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Video Article

Flow Cytometry to Estimate Leukemia Stem Cells in Primary Acute Myeloid Leukemia and in Patient-derived-xenografts, at Diagnosis and Follow Up

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Abstract

Acute myeloid leukemia (AML) is a heterogeneous, and if not treated, fatal disease. It is the most common cause of leukemia-associated mortality in adults. Initially, AML is a disease of hematopoietic stem cells (HSC) characterized by arrest of differentiation, subsequent accumulation of leukemia blast cells, and reduced production of functional hematopoietic elements. Heterogeneity extends to the presence of leukemia stem cells (LSC), with this dynamic cell compartment evolving to overcome various selection pressures imposed upon during leukemia progression and treatment. To further define the LSC population, the addition of CD90 and CD45RA allows the discrimination of normal HSCs and multipotent progenitors within the CD34+CD38- cell compartment. Here, we outline a protocol to detect simultaneous expression of several putative LSC markers (CD34, CD38, CD45RA, CD90) on primary blast cells of human AML by multiparametric flow cytometry. Furthermore, we show how to quantify three progenitor populations and a putative LSC population with increasing degree of maturation. We confirmed the presence of these populations in corresponding patient-derived-xenografts. This method of detection and quantification of putative LSC may be used for clinical follow-up of chemotherapy response (*i.e.*, minimal residual disease), as residual LSC may cause AML relapse.

Video Link

The video component of this article can be found at https://www.jove.com/video/56976/

Introduction

Acute myeloid leukemia (AML) is the most common cause of leukemia-associated mortality. Initially, AML is a clonal disease of hematopoietic stem cells (HSC) characterized by arrest of differentiation, subsequent accumulation of blast cells, and reduced production of functional hematopoietic elements. Recently, clonal heterogeneity of the blast cell population has been established essentially by using next generation sequencing (NGS) strategies and showing the existence of intra-clonal evolution of tumor cells[1].

Heterogeneity extends to the presence of leukemic stem cells (LSC). It is hypothesized that this dynamic cell compartment evolves to overcome various selection pressures imposed upon during leukemia progression and treatment. Therefore LSC are supposed to be resistant to current chemotherapeutic regimens and mediate disease relapse, with profound clinical implications[2]. Various markers have been described to characterize LSC like CD123[3], CLL-1[4], CD97[5] or TIM-3[6]. The CD34+CD38- compartment of blast cells is considered to be enriched for LSC[7] but also includes normal HSC. CD90 and CD45RA expression applied to the CD34+CD38- (P6) compartment (**Figure 1**) permits to segregate several stages of normal and malignant hematopoietic precursors[8]. Specifically, normal CD34+CD38-CD90+CD45RA- HSCs, multipotent progenitors (MPP) like CD34+CD38-CD90dimCD45RA- cells and CD34+CD38-CD90-CD45RA+ lymphoid-primed multipotent progenitor (LMPP) cells can be determined in the majority of AML cases[8,9]. The mean fluorescence intensity (MFI) of CD38 is particularly important to be considered within the CD34+ cell compartment. Because CD38 intensity defines three new cell compartments thereof: CD38 negative (P6), CD38 dim (P7) and CD38 bright (P8) (**Figure 1**). The MFI of CD38 may be determined using either hematogones and/or plasma cells as an internal positive control as these cells strongly express CD38.

The immunodeficient NOD/SCID/IL2Rγc^{null} (NSG) mouse model is widely used for engraftment of normal and malignant human hematopoietic cells[10,11]. Serial xenotransplantation assays are used to experimentally validate LSC or HSC function. These studies have been extensively

analyzed by large scale sequencing approaches. Nevertheless, less is known about the LSC and HSC immunophenotype of AML blast population engrafted into NSG mice compared to its primary AML (**Figure 2**).

The numbers of LSC are inherently low thus, identification and quantification of LSC are challenging and the method described here may be used as a flow cytometric assay at diagnosis and at clinical follow up to evaluate chemotherapy response (as residual LSC may cause AML relapse). The presence of LSC may indicate positive minimal residual disease (MRD). To date, MRD monitoring in AML mostly rely on molecular methods (*i.e.*, RT-PCR, NGS)[10,12]. However, in this protocol we detect simultaneous protein expression of several HSC/LSC markers (CD34, CD38, CD45RA, CD90) on primary blast cells of human AML by multiparametric flow cytometry[2,8,9]. This combination of antibodies may be applied in any standard flow cytometry laboratory and this flow MRD assay is particularly interesting when MRD by real-time polymerase chain reaction (RT-PCR) is not possible, *i.e.*, if leukemia specific molecular markers are not detectable in the diagnostic AML sample. Furthermore, this protocol, is complementary to the more sensitive molecular MRD techniques, as it aims to detect and quantify the abnormal hematopoietic progenitor cells which represents a functional cell characteristic (**Figure 3**).

We show how to quantify three hematopoietic progenitor populations and the putative LSC compartment with different degree of maturation by flow cytometry with antibodies available in the majority of clinical laboratories. Furthermore, we confirmed the presence of these cell compartments in corresponding patient-derived-xenografts (PDX) and at treatment follow up.

Protocol

We follow European standards for the use of animals for scientific purposes. This study was approved by the local Ethics committee (#3097-2015120414583482v3).

1. Sample preparation

1. Collect bone marrow (2 mL) from *de novo* AML in tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) at a concentration of 1.8 mg per mL of bone marrow.
2. Perform Ficoll separation, a density gradient separation to isolate mononuclear cells from red cells and granulocytes, as followed. Dilute bone marrow in 3 volumes of phosphate buffer saline (PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10mM, KH_2PO_4 1.8 mM). Carefully overlay 1 volume of Ficoll with 1 volume of diluted bone marrow. Spin 30 minutes at 300 x g without brake. Transfer the white buffy coat layer of mononucleated cells into a new sterile tube.
3. Wash cells twice in 10 mL of PBS) and centrifuge at 300 x g for 5 min, in order to remove contaminating serum components.

2. Lysis of red blood cells (RBC)

1. Add 2 mL of lysis buffer (ammonium chloride 0.8%) to the cell pellet, vortex gently and incubate at room temperature for 5 min. Centrifuge at 300 x g for 5 min.
   Note: if necessary, repeat this step.
2. Wash cells in PBS as previously described (1.3.).

3. Patient derived xenografts.

1. Obtain blast cells from bone marrow after Ficoll separation and after lymphocytes depletion using immunomagnetic negative selection (CD3 for T-, and CD20 for B-lymphocytes). Follow the manufacturer's instructions.
2. Inject 5 x 10^6 AML blast cells into the tail vein of unconditioned NSG mice. Use a restraining device to facilitate tail vein injection. Keep mice under conventional conditions and especially under specific-pathogen-free conditions at all times, by using individual ventilated cages and by manipulation under laminar flow hood. Every two weeks, take 60 µL of blood by the submandibular collection method using a hematocrit capillary. Place the capillary into a 5 mL round bottom tube. Stop the bleed by applying gentle pressure using a small sterile gauze pad. Expulse blood with a clean syringe.
3. Add 1ml of lysis buffer and incubate for 5min. Then centrifuge at 300 x g for 5min. Wash with PBS and centrifugre at 300 x g for 5 minutes. Remove supernatant, the add 100 µL PBS with 10% bovine serum albumin (BSA) stain with 3 µL anti-human CD45-FITC and 1 µL anti-murine CD45-APC and incubate at 4°C for 30 minutes.
4. Wash cell pellet two times in PBS (2 mL) with 10% BSA and centrifuge at 300 x g for 5 min.
5. Aliquot up to 1 x 10^6 cells per 100 µL of PBS into flow cytometry tubes.
6. Survey engraftment every two weeks by chimerism analysis of CD45 expression (human *versus* murine) using flow cytometry (**Figure 2A and B**).
7. At sacrifice (peripheral blast count greater than 70% or severe clinical signs of sickness), collect mononuclear blast cells from crushed spleens and from bone marrow by flushing tibias and femurs with PBS as previously described[13]. Euthanize mice by cervical dislocation following international guidelines.
8. If cell pellet contains RBC, perform lysis of red blood cells with lysis buffer (Step 2.1).
9. Wash cells pellet two times in PBS (2 mL) with 10% BSA and centrifuge at 300 x g for 5 minutes.
10. Collect cells and aliquot up to 1 x 10^6 cells per 100 µL of PBS into flow cytometry tubes.

4. Staining

1. Add conjugated antibodies (see Step 4.2) and vortex. Incubate cells for 15 minutes in the dark at room temperature.

2. Use the following panel for human primary or PDX samples consisting of 10 µL of anti-CD36-FITC, 5 µL of anti-CD19-ECD, 5 µL of anti-CD33-PC5.5, 5 µL of anti-CD90-APC, 5 µL of anti-CD34-AA700, 5 µL of anti-CD45RA-APC-H7, 5 µL of anti-CD38-Pacific Blue , 5 µL of anti-CD123-PC7 and 2.5 µL of anti-CD45-KO.
3. Remove unbound antibodies by washing the cells in 2 mL of PBS by centrifugation at 300 x g for 5 min.
4. Re-suspend cells in 450 µL of PBS for final flow cytometric analysis.

5. Gating strategy for flow cytometry analysis

1. Perform data acquisition (at least 500,000 cells) on a flow cytometer equipped with red, blue and violet lasers. Verify cytometer settings every day for 1) optical alignment, 2) fluidic system, 3) optical sensitivity, and 4) standardization using fluorospheres
2. Follow the sequential gating strategy to define CD38 expression (**Figure 1**).
    1. Use cells from normal bone marrow samples to define hematogones or plasma cells which will serve as positive control for CD38 expression.
    Note: this gate is especially important to assess as subsequent putative LSC populations depend on its definition.
    2. Define physiologic precursors cells (normal blast cells) by CD45dim/SSC (side scatter) low population criteria (**Figure 1A**). Within this blast population, define hematogones, which display a CD38++CD19+ phenotype (**Figure 1B**) and finally, use CD36 and CD33 expression to define myeloblasts, monoblasts, and erythroblasts (**Figure 1C**). Determine CD34 expression on hematogones as shown in (**Figure 1E**). Assess several subpopulations of precursors within the blast cells defined as P6-P10 (**Figure 1D**). Separate the CD34 compartment of blast cells into P6, P7, P8 progenitor subpopulations based on preset CD38 gates. Ensure that the P8 compartment contains the blasts that have the same CD38 intensity as the hematogones, that P6 includes cells which are CD38-based on the CD38 fluorescence minus one (FMO) control and that P7 cells are in between the two extremes with regard to CD38 expression (**Figure 1D**).

3. From the CD34+38- (P6) gated cells, separate HSC from putative LSC using CD90 and CD45RA expression (**Figure 1F**).
Note: the HSC phenotype is CD34+CD38-CD90+CD45RA- and the multipotent progenitors (MPP) are defined as CD34+CD38-CD90-CD45RA-. The putative LSC phenotype is CD34+CD38-CD90dimCD45RA+ and the more immature downstream population are the lymphoid-primed progenitors (LMPP) defined as CD34+CD38-CD90-CD45RA+.

6. MRD monitoring by RT-PCR

1. Monitor MRD levels by RT-PCR as described previously[14].

Representative Results

Here we present a method to determine progenitor populations in normal and malignant human bone marrow samples. We collected bone marrow and spleen from a successful PDX and performed multiparametric flow cytometry as described above. We compared several subpopulations of blast cells between the diagnostic patient sample and the corresponding PDX and particularly, the CD34+CD38- progenitor compartment, notably enriched in putative LSC. We have found that the CD34+CD38- blast fraction was higher in PDX compared to the diagnostic patient sample (3.48% vs. 0.53%, **Figure 2C**, **D**). Interestingly, we found a higher percentage of putative LSC (displaying a CD34+CD38-CD90-CD45RA+ phenotype) in the PDX compared to the primary patient sample (2.76% vs. 0.48%, **Figure 2E**, **F**), suggesting a possible enrichment of malignant progenitor cells in hematologic tissues in this mouse model. The frequency of normal progenitor cell fractions did not differ between the PDX and the patient sample.
Furthermore, we studied the blast cell progenitor populations based on the expression of CD34, CD38, CD90, and CD45A (as described in this protocol) in two different patient samples, at diagnosis and at treatment follow-up to determine the level of MRD. In the first patient, the CD34+CD38- (P6) fraction increased from 0.06% at diagnosis to 3.33% at the first treatment follow-up (**Figure 3A**, **B**). In contrast, the CD34+CD38- (P6) fraction decreased in the second patient from 7.8% at diagnosis to 2.27% at the first treatment follow-up (**Figure 3C**, **D**). Accordingly, the putative LSC fraction increased in the first patient from 0% at diagnosis to 0.65% (**Figure 3A'**, **B'**) and decreased in the second patient from 6.57% at diagnosis to 1.44% at treatment follow-up (**Figure 3C'**, **D'**). Similarly, MRD monitoring by molecular markers (i.e., NPM1 mutation [first patient] and CBFB-MYH11 translocation [second patient]) showed that molecular MRD decrease less in the first patient (0.8%) compared to the second patient (0.05%) for the first treatment follow-up point (**Figure 3E**, **G**). Finally, to validate that putative P6 LSC are malignant, expression of leukemia associated antigens CD123 and CD33 were estimated, showing abnormal expression of CD33 for both patients (**Figure 3F**, **G**).

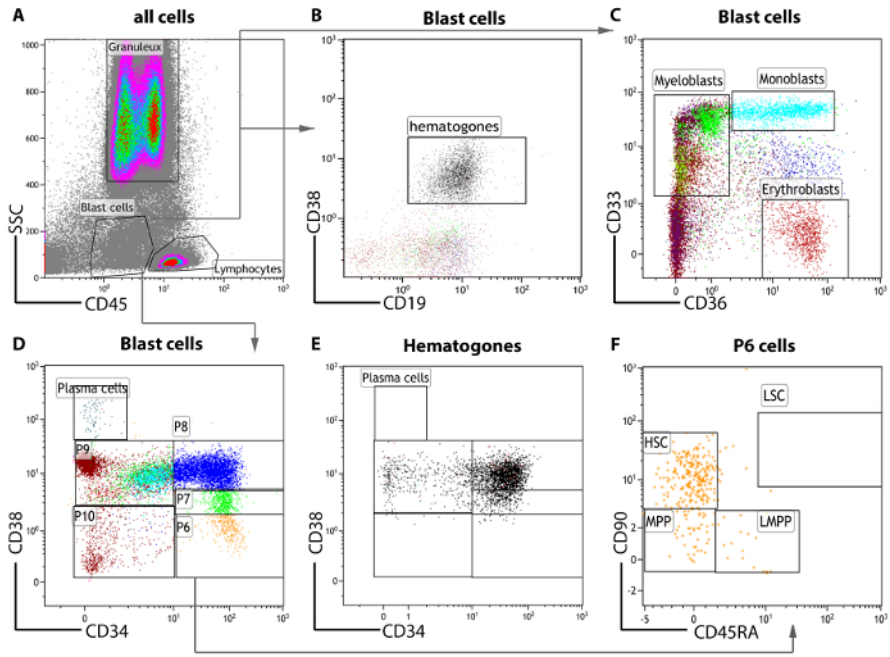

Figure 1: Gating strategy used for physiologic precursors and AML blasts. Density plot with gating of physiologic precursors shown for a normal bone marrow. (**A**) Gating of blast cells based upon CD45dimSSClow phenotype on all mononucleated cells. (**B**) Gating of hematogones (CD38+CD19+) for verification of CD38 positivity. (**C**). Gating strategy of normal myeloblasts, monoblasts and erythroblasts based on CD33 and CD36. (**D**) Blast cells can be separated into CD34+ progenitor subpopulations and using CD38 expression into P6 (CD34+CD38-), P7 (CD34+CD38dim), P8 (CD34+CD38+) cell compartments. (**E**) Gating of hematogones based on CD34 and CD38 expression, verify CD38 positivity. (**G**) Determination of HSC, MPP, LMPP, and putative LSC subpopulations using expression of CD90 and CD45RA as previously described[9]. Of note, no detection of LSC as this is a normal bone marrow sample. Please click here to view a larger version of this figure.

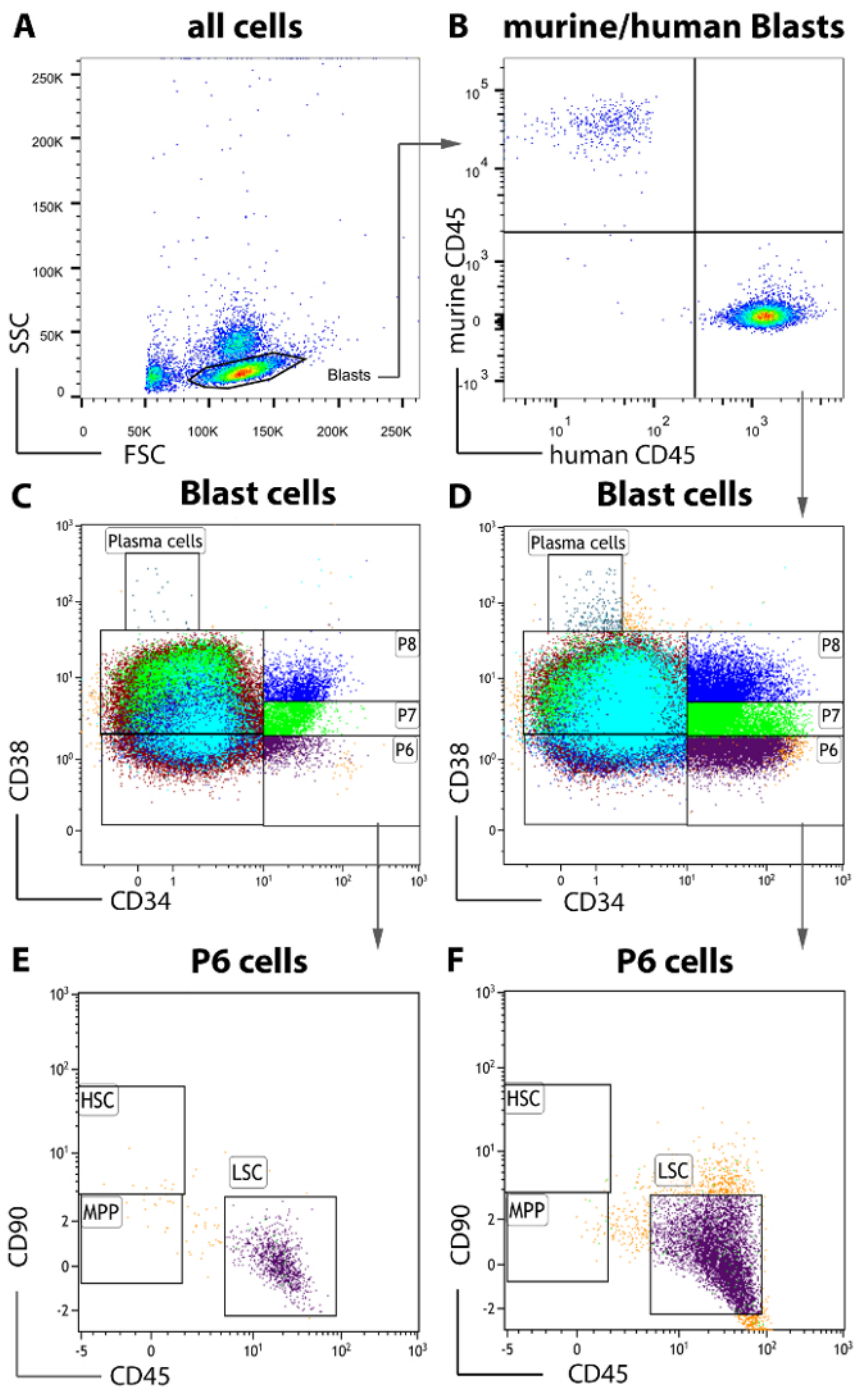

Figure 2: Blast cell progenitor populations in a patient and in corresponding patient-derived-xenograft (PDX) by multiparametric flow cytometry. (**A-B**) Chimerism analysis of PDX. (**A**) Murine and human blasts are first defined by size and structure (FSC, SSC). (**B**) Subsequently, the percentage of human versus murine CD45 staining is indicative of xenoengraftment and leukemia burden. Comparison of CD34+CD38- blast cells percentage between (**C**) primary diagnostic and (**D**) PDX AML sample. Comparison of putative LSC (CD34+CD38-CD90-CD45RA+) between (**E**) primary AML and (**F**) PDX. Please click here to view a larger version of this figure.

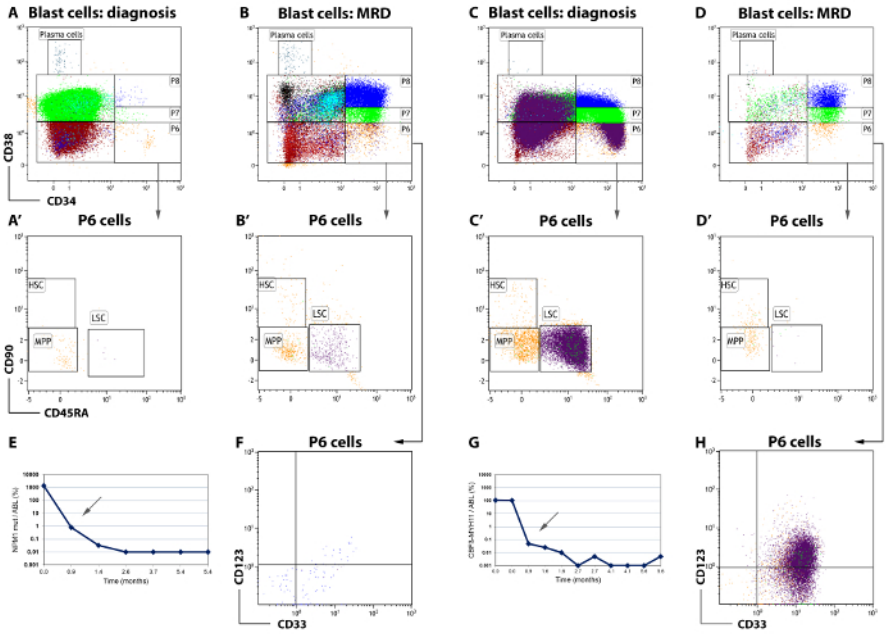

Figure 3: Blast cell progenitor populations in two patients at diagnosis and after remission induction therapy for minimal residual disease (MRD) detection. Flow cytometric analysis of CD34+CD38- blast cell subpopulations in two representative patients (**A, C**) at diagnosis and (**B, D**) after remission induction therapy. Figures (**A'-D'**) illustrate respective P6 gates containing putative LSC for the two patients at diagnosis and after initial treatment. Shown in (**E, G**) is the minimal residual disease (MRD) analysis by molecular markers used for these two patients over a period of five months (NPM1 mutation and CBFß-MYH11 gene fusion, respectively). (**F, G**) Expression of leukemia associated antigens CD123 and CD33 on putative P6 LSC, showing abnormal expression of CD33 for both patients. Please click here to view a larger version of this figure.

Discussion

An accurate and reproducible assessment of membrane or cytoplasmic markers by flow cytometry requires that instrument settings are verified every day for optical alignment, proper functioning of the fluidic system, optical sensitivity, and standardization using calibration beads.

Detection of blast cell populations in AML using CD90 and CD45RA by multi-parametric flow cytometry analysis is a relatively simple and reliable method. A critical step in this analysis is an accurate assessment of CD38 expression on blast cells, in order to perform correct gating of the CD34+CD38- population. CD38 intensity is defined using normal bone marrow cells specifically on hematogones and/or plasma cells (CD38+). Intensity of other markers is determined using isotype controls.

The addition of CD45RA and CD90 is used to distinguish HSC from putative LSC. The latter and not HSC maybe the cause of disease relapse, thus this protocol is of interest for minimal residual disease monitoring using flow cytometry panels. Monitoring of AML during treatment is essential to improve risk stratification and to guide AML therapy. Unfortunately, appropriate molecular markers are not available for all patients. Therefore, MRD monitoring by flow cytometry may be feasible for all AML cases. Even though this method may not be exhaustive as it focalizes on the majority of AML cases which have a CD34+CD38- progenitor and a putative LSC population. Of note, it is possible that the putative LSC compartment may include normal progenitors, i.e., LMPP). Furthermore, we cannot rule out that there are rare cases with functional LSC not following this abnormal expression pattern. Of note, even in NPM1-mutated AML which are considered CD34 negative, we are able to detect putative LSC. Therefore, despite the heterogeneity and complexity of the AML LSC compartment, our method may be used as a biomarker of chemotherapy response during follow up of AML.

Disclosures

The authors have nothing to disclose.

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