurrent modulation of one or several antigens.

In this study we have not investigated directly the possible mechanism unlocking the IM, although we favour the hypothesis that it could be drug-induced, specifically by the corticosteroids, which are known regulators of gene transcription. In line with this, we observed IM as early as on day 8 in PB (after 7 days of prednisone and a single intrathecal injection of methotrexate). Moreover normal mature B cells in our study were also subjected to IM to some extent and these findings are in line with those of earlier studies.²³

A possible demerit of the study is the difference in the expression of CD45, CD19 and CD58 on day 33, compared to those found in earlier studies.²²⁻²⁴ We assume that the relatively low number of MRD-positive patients at the end of the

induction could have biased these specific results and warrant further research. On the other hand, variable CD binding, diverse antibody conjugates, distinct lysing procedure and the flow cytometer type with its compensation settings could also lead to such changes in measured MFI-values. High quality analyses require strict implementation of the internationally accepted standards for flow cytometric ALL MRD detection.²⁵⁻²⁹ That is why we applied uniform staining and lysing procedure, by using commercially available monoclonals and lysing solution in quantities recommended by the manufacturer. Stability of the flow cytometer was checked weekly using standard BD FACS CST kit and BD FACS 7-Color setup beads and showed no faults. Uniform gating strategy was accepted. In our case divergent results could probably be explained by the different cell clones used. Unfortunately, we could not prove it by simultaneous staining with another conjugates of the same CDs due to limited resources.

An advantage of our panel, similarly to Dworzak et al., 2002, is the acquisition based on the specific live-cell-permeant nucleated-cell dye Syto41® positivity, which together with the FSC and SSC parameters ensures that changes in the expression of antigens (hence TIM) are not a consequence of non-specific abstraction of fluorophores by debris. Additionally, we also looked for and visually ruled out MRD-subclone selection.

Chemotherapy leads to changes in the antigen expression of both the leukemic population (CD10+/+, CD20low/-, CD38++, CD34+/+, CD45low/-), and the remaining mature B-lymphocytes (CD10 low/-, CD20++, CD38-, CD34-, CD45++, CD19+).^{23,32} In the present study, we found a five-fold higher level of upmodulation of CD20, two-fold higher upmodulation of CD45 and significant downmodulation of CD58 expressed by the B-lymphocytes when compared to that of the leukemic cells. This favoured the visual differentiation of the MRD-population that remained outside the areas usually occupied by the "normal" cells in all dot plots. It has been shown that increasing the number of CD-antibodies (≥ 6) in a single strategic, one-tube combination, could further increase the specificity and sensitivity of the analysis up to 1×10^{-5} and reduce the risk of false negativity due to the IM or cell loss.^{2,25,31} Based on the variegated immunophenotypic modulation of malignant and normal B-lymphocytes during the early induction therapy and by using 8-color flow cytometry we were able

to unambiguously distinguish the MRD-population in all samples relying on at least 4 different CD markers with known pattern of modulation.

CONCLUSIONS

Our results confirm the presence of specific immunophenotypic changes occurring during the induction treatment of children with ALL. Dominating steroids in this phase could have triggered this phenomenon and researchers and routine laboratory workers should take it into account. The proposed standardized 8-color combination is suitable for multiparametric FCM detection of MRD in children with ALL, while minimizing the negative impact of TIM, and increasing the sensitivity of the analysis. Further identification of new leukemia markers which are easily detectable and are stably expressed in ALL cases could help discriminate reliably leukemic blasts from normal regenerating and mature cells, and therefore could improve MRD studies and extend the benefit to all patients.

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Иммунофенотипическая модуляция бластных клеток при обнаружении минимального остаточного заболевания детским острым лимфобластным лейкозом

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Раннее обнаружение лейкозных клеток во время индукционной терапии детского острого лимфобластного лейкоза (ALL) является основой оптимизации лечения. В настоящее время наиболее широко используемые методы обнаружения остаточных злокачественных клеток в костном мозге и/или периферической крови при минимальной остаточной болезни (МОБ) являются ПЦР и проточная цитометрия (FCM). Иммунофенотипическая модуляция (ИМ) является хорошо известным фактором, который может воспрепятствовать точному анализу при FCM.

Цель: Сообщить об ИМ, обнаруженной с помощью 8-цветной проточной цитометрии во время индукции ремиссии BFM-типа в течение 24 последовательных МОБ-положительных образцов, полученных от детей с В-клеточным острым лимфобластным лейкозом из клеток-предшественников и возможных последствий для обнаружения МОБ.

Материал и методы: За период 2010 - 2012 годов мы, в соответствии с нашими предположениями, проводили последовательное наблюдение МОБ на 15 и 33 день индукционной терапии в костном мозге

и на 8 день - в периферической крови. ИМ оценивали с помощью сравнительного анализа изменений средней интенсивности флуоресценции 7 исключительно значимых антигенов, выраженных лейкозными клетками и нормальными В-лимфоцитами.

Результаты: ИМ обнаруживалась, в разной степени, во всех анализированных пробах костного мозга на 15 день и в большинстве случаев на 33 день. Наблюдалась статистически значимые изменения уровней средней интенсивности флуоресценции четырех CD (определителей классификации), выраженных лейкозными бластами: понижение уровня модуляции CD10, CD19 и CD34 и повышение уровня модуляции CD20. Никаких изменений в выражении CD38, CD58 и CD45 замечено не было.

Заключение: Измерение МОБ с помощью стандартизированной 8-цветной проточной цитометрии помогает улучшить контроль заболевания, что приводит к улучшению результатов лечения. Тем не менее, ИМ различных антигенов, выраженных лейкозными бластами, должны приниматься во внимание и осторожно анализироваться.

Ключевые слова: МОБ, проточная цитометрия, иммунофенотипическая модуляция, детский острый лимфобластный лейкоз