Aberrant Underexpression of CD81 in Precursor B-Cell Acute Lymphoblastic Leukemia

Utility in Detection of Minimal Residual Disease by Flow Cytometry

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Abstract

We studied CD81 expression by flow cytometry (FC) on benign precursor B cells (hematogones) and leukemic blasts in precursor B-cell acute lymphoblastic leukemia (pre-B-ALL) and established its usefulness in minimal residual disease (MRD) assays. Hematogones showed uniformly bright CD81 expression. In 98 pre-B-ALLs at diagnosis or overt relapse, 80 (82%) showed aberrantly decreased CD81 intensity. We used hematogones in 139 MRD– specimens to set a lower threshold for normal CD81 expression. In 133 specimens positive for residual pre-B-ALL, 87.2% showed increased CD81-dim immature B cells (>10%) and/or a discrete cluster of CD81-dim cells in a background of hematogones. Only 1 of 139 MRD– specimens showed more than 10% CD81-dim cells. Decreased CD81 expression was maintained in 91% of aberrant cases analyzed before and after chemotherapy. Decreased CD81 expression is a sensitive and specific marker for residual pre-B-ALL, even in a background of hematogones, making CD81 a useful addition to a panel for MRD detection by FC.

The morphologic threshold for unequivocal detection of residual disease in patients with precursor B-cell acute lymphoblastic leukemia (pre-B-ALL) is generally considered to be a blast count of 5% of total nucleated cells in bone marrow aspirate smears. Flow cytometry (FC) is a rapid, convenient, and generally applicable technique for detecting minimal residual disease (MRD), defined as disease not detectable by morphologic examination, in pre-B-ALL. It can be more than 100 times more sensitive than morphologic examination.1-4 Postchemotherapy MRD has been shown to be an independent and powerful prognostic factor for predicting relapse in patients with pre-B-ALL.5-11 The results of MRD analysis are used in some treatment protocols to determine the need for further treatment, including treatment intensification. Patients with no detectable MRD may be candidates for less intensive treatment regimens, thus avoiding unnecessary toxic effects.

Leukemic blasts in pre-B-ALL often show aberrant gain or loss of surface antigens or changes in the pattern or intensity of antigen expression, giving rise to an immunophenotypic profile distinct from normal cells. Thus, FC can be used to differentiate leukemic cells from their normal immature B-lineage counterparts, termed hematogones. However, the immunophenotypic differences between hematogones and leukemic blasts in some cases may be subtle, and extensive experience may be required for reliable interpretation of some antigens or for detecting very low-level disease. In addition, hematogones are often abundant in the bone marrow of young children and in postchemotherapy and posttransplantation settings.12 As a result, the evaluation of MRD in these circumstances can be problematic. The ideal FC marker for detecting MRD in pre-B-ALL should show
a consistent pattern of expression in hematogones and an aberrant expression pattern in leukemic cells in a high proportion of cases. There should be minimal overlap in expression between benign and leukemic cells, and the level of expression in leukemic cells should be stable over time.

CD81 is an integral surface membrane protein that is a member of the tetraspanin family. CD81 is associated with CD19 to form a CD19-CD21-CD81 multimolecular complex that is involved in signal transduction in B cells.13-15 Barrena and colleagues16 reported CD81 expression to be aberrantly dim in a small series of pre-B-ALL cases and suggested the possible usefulness of CD81 expression in distinguishing pre-B-ALL cells from hematogones. The purposes of the present study were to examine the expression of CD81 on a larger range of pre-B-ALL cases and to establish a practical method of analyzing FC data for aberrant CD81 expression in MRD specimens.

Materials and Methods

Samples

Bone marrow aspirate samples were submitted to the clinical FC laboratory at The University of Texas M. D. Anderson Cancer Center, Houston, between March 2006 and March 2007. Two different groups of cases were used in this study. A total of 98 cases of overt pre-B-ALL (>5% blasts) submitted for initial diagnosis (73 cases) or during relapse (25 cases) formed the first group. In every case, the diagnosis of pre-B-ALL was established using World Health Organization criteria17 and included flow cytometric immunophenotypic data derived from an extensive panel of myeloid and lymphoid markers.

The second study group included 272 bone marrow aspirate specimens from 148 patients with pre-B-ALL that had sufficient CD19+/CD34+ cells for CD81 analysis (>100 cells). In these cases, MRD was assessed using a panel of markers, including CD9, CD10, CD13, CD19, CD20, CD22, CD33, CD34, CD38, CD58, CD66c, and cytoplasmic terminal deoxynucleotidyl transferase. MRD was identified in comparison with the known patterns of antigen expression by normal maturing CD19+ B cells using an approach similar to that described by Weir et al.18 A distinct cluster of at least 20 cells showing altered antigen expression was regarded as an aberrant population. We required aberrant expression of at least 2 antigens to make a diagnosis of MRD. The 272 follow-up bone marrow aspirate specimens included 133 positive for residual pre-B-ALL and 139 cases negative for MRD. As control samples for CD81 expression, 12 apparently normal bone marrow aspirate specimens submitted for lymphoma staging workup but negative for disease were also assessed.

Flow Cytometry

For analysis of CD81 expression in initial diagnostic specimens, the antibody combination used was CD34–fluorescein isothiocyanate (FITC), CD81–phycoerythrin (PE), CD45–peridinin chlorophyll protein (PerCP)–cyanine (Cy)5.5 (all from BD Biosciences, San Jose, CA) and CD10–allophycocyanin (APC; Beckman Coulter, Fullerton, CA). For the 272 follow-up specimens, the antibody combination CD10–FITC, CD81–PE, CD34–PerCP-Cy5.5, and CD19–APC was used. 1 × 10^6 bone marrow cells were stained using a standard stain-lyse-wash procedure, using Ortho Lyse buffer (BD Biosciences). Data were acquired on FACSCalibur cytometers using CellQuest software (BD Biosciences). In most cases, 3 × 10^4 cells were acquired at initial diagnosis of overtly leukemic specimens. For follow-up specimens, at least 2 × 10^5 total cells were acquired when specimen quality permitted. Data on B cells were selectively acquired by gating on CD19+ cells with low side scatter. Data were retrospectively analyzed using FlowJo software (Treestar, Ashland, OR). For initial diagnostic specimens, leukemic blasts were gated on the basis of CD45 and side scatter. For follow-up and control specimens, CD19+/CD34+ cells were gated.

Data Analysis

Patients’ clinical charts were reviewed, and data were collected with regard to demographic data, date of diagnosis, dates of flow cytometric immunophenotypic analysis, and dates of chemotherapy administration. The 2-tailed Student t test was used for numeric comparison between 2 groups. The Fisher exact test was applied for categorical variables. Differences between 2 groups were considered statistically significant if P values were less than .05 in a 2-tailed test.

Results

The pattern of CD81 expression in normal bone marrow cells is shown in Image 1. Hematogones, CD19+ with dim CD45 expression and low side scatter, showed the brightest CD81 expression. Lymphocytes, monocytes, myeloid blasts, and a subset of granulocytes all showed dim to moderate expression of CD81. Among CD19+ B cells, all CD10+ precursors showed bright CD81 expression Image 2A and Image 2B, whereas the more mature CD10– B cells showed moderate CD81 expression. In normal bone marrow, only very rare immature CD19+/CD34+ cells or CD19+/CD10+ cells showed decreased CD81 expression (in boxed areas in Images 2A and 2B). In contrast, in most cases of pre-B-ALL, many CD19+/CD34+ leukemic blasts showed decreased expression of CD81 Image 2C.

The median fluorescence intensity (MFI) of CD81 expression by benign CD19+/CD34+ immature B-cell precursors in control bone marrow samples showed a narrow range, and 11

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of 12 cases had a CD81 MFI greater than 1,000. Figure 1. In 98 cases of pre-B-ALL, analyzed at the time of diagnosis or overt relapse, CD45 dim leukemic blasts showed decreased CD81 expression in 80 of 98 (82%) cases (Figure 1). In these cases the CD81 MFI was less than 1,000. The difference between the range of MFI of immature hematogones (mean, 1,460; standard error of mean [SEM], ±114) and the range of MFI of leukemic blasts (mean ± SEM, 647 ± 94) was statistically significant (P = .0034; Student t test). Rare cases of pre-B-ALL showed high levels of CD81 expression; 5 (5%) of 98 had an MFI outside the normal range (mean plus 2 SDs) based on the 12 normal cases.

The frequency of aberrantly decreased CD81 expression was higher among pre-B-ALL cases that were CD34+ (>20% of blasts) than among CD34− pre-B-ALL cases. Of 85 CD34+ cases, 76 (89%) showed a decreased CD81 MFI, whereas only 4 (31%) of 13 CD34− cases had a decreased CD81 MFI. This difference was statistically significant (P < .0001; Fisher exact test). Of 5 pre-B-ALL cases with high levels of CD81 expression, 3 were negative for CD34.

Since CD81 vs CD34 plots appeared to provide the greatest potential separation between normal bone marrow elements and the leukemic blasts in the majority of pre-B-ALLs, we selected CD34+ cases for our analysis of 272 follow-up MRD specimens. CD81 expression by CD19+/CD34+ cells is shown in Image 3A for 6 representative follow-up bone marrow specimens submitted for pre-B-ALL MRD analysis. In cases negative for MRD, benign immature hematogones showed bright CD81 expression (Image 3A). In most specimens positive for residual pre-B-ALL, the CD19+/CD34+ leukemic blasts showed decreased CD81 expression (Image 3B). In a subset of positive specimens, the leukemic blasts showed very bright CD81 expression (Image 3C), similar to or even brighter than that of benign immature hematogones. In occasional positive
specimens, most leukemic blasts were relatively bright, but CD81 expression was more variable than seen in benign hematogones (Image 3D), so that many blasts overlapped with hematogones but a subset showed decreased CD81 expression. A number of specimens positive for MRD showed distinct population clusters on CD34 vs CD81 plots (Image 3E). Based on the aberrant expression of other markers in the MRD panel, such specimens were seen to contain a population of residual leukemic blasts in a background of hematogones. Image 3F shows a subsequent specimen from the same patient as Image 3E, now in overt clinical relapse, with numerous leukemic blasts demonstrating dim CD81 expression.

As these examples illustrate, a threshold for MRD detection based on the CD81 MFI of total CD19+/CD34+ cells would lack sensitivity, particularly for specimens containing both leukemic blasts and hematogones. In fact, the positive cases shown in Images 3D and 3E had normal or only borderline low values for CD81 MFI. Therefore, we sought to establish a threshold gate for MRD detection based on the lower limit of CD81 expression by normal immature B-cell precursors.

Based on the other markers in the MRD panel, 139 follow-up bone marrow specimens were negative for residual pre-B-ALL, and, thus, the CD19+/CD34+ cells in these specimens were apparently benign hematogones. We adjusted the CD81 threshold so as to maximize the specificity of MRD detection (ie, to minimize the possibility of a false-positive diagnosis). Using an MFI threshold of 388 for CD81-PE expression (as shown in Image 3), the percentage of CD19+/CD34+ cells with decreased CD81 levels was more than 10% in only 1 of 139 cases negative for MRD Figure 2A. The 1 patient with an increased percentage of CD81-dim cells had received rituximab therapy for CD20+ pre-B-ALL and showed a proportional increase in CD34+ early B-cell precursors. For this patient, the original leukemic blasts showed multiple aberrancies in the expression of other markers, as did the blasts in a subsequent extramedullary relapse 6 months later, but none of these aberrancies were seen in the MRD specimen.

In contrast, 113 (85.0%) of 133 specimens positive for pre-B-ALL, as assessed by standard markers in the MRD panel, had more than 10% of cells with decreased expression of CD81 Figure 2B. The difference in the percentage of CD81-dim cells in cases negative for MRD (mean ± SEM, 1.93 ± 0.21) and cases positive for residual disease (mean ± SEM, 59.67 ± 3.10) was statistically significant (P < .0001).

In addition, 3 of the MRD+ specimens with fewer than 10% CD81-dim cells showed diagnostically aberrant CD81 staining as they contained discrete clusters of leukemic blasts with decreased CD81 expression in a background of CD81-bright hematogones (indicated by asterisks in Figure 2B). Thus, by using a threshold of more than 10% of CD19+/CD34+ cells with decreased expression of CD81 or the presence of a discrete cluster of cells with dim CD81 as
criteria for a positive diagnosis, the sensitivity and specificity for detecting residual pre-B-ALL were 87% and more than 99%, respectively.

The specimens positive for residual pre-B-ALL were qualitatively assessed for aberrant expression of CD10 and CD34, 2 widely used markers for MRD detection by FC. Benign hematogones in the MRD– specimens showed a consistent pattern of CD10-FITC vs CD34-PerCP-Cy5.5 expression on CD19+ bone marrow cells, although the range of normal expression was broader for both markers than seen with CD81. In contrast, leukemic blasts showed comparatively increased or decreased levels of CD10 and/or CD34 in most specimens (107/133 [80.5%]). Of the remaining 26 specimens (from 19 patients) showing residual pre-B-ALL with unremarkable levels of CD10 and CD34 expression, 20 showed aberrantly decreased CD81 expression, using the criteria described.

Qualitative assessment of CD38 expression also showed an aberrant decrease in most specimens positive for residual pre-B-ALL (118/133 [88.7%]). A few positive specimens showed bright (nondiagnostic) CD38 expression but decreased CD81 expression (9/133 [6.8%]), and a few showed the reciprocal pattern, with bright (nondiagnostic) CD81 expression but aberrantly decreased CD38 (11/133 [8.3%]). Specimens with normal expression levels of both CD38 and CD81 were rare (6/133 [4.5%]). Leukemic blasts in all 133 positive specimens showed aberrant levels of expression in 1 or more of the 4 antigens, CD10, CD34, CD38, and CD81.

We examined the stability of CD81 expression through time and following chemotherapy. For 25 patients, CD81 expression data were available from the original diagnostic workup and there were subsequent specimens positive for residual disease. Of the 25 patients, at the time of MRD assessment, 10 were undergoing induction therapy or were immediately postinduction, 8 were in consolidation or maintenance phase, and 7 had completed maintenance therapy. In 23 of the 25 patients, leukemic blasts showed aberrantly decreased CD81 expression at time of initial diagnosis. The CD81 MFI remained aberrantly dim through 1 or more subsequent time points in 21 (91%) of 23 patients. The CD81 MFI for 6 representative patients with multiple follow-up samples is shown in Figure 3.

Discussion

We have shown that CD81 is aberrantly decreased in a large majority of pre-B-ALL cases, particularly in CD34+ cases, and that this finding can be used in FC assays for the detection of MRD. A subset of pre-B-ALL cases show bright CD81 expression, which may occasionally be brighter than benign hematogones. Barrena et al16 first characterized CD81 expression during normal B-cell maturation and examined CD81 expression in a variety of B-lineage neoplasms as part of a broader study of tetraspanin molecule expression in benign and neoplastic B cells. They demonstrated uniformly high levels of CD81 expression in early-stage CD34+/CD10+ and late-stage CD34–/CD10+ hematogones. CD81 was aberrantly underexpressed in 9 (75%) of 12 pre-B-ALL cases in their series. Our findings confirm and extend these results.

CD81 is particularly suitable for flow cytometric detection of MRD in pre-B-ALL because, in most cases, at least
some leukemic blasts fall outside the normal range of CD81 expression for hematogones. Thus, when displaying CD19+ bone marrow cells on a CD34 vs CD81 plot, leukemic blasts form a cluster in a region devoid of normal bone marrow cells. Even very small numbers of leukemic blasts may be identified, despite the presence of a predominant population of hematogones, as the aberrant cells often form a discrete second cluster.

To analyze cases with a single population cluster of B cells on CD34 vs CD81 plots, we established a lower threshold for normal CD81 expression. Most specimens with residual pre-B-ALL showed an increased percentage of aberrant CD81-dim cells. By using dual criteria for a positive diagnosis, with increased CD81-dim cells or a discrete CD81-dim cluster, we identified 87.2% of specimens with residual pre-B-ALL. Our approach should be generalizable to other testing sites because normal bone marrow specimens may be used to establish laboratory-specific lower thresholds for CD81 expression.

Rare, apparently MRD−, follow-up specimens may show a mild decrease in CD81 expression. The single such specimen in our series (1/133) showed markedly left-shifted hematogone maturation, with all B cells positive for CD10 and at most only dim to moderate CD20 expression, in a patient who had received rituximab therapy for CD20+ pre-B-ALL. We suggest caution in interpreting an isolated decrease in CD81 expression in the setting of rituximab administration or during very early recovery from chemotherapy. In our laboratory, we require aberrances in the expression of at least 2 markers for a diagnosis of MRD, and this approach should also avoid potential false-positive diagnoses based on only dim CD81.

CD38 is another extremely useful marker for pre-B-ALL MRD detection, with very similar properties to CD81: hematogones show uniform bright expression, and most pre-B-ALLs are aberrantly dim for CD38. In our series, CD38 expression was also easily interpretable and highly sensitive, with 89% of specimens positive for residual pre-B-ALL demonstrating aberrant CD38 expression. CD81 was complementary to CD38 in MRD detection, as more than half of the specimens with nondiagnostic CD38 levels showed aberrant dim CD81 expression. Thus, 127 (95.5%) of 133 positive specimens could be identified using a combination of CD81 and CD38 expression. CD81 was similarly complementary to CD10 and CD34 in MRD detection.

It is well known that phenotypic shifts in pre-B-ALL can occur with some markers between diagnostic and postchemotherapy or relapse specimens on leukemic blasts and on background hematogones in up to 72% of cases.10-24 In our study, we analyzed CD81 expression in residual blasts in de novo cases, in cases in relapse, and in various cases at the end of induction after consolidation and during maintenance chemotherapy. We found that in nearly all cases, aberrant blasts retained their decreased expression of CD81 at all stages of therapy examined. The MFI for CD81 expression was stable (varying by <4-fold) in 91% of cases with sequential samples in this study.

The biologic significance of aberrant CD81 underexpression in pre-B-ALL is unknown. CD81 is an integral surface membrane protein with 4 transmembrane domains and is a highly conserved member of the tetraspanin family. CD81 is expressed to some degree on almost all cell types and is involved in cell morphology, adhesion, motility, invasion, fusion, and signaling.25 Like other tetraspanins, CD81 associates laterally with integrins in the cell membrane to form multimolecular complexes in the form of tetraspanin-enriched microdomains, also known as the tetraspanin web. In B cells, CD81 associates with CD19 to form a CD19-CD21-CD81 multimolecular complex that carries out signal transduction and is crucial for B-cell development and the humoral immune response.13,14,26 In some carcinomas, CD81 appears to have antimetastatic effects (reviewed by Zoller). It is not known at present whether the partially decreased CD81 expression in many pre-B-ALLs could affect CD19 signaling and/or the adhesion of leukemic blasts to the marrow microenvironment.

In this study, we have shown that CD81 is aberrantly decreased in leukemic blasts of pre-B-ALL cases compared with benign hematogones, particularly in CD34+ cases. With our gating strategy, we achieved a high sensitivity and specificity for MRD detection in our cohort of CD34+ cases. CD81 was particularly useful in cases in which a small number of...
leukemic blasts were admixed with numerous hematogones. The MFI for CD81 expression was also stable in 91% of cases with sequential samples in this study. These results indicate that aberrant CD81 expression is a robust marker for leukemic blasts in pre-B-ALL and is a useful addition to flow cytometric panels used to assess for MRD.

References


