Evaluating New Markers for Minimal Residual Disease Analysis by Flow Cytometry in Precursor B Lymphoblastic Leukemia

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Received: 27 December 2016 / Accepted: 27 June 2017 / Published online: 3 July 2017
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Abstract Minimal residual disease is currently the most powerful prognostic indicator in Precursor B lymphoblastic leukemia. Multiparameter flow cytometry is the most commonly used modality. Seventy three B ALL cases and 15 normal marrows were evaluated for expression patterns of leukemia markers (CD38, CD58, CD73) in all 73 cases and CD66c, CD86 and CD123 in 23 cases. CD73 was aberrantly expressed in 90.41% cases and CD86 in 60.87% B ALL cases. Thus addition of these markers in MRD panels can increase the sensitivity of the assay.

Keywords Acute leukemia · MRD · Hematology

Introduction

Minimal residual disease (MRD) is currently the most powerful prognostic indicator in acute lymphoblastic leukemia. MRD analysis can be done by either flow cytometric or molecular techniques. Flow cytometric detection holds potential for wider applicability than molecular techniques because flow cytometric methods for leukemia diagnosis are already established at most cancer centers worldwide.

Flow cytometric detection of MRD is based on the principle that ALL cells express immunophenotypic features that can be used to distinguish them from normal hematopoietic cells, including hematogones and activated lymphocytes commonly referred to as Leukemia associated immunophenotype (LAIP). In virtually all patients with ALL, leukemia-associated immunophenotypes can be defined at diagnosis and then used to monitor MRD during treatment [1, 2]. The reliability of flow cytometric MRD assays depends on several factors. The most important being the correct marker combination in use. Applicability is limited in some cases by the lack of suitable leukemia associated immunophenotype (LAIP) with the currently used markers and also antigen immunomodulation post treatment. Therefore, the identification of new leukemia markers that are easily detectable and are stably expressed in a large proportion of ALL cases should simplify the application of MRD studies, help extend their benefit to all patients and possibly enhance the sensitivity of MRD detection.

Materials and Methods

All newly diagnosed cases of precursor B lymphoblastic leukemia presenting to our laboratory between May 2014 to April 2015 (n = 73) were analysed additionally for the expression of leukemia markers (to determine Leukemia Associated Immunophenotype LAIP). All 73 cases were analysed for the expression of CD38, CD58 and CD73 (Tube 1). Twenty three of these cases were additionally analysed for the expression of CD66c, CD86 and CD123 (Tube 1 and tube 2). The panel was as follows (Tube 1: CD38 FITC, CD58PE, CD34 PerCP, CD10 PE-CY7, CD19 APC, CD45 APC-H7, CD73BV421, CD20 V500-C; Tube

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2: CD66c FITC, CD123PE, CD34 Per CP, CD10 PE-CY7, CD19APC, CD45 APC-H7, CD86 BV421). In addition 15 normal bone marrow samples were analysed for the expression pattern of normal B precursors (Hematogones). Based on the expression patterns on hematogones, normal templates were prepared (Fig. 1). Marrows selected for preparing normal templates comprised of Staging marrows of hodgkin's lymphoma ($n = 3$), solid tumors ($n = 6$), AML remission ($n = 4$), suspected ITP ($n = 2$). In twelve cases post induction bone marrow aspirate sample was analysed for stability of leukemia markers. Out of these 12 cases 5 were positive for minimal residual disease at the end of induction (i.e. $>0.01\%$) and 7 were negative. Figures 3 and 4 illustrate 3 such cases where diagnostic and end induction MRD samples were available.

Whole blood Stain Lyse Wash method was used. 100 μ l of Bone marrow sample was taken in BD Falcon Tubes. Pretitrated Volume of antibodies were added and mixed by vortexing. The Tubes were incubated in dark at room

temperature for 10–12 min. BD Pharm Lyse (3 ml) was added followed by mixing by vortexing. The tubes were incubated in dark at room temperature for 15–20 min. The tubes were centrifuged at 200–300 g for 5 min. After discarding the supernatant, the pellet was broken and resuspended in 2 ml sheath fluid. After vortexing the tubes were centrifuged again at 200–300 g for 5 min. The washing with sheath fluid was repeated twice. After the final wash the pellet was resuspended in 500 μ l sheath fluid. The sample was acquired on a precallibrated flow cytometer. Analysis was done using BD FACS DIVA software version 7.0.

Results

Out of the 73 patients there were 33 males and 40 females. The age was in the range of 1–40 years (median 22). Amongst the cases analysed, the abnormal expression of

Fig. 1 Figure depicts the templates prepared for Tube 1 (using normal bone marrow samples). The drawn outlines indicate the regions defined as normal (expression patterns of hematogones). In

this case there was aberrant expression of CD38, CD58 and CD73 by leukemic blasts (LAIPs for this case)

leukemia markers i.e. CD38 downregulation, CD58 overexpression, and CD73 overexpression were seen in 82.19, 58.9 and 90.41% respectively (Table 1). CD66c overexpression was observed in 52.17%, CD86 overexpression in 60.87% and CD123 in 52.17% (Table 1). In the 10 cases where post induction sample was analysed for the stability of leukemia markers, the leukemia markers were found to be stably expressed. Figures 1 and 2 depict the abnormal

expression of CD38, CD58, CD73 and CD66c, CD86 and CD123 respectively (Figs. 3, 4).

Discussion

Over the past three decades remarkable progress has been achieved in the treatment of acute lymphoblastic leukemia. Technological advancement has led to introduction of the concept of minimal residual disease which has challenged the conventional definition of “remission” and has proven over time to have an independent clinical relevance [2–5]. Acute leukaemia patients who achieve clinical remission after induction therapy may harbour as many as 10 [5] tumour cells. These cells which cannot be detected by routine cytomorphological methods constitute the minimal residual disease (MRD). The level of MRD provides a measure of kinetics of tumor reduction, giving the insight into in vivo resistance of overall leukemic population to the

Table 1 The leukemia markers abnormally expressed in B ALL

Leukemia Marker	Expression	Number	Percentage
CD38	Underexpressed	60/73	82.19
CD58	Overexpressed	43/73	58.90
CD73	Overexpressed	66/73	90.41
CD66c	Overexpressed	12/23	52.17
CD86	Overexpressed	14/23	60.87
CD123	Overexpressed	12/23	52.17

Fig. 2 Figure depicts the templates prepared for Tube 2 (using normal bone marrow samples). The drawn outlines indicate the regions defined as normal (expression patterns of hematogones). In

this case there was aberrant expression of CD66c, CD123 and CD86 by leukemic blasts (LAIPs for this case)

Fig. 3 Figure depicts expression patterns of CD73 and CD86 in a 1 year old child at diagnosis (a) and MRD sample at the end of induction (b). CD73 and CD86 are overexpressed at diagnosis and both served as useful markers for MRD analysis post induction

Fig. 4 a Depicts CD73 overexpression in a 39 year old male patient with B ALL at the time of diagnosis (left column) and MRD analysis at the end of induction (right column). b depicts CD73 overexpression

in a 16 year old male patient with B ALL (left column) and MRD analysis at the end of induction (right column)

chemotherapeutic agents. MRD levels can be monitored by flow cytometry which usually targets the leukaemia associated immunophenotype (LAIP) [1].

The prognostic value of MRD testing in childhood ALL was demonstrated most convincingly by three large prospective studies reported in the late 1990s by the

European Organisation for Research and Treatment of Cancer (EORTC) [6], St Jude [7], and BFM [8] groups.

Dhedin et al. reassessed the role of allogeneic stem cell transplantation (SCT) in patients treated in the GRAALL-2003 and GRAALL-2005 trials. In this study they report relatively good results associated with myeloablative SCT

in CR1 in adults with Ph-negative high-risk ALL treated with a pediatric-inspired protocol. The authors confirmed in a large prospective study that early MRD response is the best and maybe the unique tool to optimally select the patients who may currently benefit from SCT in CR1 [9].

Minimal residual disease is thus emerging as the most important predictor of prognosis in treatment of acute lymphoblastic leukemia. To be able to do MRD studies in hematopathology laboratories is thus extremely important. This is a challenging task particularly in resource restricted setting like ours. There is a constant endeavour to find new markers of MRD in ALL so as to increase the sensitivity and by using the most sensitive markers in our panels, the cost can be significantly reduced without compromising the results.

Coustan Smith et al. [5] searched for new markers of MRD in B-lineage ALL by defining differences in genome-wide gene expression between leukemic and normal cells. They focused their analysis on normal BM cells with immunophenotypic features similar to those of leukemic lymphoblasts, i.e. BM CD19+CD10+cells, which are the most difficult to distinguish from ALL lymphoblasts during MRD studies by flow cytometry. Among genes differentially expressed by genome-wide expression array analysis, they selected 30 for further studies based on their highly abnormal expression at the mRNA level in a substantial proportion of ALL cases and on the availability of specific Abs suitable for routine flow cytometric studies. They found that 22 of the 30 markers were also differentially expressed by flow cytometry. The top 16 differentially expressed markers listed in were included for a total of 258 tests (number of tests for each marker: CD44, 15; BCL2, 18; HSPB1, 32; CD73, 15; CD24, 7; CD123, 34; CD72, 10; CD86, 21; CD200, 29; CD79b, 5; CD164, 7; CD304, 12; CD97, 20; CD102, 10; CD99, 8; CD300A). In this study CD73 overexpression was observed in 54.5%. CD86 overexpression in 46.7% and CD 123 overexpression in 50.7% cases.

Wang et al. [10] analysed the expression of CD73 by flow cytometry (FC) in bone marrow (BM) specimens of B cell acute lymphoblastic leukemia (B-ALL) with or without minimal residual disease (MRD), and its advantages were evaluated using the MRD assay. The authors recommended CD73 as an optional MRD marker for B-ALL patients by using FC.

There are limited studies from India about ALL minimal residual disease. Patkar et al. [11] studied 42 B ALL cases for MRD testing. The antibodies used included CD45, CD11a, CD38, CD20, CD10, CD19, CD58, CD34, CD123, and CD22.

Tembhare et al. [12] analysed expression-patterns of six new markers, i.e. CD24, CD44, CD72, CD73, CD86, and CD200 in leukemic-blasts from ninety childhood BCPALL

patients and in hematogones from 20 uninvolved staging bone marrow (BM) and ten postinduction non-BCPALL BM samples using eight-color MFC. Frequencies of LAIPs of CD73, CD86, CD72, CD44, CD200, and CD24 in diagnostic samples were 76.7, 56.7, 55.6, 50, 28.9, and 20%, respectively. In MRD-positive samples, CD73 showed the maximum (83%) frequency of LAIP and CD86 showed the highest (100%) stability of aberrant expression. Inclusion of CD73 and CD86 increased the applicability of MFC-MRD assay to 98.9% MRD samples.

In the present study we report abnormal expression of CD73 (overexpression) in more than 90% and CD86 overexpression in more than 60% precursor B ALL cases. The addition of these markers in B ALL MRD panels can contribute to increasing the sensitivity of the assay. The results should be corroborated in studies involving more number of patients. We hereby suggest that a 8 color tube similar to the tube 1 in our study (incorporating CD38, CD73, CD58 as leukemia markers) can serve as a sensitive single tube useful in a sizeable proportion of B ALL cases. The second tube incorporating CD86, CD123, CD66c can be used as an optional tube in cases which lack LAIP in the first tube. The above suggestions should be tested in a large cohort of B ALL cases and sensitivity and predictive values tested.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval Approval from Institute board for conducting the study was taken.

Human and Animal Rights No animals were included in the study.

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