



## Original Article

## Multidimensional Flow Cytometry for Detection of Rare Populations in Hematological Malignancies

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### Article info

#### Article history:

Received: April 30, 2008

Revised: May 23, 2008

Accepted: June 11, 2008

#### Keywords:

Flow cytometry

Leukemia

Lymphoproliferative disorder

Minimal residual disease

Myelodysplastic syndrome

### Abstract

**Objective:** Flow cytometry is becoming an important tool in the characterization of different hematological disorders. The aim of our study was to detect very rare populations in hematological malignancies using the comprehensive concept of immunophenotyping.

**Patients and Methods:** Six patients, including three with acute myeloid leukemia, one with acute lymphoblastic leukemia, one with myelodysplastic syndrome, and one with B-cell lymphoma, were enrolled in this study. Serial bone marrow aspirates were analyzed by three-color multidimensional flow cytometry.

**Results:** The core principles for the use of flow cytometric analysis are understanding deviations in abnormal antigen expression from normal cellular differentiating pathways and characterizing different patterns of antigenic aberrancy for each patient. Our results demonstrate that multidimensional flow cytometry can be used to: (1) monitor minimal residual disease after treatment; (2) aid in the differential diagnosis of cases, which are difficult to evaluate using conventional morphology; and (3) help in the staging of lymphoproliferative disorders.

**Conclusion:** After selecting appropriate combinations of monoclonal antibodies, and applying advanced knowledge in immunophenotyping, flow cytometry is very sensitive and specific for the detection of rare populations in hematological disorders. (*Tzu Chi Med J* 2009;21(1):40–51)

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## 1. Introduction

Analysis of human bone marrow aspirates is complicated by the presence of multiple cell lineages, each

of which are at a variety of maturational stages. Traditionally, microscopic examination can be used to identify most hematological disorders. However, it is difficult to discriminate very rare populations of

abnormal cells from a heterogeneous marrow environment. For example, patients with fewer than 5% morphologically identifiable blasts in bone marrow samples are judged to be in hematological remission, although they may still harbor as many as  $10^{11}$ – $10^{12}$  leukemic cells. The use of polymerase chain reaction to identify fusion transcripts and clonal antigen-receptor gene rearrangements is attractive because of its high sensitivity of one target cell per  $10^5$ – $10^6$  cells. However, only a minority of hematological diseases have specific genetic abnormalities available for clinical uses.

Immunophenotyping has become a powerful tool for characterizing different kinds of hematological disorders including acute and chronic leukemias, lymphoproliferative disorders, and paroxysmal nocturnal hemoglobinemia. Immunophenotyping can even aid as an adjunct in the diagnosis of myelodysplastic syndrome (MDS) and other myeloproliferative disorders. It has been shown that maturation of myeloid and lymphoid cells can be assessed by multidimensional flow cytometry (1–3). This technique permits the identification of cellular lineage and maturational stage in heterogeneous cell populations, and allows the detection of potential deviations from normal cellular differentiating pathways (4–7).

The development of multidimensional flow cytometry has increased the sensitivity and specificity for the detection of minimal residual hematological malignancies after treatment. The concept of minimal residual disease (MRD) was introduced to more accurately estimate the true number of malignant cells and, in turn, to improve clinical management and curative rates. There is evidence that the presence of MRD significantly increases the rate of relapse and reduces overall survival for acute myeloid leukemia (AML) (8–11) and acute lymphoblastic leukemia (ALL) (12–14).

Here, we describe this comprehensive concept of multidimensional high resolution flow cytometry by the demonstration of six patients with various hematological diseases. The three goals of this study were: (1) to detect minimal residual disease after treatment; (2) to aid in the initial differential diagnosis of cases, which are difficult to evaluate using conventional morphology; and (3) to help in the staging of lymphoproliferative disorders.

## 2. Patients and methods

### 2.1. Patients

Six patients with various hematological disorders were enrolled in this analysis. Diagnostic bone marrow was obtained for morphological evaluation, cytogenetic study and flow cytometric analysis. Serial follow-up bone marrow aspirates for those patients who received

treatment were also sent for analysis. All six patients signed informed consent to undergo bone marrow examination and flow cytometric tests.

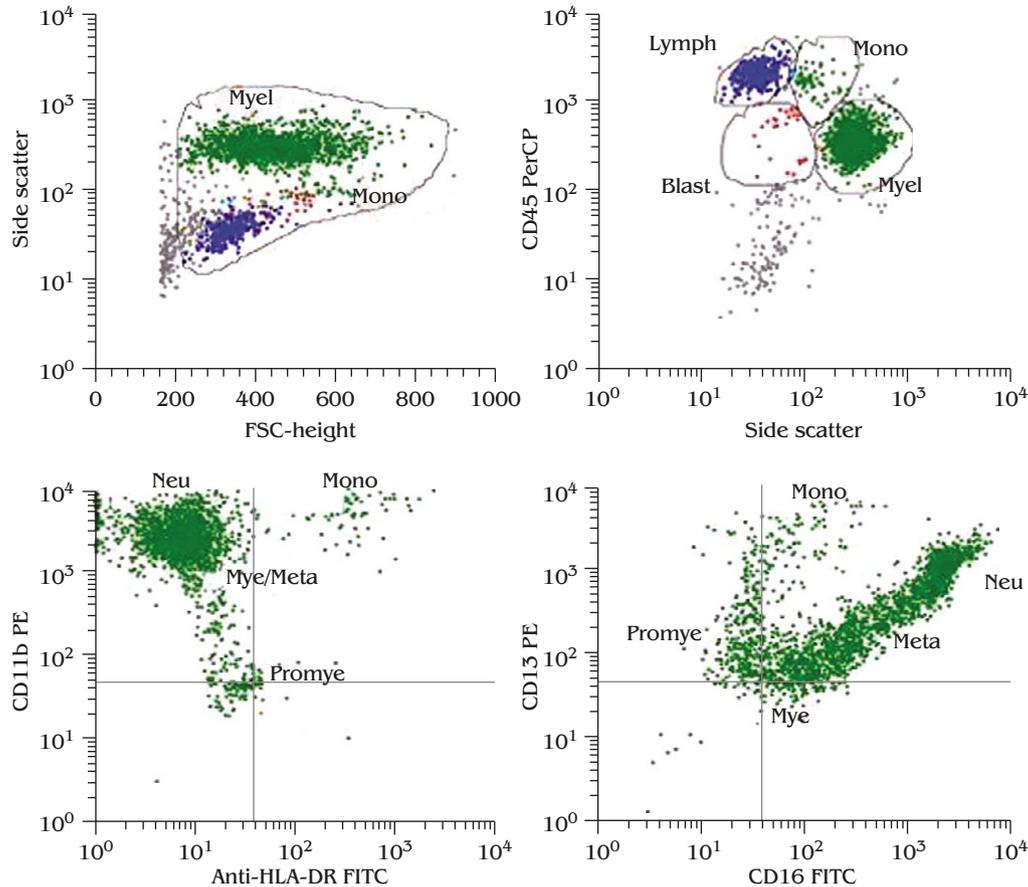
### 2.2. Cell preparation and multidimensional flow cytometric analysis

The procedure for labeling cells has been previously described (15). A working dilution of monoclonal antibodies titrated for maximum fluorescence, CD45 peridinin-chlorophyll-a protein (CD45 PerCP), and 0.1 mL of well-mixed heparinized bone marrow were incubated for 20 minutes at room temperature in the dark. The erythrocytes were lysed by adding 3 mL  $\text{NH}_4\text{Cl}$  (0.83%, buffered with  $\text{KHCO}_3$ , pH 7.2) for 5 minutes at  $37^\circ\text{C}$ . The cells were then pelleted, washed twice in phosphate buffered saline (PBS), pelleted again and re-suspended in PBS with 1% paraformaldehyde.

Specimens were analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) flow cytometer. The three color fluorescence used for monoclonal antibody conjugate included peridinin-chlorophyll-a protein (PerCP), phycoerythrin (PE), and fluorescein isothiocyanate (FITC). The instrument set-up was first standardized using lymphocytes as a reference, which was accomplished by gating on the unstained lymphocytes. This was followed by adjustment of the light scattering detectors to place the lymphocytes in a standard position in the correlative display of forward light scattering and orthogonal light scattering. Lymphocytes and maturing myeloid cells were then stained for further standardization using a series of fluorescence-conjugated monoclonal antibodies, which included CD45-PerCP, CD4-FITC and CD8-PE for lymphocytes and CD45-PerCP, CD11b-PE and CD16-FITC for myeloid cells.

For each combination of antibodies, a total of at least 10,000 events were recorded. List mode data were analyzed with WinList software (Verity Software, Topsham, ME, USA) in accordance with previously published techniques (15–17). For each sample, a computerized region was created to limit the analysis to viable cells with a forward and right-angle light scatter pattern characteristic of lymphocytes, monocytes, maturing myeloid elements, and blast-sized cells. Secondary gating was performed to limit further analysis to different groups of cells based on characteristic CD45 versus log side scatter properties.

Monoclonal antibodies used in this study were obtained from the indicated sources: fluorescein isothiocyanate (FITC-conjugated): CD16 from Pharmingen International (San Jose, CA, USA); CD14, CD15, CD33, CD34, CD7, CD5, CD38, HLA-DR, CD3, CD5, CD56, CD20, CD4, CD22, FMC-7, kappa, lambda, CD2 from Becton Dickinson Immunocytometry Systems



**Fig. 1** — Development of myeloid cells during normal hematopoiesis. Myeloid elements and monocytes are both shown in green. Four populations of lymphocytes, monocytes, maturing myeloid elements and blasts can be clearly identified based on different characteristics among CD45, and forward and side light scatters. On the panel of CD11b vs. HLA-DR, promyelocytes show very low expression of both antigens. During the process of differentiation toward neutrophils, HLA-DR is entirely lost, but more and more CD11b is acquired. On the panel of CD13 vs. CD16, CD13 is initially shown on promyelocytes but it is decreased to the lowest level on myelocytes. In the later stages of development toward neutrophils, the intensity of CD13 and CD16 increase to maximal levels, which creates a characteristic picture of a “concave”-shaped maturation pathway. Monocytes are both CD11b/HLA-DR and CD13/CD16 positive. FSC = forward light scatter; Lymph = lymphocytes; Mono = monocytes; Myel = maturing myeloid elements; Promye = promyelocytes; Mye = myelocytes; Meta = metamyelocytes; Neu = neutrophils.

(San Jose, CA, USA). Phycoerythrin (PE-conjugated): CD11b, CD13, CD33, CD14, CD19, CD34, CD56, CD38, CD5, CD23, CD8, CD25 from Becton Dickinson; CD10 from Pharmingen International; peridinin-chlorophyll-a (PerCP-conjugated) CD45 from Becton Dickinson.

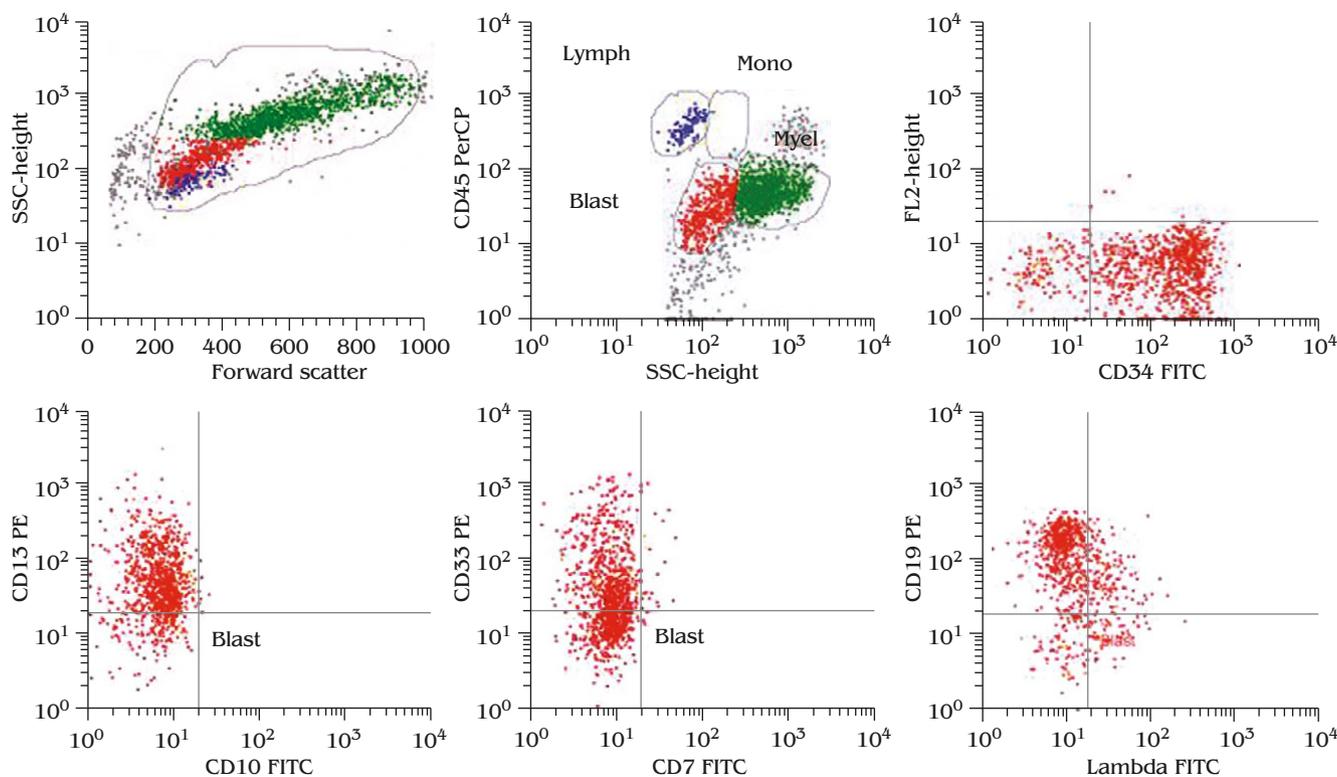
Fig. 1 shows an example of the normal maturational pathway for the myeloid elements.

### 3. Results

#### 3.1. Detection of minimal residual disease after treatment

Case 1 was a woman aged 40 years with acute myeloid leukemia. Diagnostic flow cytometry of her bone marrow aspirate demonstrated 22.5% blasts. As shown

in Fig. 2, the leukemic blasts revealed intermediate signals on side scatter and forward scatter. The blasts had low expression of CD45 but had high expression of CD34, HLA-DR, CD13 and CD33. Of importance, aberrant CD19 expression was found at a high level. Cytochemical staining of myeloperoxidase was also positive on the morphological examination. This was classical AML with aberrant CD19 expression. After induction chemotherapy with 3 days of idarubicin plus 7 days of Ara-C (I3A7), the blasts remained at 14.0% on day 16. Additional chemotherapy with 2 days of mitoxantrone and 5 days of Ara-C (N2A5) was given immediately. Nineteen days later, flow cytometric analysis revealed almost no residual leukemia based on the gating of CD19. Because complete remission was successfully achieved, consolidation chemotherapy with high-dose Ara-C (HD-AC) was given. Unfortunately, after



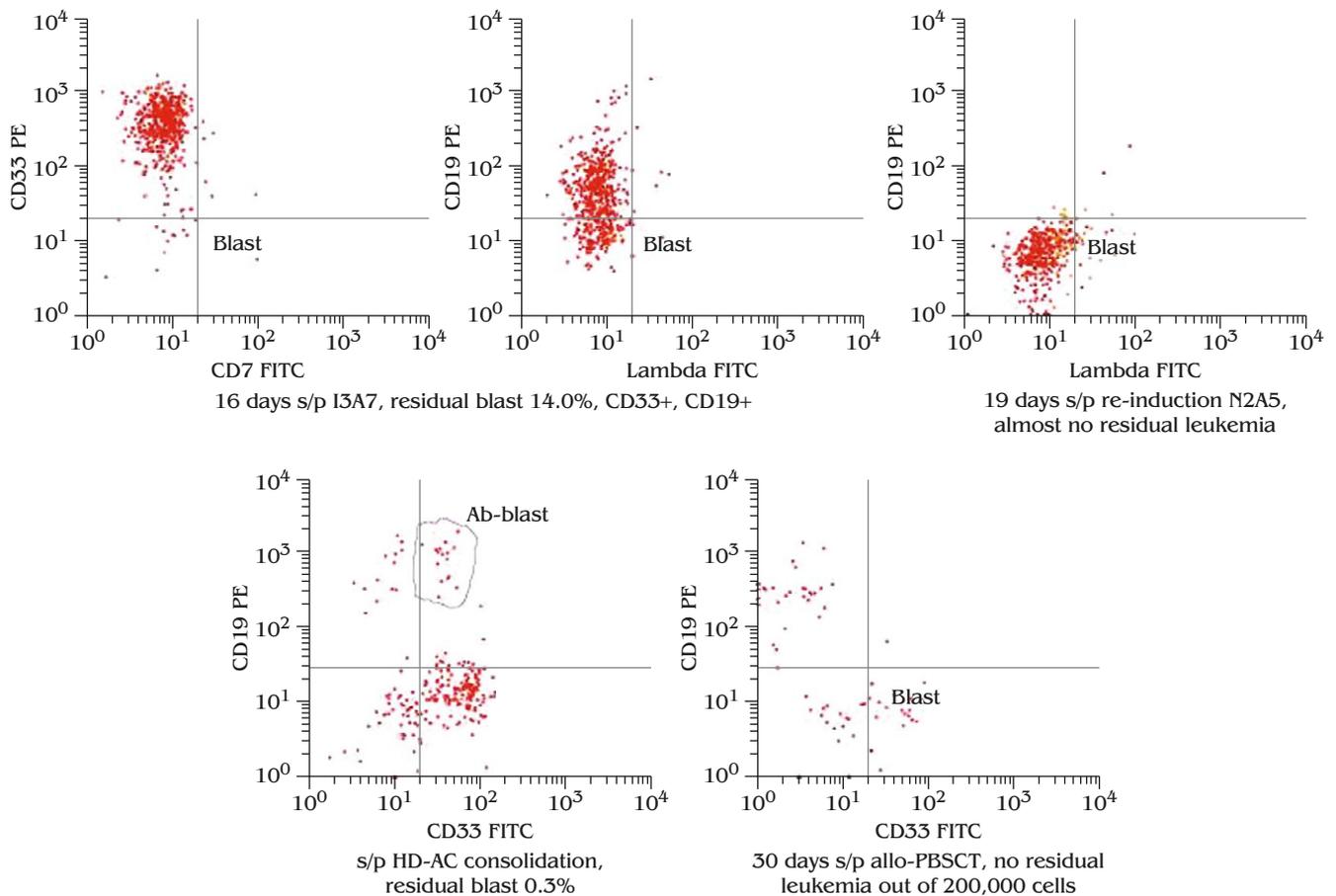
**Fig. 2 — Case 1: A woman aged 40 years with AML. Diagnostic flow cytometry shows 22.5% blasts, which showed low CD45+, CD34+, CD13+ and CD33+, and aberrantly high levels of CD19.**

recovery of the bone marrow cells, flow cytometry showed a small population of blasts that co-expressed CD33 and CD19 at only 0.3%, which indicated early relapse of AML. This patient then received allogeneic peripheral blood stem cell transplantation from her sibling. As shown in Fig. 3, on the day of stable engraftment, flow cytometry showed no leukemic blasts out of 200,000 marrow cells, which were analyzed in correlation with the previous blast region based on CD33/CD19 gating.

Case 2 was a woman aged 45 with acute promyelocytic leukemia (APL). Bone marrow morphology showed characteristics of hypergranular promyelocytes with multiple intra-cytoplasmic Auer rods. A cytogenetics study showed classical t(15;17). Diagnostic flow cytometry, as shown in Fig. 4, revealed abnormal leukemic cells located between the usual blast and maturing myeloid region. There was a marked increase in the signal on the side scatter, which indicated its hypergranular nature. The leukemic cells showed autofluorescence, high CD13 and CD33 expression and a lack of CD34 and HLA-DR, typical features of promyelocytes. The aberrant loss of CD15 expression also indicated its dysregulated nature. She was treated with standard all-trans-retinoic-acid (ATRA) plus idarubicin for 3 days. Complete remission was achieved with normalization of morphology, flow cytometry and cytogenetics. She was given one course of consolidation

chemotherapy with daunorubicin. Just before the next cycle of consolidation chemotherapy, follow-up flow cytometry showed a 4.2% abnormal population of cells that were located close to the usual blast region, although the other maturing myeloid cells showed orderly differentiation. These were not normal blasts because they did not express HLA-DR or CD34. As shown in Fig. 5, only several dots of normal blasts expressed CD34 and HLA-DR. These abnormal cells also highly expressed CD13 and CD33, indicating early relapse of promyelocytic leukemia. After carefully reviewing the bone marrow morphology, a small area of blast-like promyelocytes aggregates could be seen, which was correlated to reduced granularity on the relapsed flow cytometric figure. A subsequent cytogenetics study confirmed re-emergence of t(15;17). After salvage treatment with arsenic trioxide, the small population of relapsed leukemic cells disappeared completely on flow cytometric monitoring.

Case 3 was a woman aged 54 with acute myeloid leukemia. As shown in Fig. 6, diagnostic flow cytometry revealed 94.0% blasts with decreased CD45 and side scatter signals. The leukemic cells revealed high CD13 and CD33 expression but a lack of CD34 and HLA-DR (CD13 and CD34 are not shown in the figure). One striking feature is that the blasts aberrantly expressed high amounts of CD56. A follow-up bone marrow examination was performed 25 days after



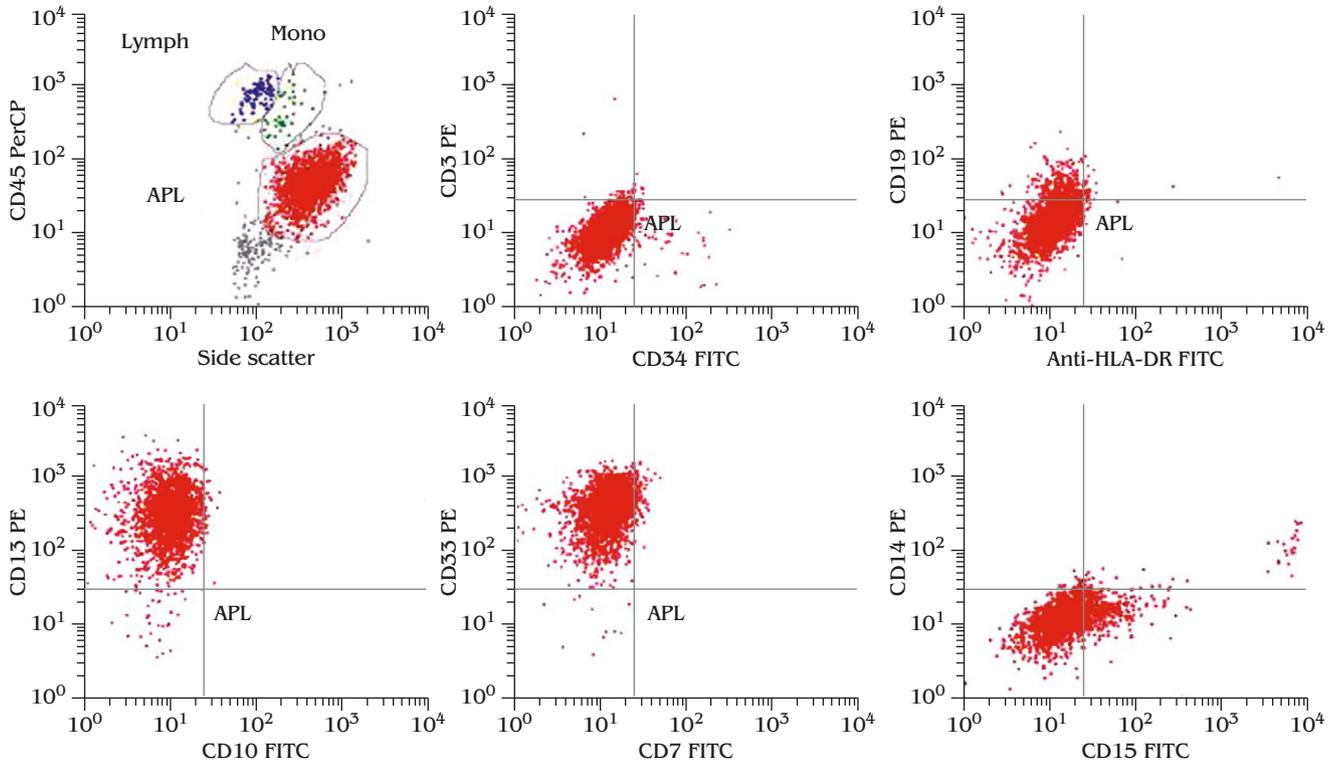
**Fig. 3 — Case 1: A woman aged 40 years with AML. Upper left & middle: Sixteen days after induction chemotherapy with I3A7, residual blasts are at 14.0% and express CD33 and CD19. Upper right: Nineteen days after re-induction chemotherapy with N2A5, no residual leukemia is seen based on CD19 gating. Lower left: Relapse of leukemia with MRD of 0.3% after high-dose Ara-C (HD-AC) consolidation chemotherapy. There is co-expression of CD33 and CD19. Lower right: No MRD is seen after allogeneic peripheral blood stem cell transplantation (allo-PBSCT).**

induction chemotherapy. Although morphological remission was achieved, minimal residual disease with 1.2% leukemic blasts was clearly demonstrated on the flow cytometry. The key clues were a small population of blasts which had aberrantly lost HLA-DR and CD34 expression. Again, both HLA-DR and CD34 are universally expressed by normally recovering myeloblasts or lymphoblasts. In addition, the same population of leukemic cells revealed high CD56 expression, as at diagnosis. All residual leukemia was gone after re-induction chemotherapy.

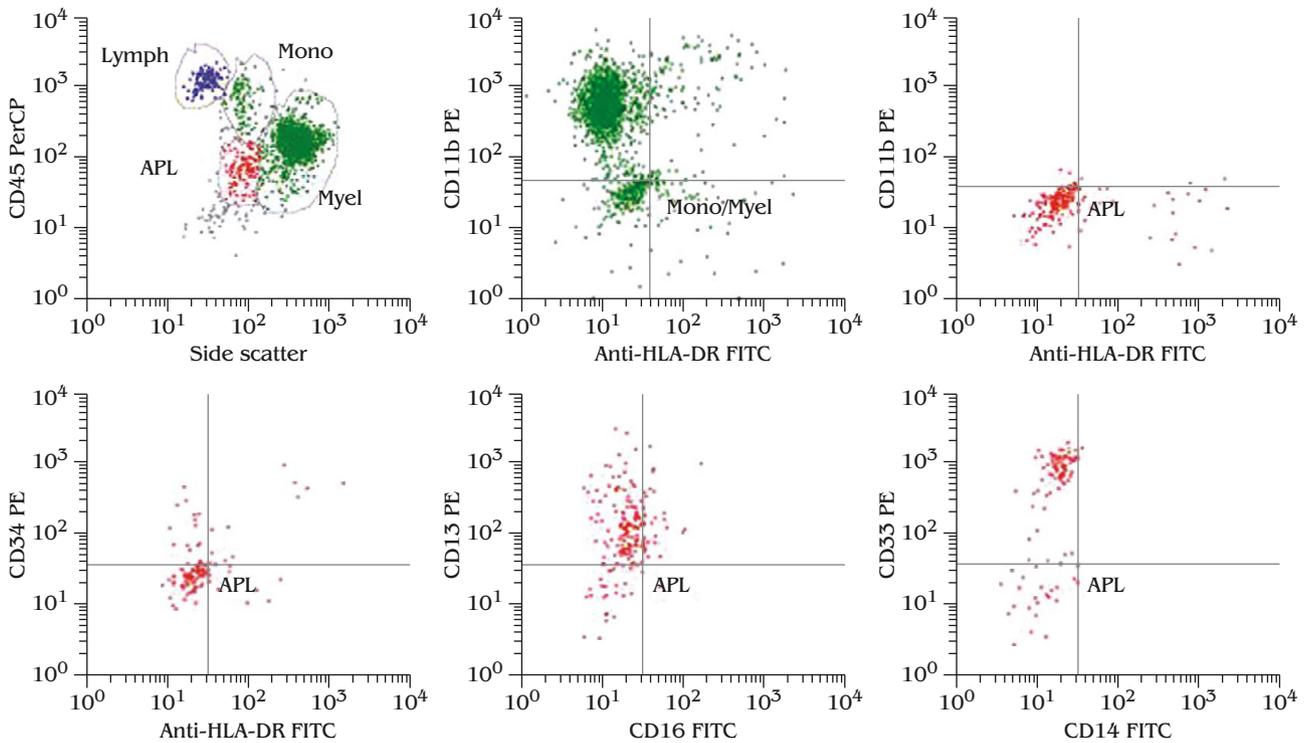
### **3.2. Multidimensional flow cytometric analysis as an aid in initial differential diagnosis of cases which are difficult to evaluate by conventional morphology**

Case 4 was a boy aged 15 years with a presentation of fever and chills. Extreme leukocytosis was found

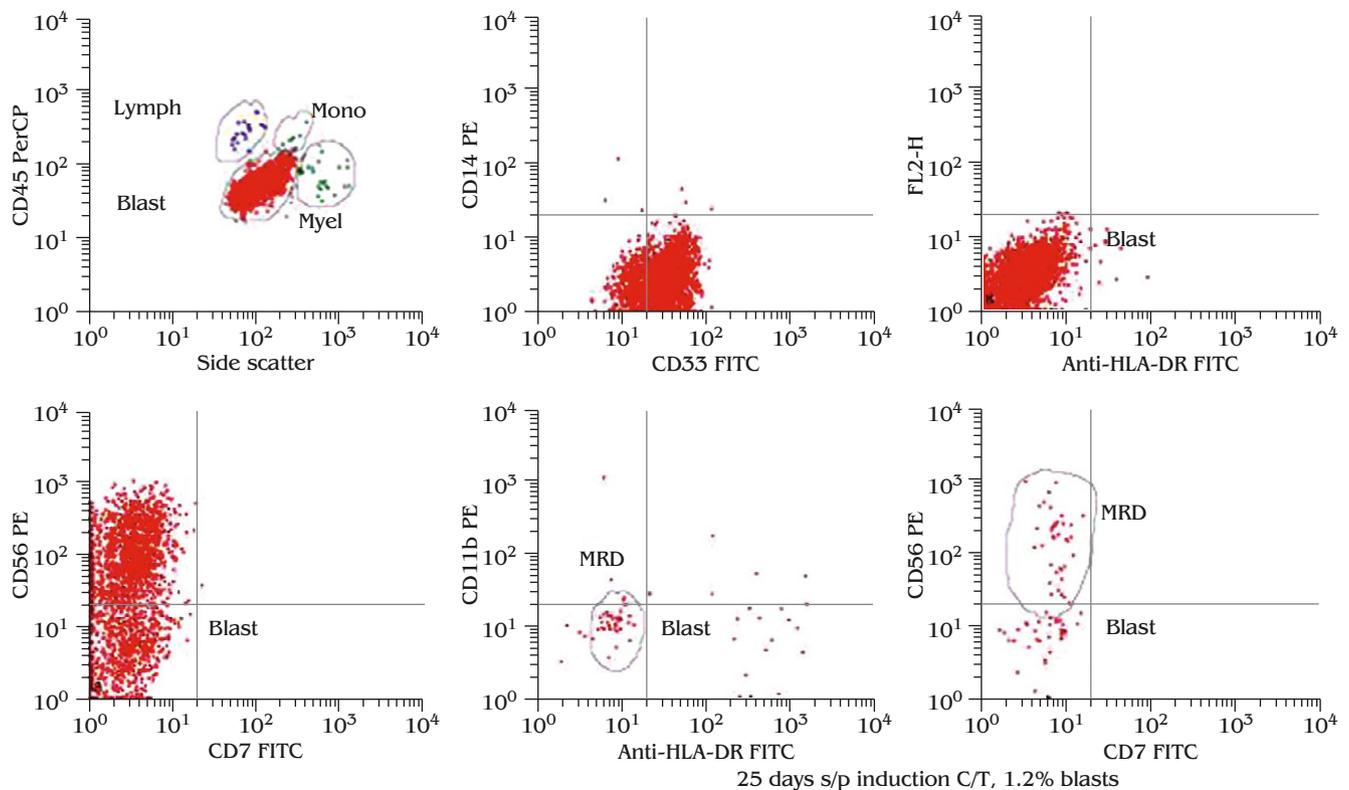
in the peripheral blood circulation. Bone marrow examination revealed leukemic marrow. The blasts were intermediate in size with multiple intra-cytoplasmic vacuolizations, as shown in Fig. 7. These findings raised concerns of Burkitt's leukemia, a mature B-cell leukemia. Some specific chemotherapeutic regimens should be chosen for Burkitt's leukemia rather than for non-Burkitt's leukemia. As shown in Fig. 8, the intermediate flow cytometry revealed that the leukemic blasts showed markedly decreased CD45 expression, a striking feature of acute lymphoblastic leukemia. The enhanced CD19 expression indicated its B-cell origin. The lack of clonal immunoglobulin light chain expression on the panels of CD19 vs. kappa and CD19 vs. lambda definitely excluded the possibility of mature B-cell leukemia or lymphoma. High CD10 expression was strong evidence of precursor B-cell ALL. Chromosome study showed t(1;19), which corresponds to cytogenetics for non-Burkitt's lymphoma. Flow cytometric analysis after induction chemotherapy showed no residual leukemic cells present on the lower CD45



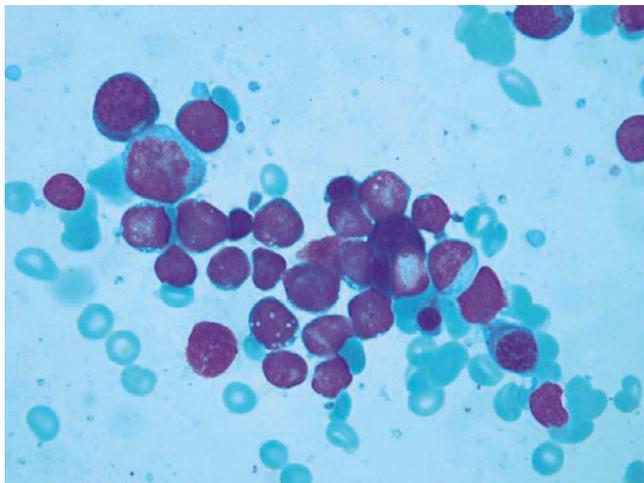
**Fig. 4 – Case 2: A woman aged 45 years with acute promyelocytic leukemia (APL). Diagnostic flow cytometry shows the characteristics of autofluorescence, bright CD13+, CD33+CD34-, and HLA-DR-. CD15 is aberrantly lost on the dysregulated promyelocytes.**



**Fig. 5 – Case 2: A woman aged 45 years with APL, which has relapsed. The relapsed APL shows less granularity on the CD45 vs. side scatter panel. The maturing monocytes and myeloid elements reveal orderly differentiation. The 4.2% of relapsed APL shows characteristic HLA-DR- and CD34-, but high CD13+ and CD33+.**



**Fig. 6 — Case 3: A woman aged 54 years with AML. Diagnostic flow cytometry shows 94.0% blasts, which reveal low CD45, high CD33+, but a lack of HLA-DR, and aberrant expression of CD56. Lower middle & right: Twenty-five days after induction chemotherapy, minimal residual disease (MRD) remains at 1.2% of leukemic blasts with characteristic HLA-DR- and CD56+.**



**Fig. 7 — Case 4: A boy aged 15 years with ALL. Diagnostic bone marrow tests show that the leukemic blasts are intermediate in size with multiple intra-cytoplasmic vacuolizations, findings with which it is difficult to differentiate between Burkitt's and non-Burkitt's leukemia.**

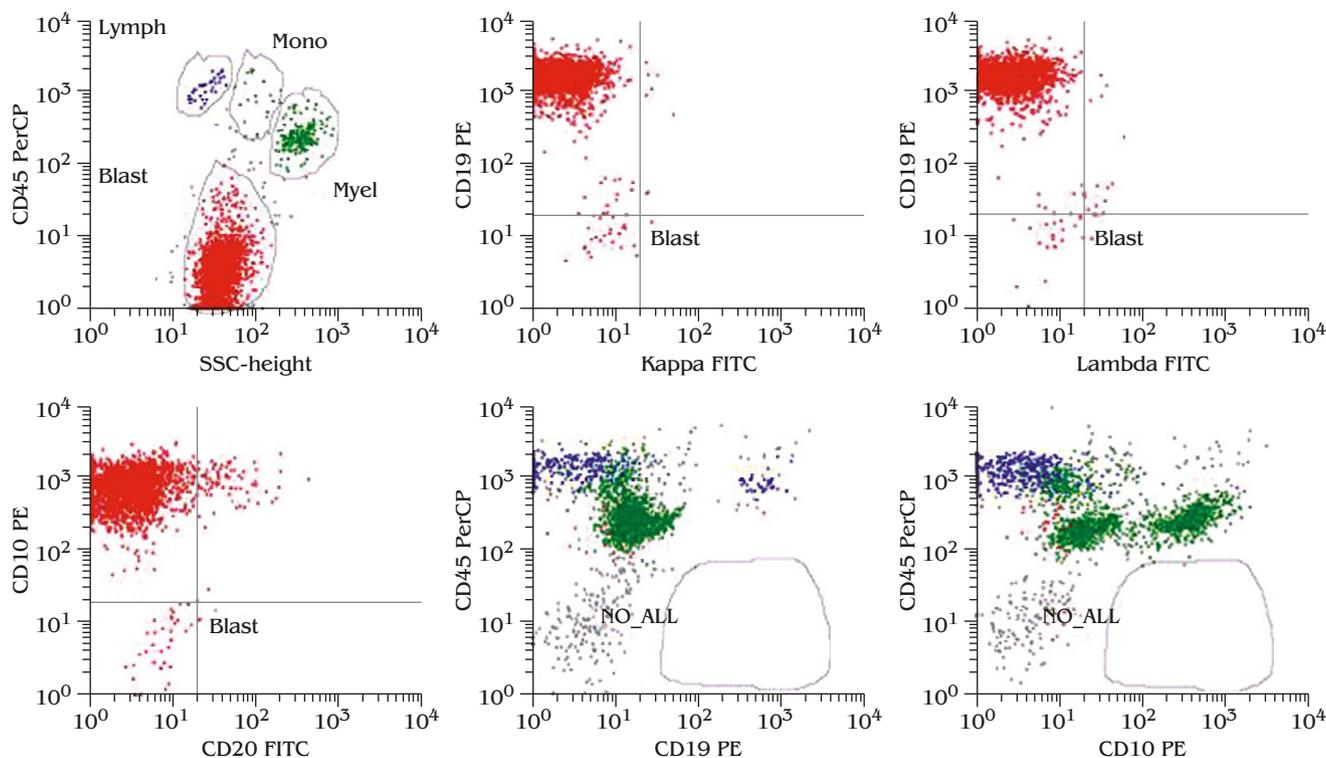
*vs.* higher CD19 or CD10 region, an area that is characteristic of the presence of precursor B-ALL.

Case 5 was a woman aged 53 years with pancytopenia of an unknown cause. Bone marrow morphology

showed hypocellular marrow. Only a few subtle changes were seen on the three blood cell lineages, which made the differential diagnosis between aplastic anemia and myelodysplastic syndrome difficult. Flow cytometry revealed that the number of blasts was within the normal range at 1.4%. As shown in Fig. 9, the four populations of lymphocytes, monocytes, maturing myeloid cells and blasts were in the proper positions on the side scatter *vs.* forward scatter and the CD45 *vs.* side scatter panels. However, a small but significant portion of CD56 is aberrantly expressed on blasts, monocytes and maturing myeloid elements. This condition is consistent with aberrant antigen expression of myelodysplasia. A cytogenetics study showed complex chromosome changes that confirmed the flow cytometric diagnosis of myelodysplastic syndrome.

### 3.3. Help in the staging of lymphoproliferative disorders

Case 6 was a man aged 66 years with MALToma of the left lacrimal gland. Endoscopic examination of the stomach revealed no gastric MALToma. A whole-body computed tomography scan showed no involvement



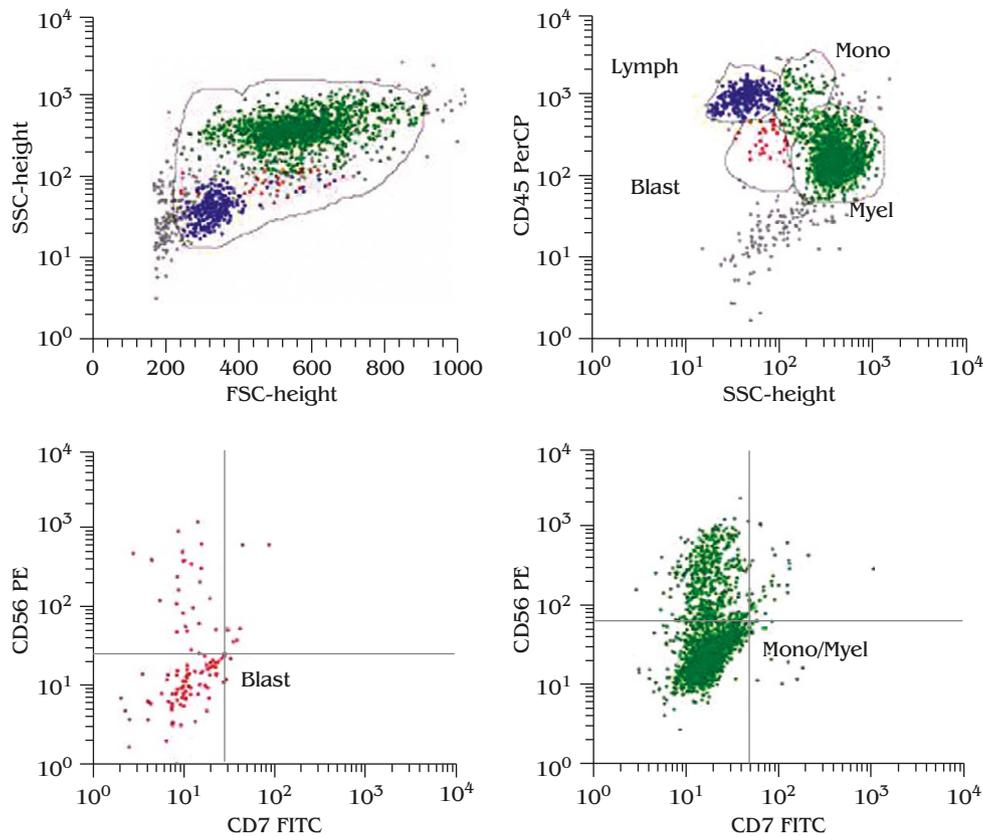
**Fig. 8 — Case 4: A boy aged 15 years with ALL. Diagnostic flow cytometry shows the blasts have decreased CD45, high CD10+, and CD19+ without surface kappa or lambda expression, featured diagnostic of precursor B-cell ALL. Lower middle & right: No MRD is detected after induction chemotherapy based on gating on the CD45 vs. CD19 & CD10 panels.**

of lymphoma in the visceral organs or other lymphoid structures. A bone marrow examination was performed for morphological survey. The marrow aspirate did not show any abnormal aggregates of lymphocytes. The pathology of the biopsy sample revealed a few suspicious areas that may have had lymphoma involvement, as shown in Fig. 10A. Careful examination of the flow cytometric analysis of the bone marrow aspirate revealed a small population of lymphocytes at 7.0% that showed low CD45 expression on the CD45 vs. side scatter panel. As shown in Fig. 11, these cells were smaller and less granular than normal T-cells. These lymphocytes revealed high CD19 expression with clonal surface kappa expression. CD5 was negative, which excluded chronic lymphocytic leukemia and mantle cell lymphoma. CD10 was negative, which excluded follicular center B-cell lymphoma. They were small in size, which excluded large B-cell lymphoma. Other markers showed CD20+, HLA-DR+ and subpopulations of CD23+, CD22+, and FMC-7+. All flow cytometric data were consistent with marginal zone origin of B-cell lymphoma, which was in accordance with MALToma involvement in the bone marrow. A cytochemical L-26 (CD20) stain of the marrow biopsy sample also confirmed the involvement of B-cell lymphoma, as shown in Fig. 10B.

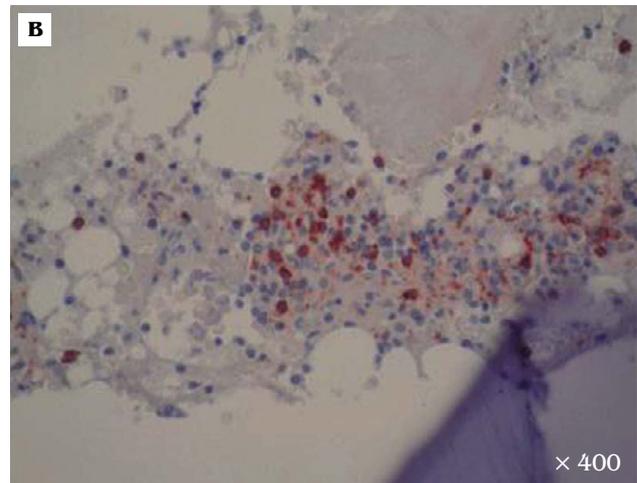
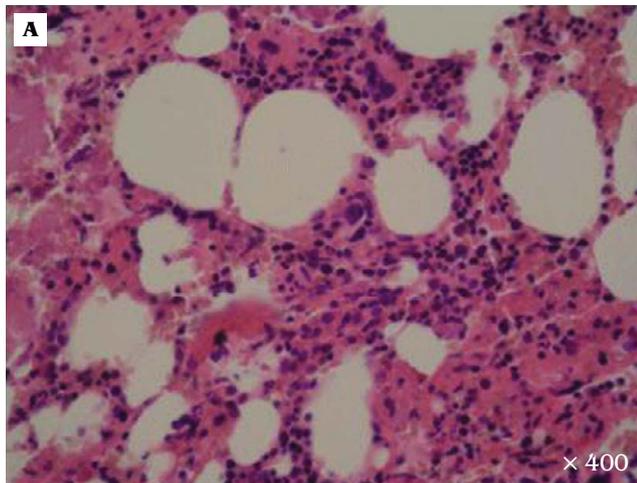
#### 4. Discussion

Our current study shows that multidimensional flow cytometry is a very sensitive and specific tool in the diagnosis of hematological disorders and for the monitoring of minimal residual disease after treatment. It aids the selection of a specific therapeutic strategy for individual patients. To apply this highly sophisticated flow cytometry method, one should first be aware of the normal maturation pathways of blood cells demonstrated on flow cytometric figures. The normal myeloid and lymphoid cellular differentiation in bone marrow has been extensively studied using flow cytometry (1–3). Second, it is necessary to carefully compare the deviations in various hematological disorders from the normal cellular differentiating pathways on flow cytometry (4–7). Thus the most important figures shown by multidimensional flow cytometry are to characterize the different “patterns” between normal and abnormal cells, but not just to quantify the expression of one specific surface antigen. After applying these core principles of immunophenotyping, various hematological disorders can be illustrated clearly.

Using multidimensional flow cytometry for these six unique cases, we were able to demonstrate our



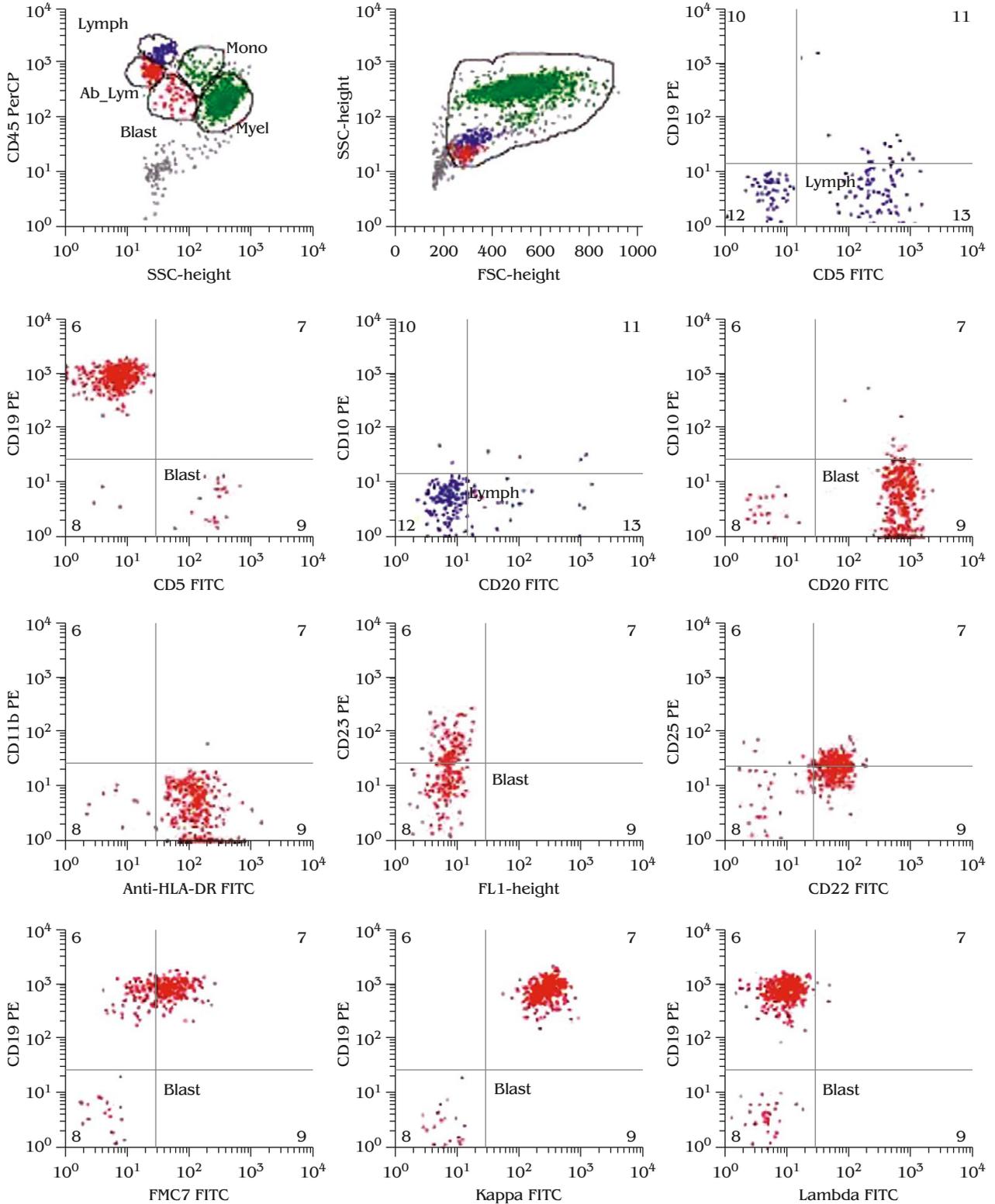
**Fig. 9 — Case 5: A woman aged 53 years with myelodysplastic syndrome. Diagnostic flow cytometry shows aberrant CD56 expression on blasts, monocytes and maturing myeloid elements.**



**Fig. 10 — Case 6: A man aged 66 years with MALToma of the left lacrimal gland. (A) A bone marrow biopsy with hematoxylin-eosin staining shows a few suspicious small lymphoid aggregates. (B) L-26 staining (brown) confirms interstitial involvement of B-cell lymphoma.**

three goals, as follows: (1) detection of minimal residual disease after treatment; (2) aid in initial differential diagnosis of cases which are difficult to evaluate by conventional morphology; (3) help in the staging of lymphoproliferative disorders.

Cases 1–3 are characteristic examples of the detection of MRD in patients with AML. As described in previous studies, there are four types of aberrant antigen expression on myeloid leukemic cells: (1) expression of non-myeloid antigens; (2) asynchronous



**Fig. 11 — Case 6: A man aged 66 years with MALToma. Diagnostic flow cytometry shows 7.0% abnormal lymphocytes (red), which show low CD45 expression, decreases in size and granularity, enhanced CD19+, surface kappa+, lambda-, CD5-, CD10-, CD20+, HLA-DR+, and subpopulation of CD23+, CD22+, and FMC-7+, feature consistent with the marginal zone origin of B-cell lymphoma.**

expression of myeloid associated antigens; (3) over-expression of myeloid associated antigens; and (4) absence of expression of myeloid associated antigens (4,18). Aberrant expression of non-myeloid antigens in AML is the so-called "lineage infidelity". AML expressing both lymphoid (CD2, 5, 7, 10, 19, and 25) or natural killer (NK) antigens (CD56) and myeloid (CD13, 14, 15, and 33) antigens allows the detection of unusual phenotypes in the presence of normal hematopoietic elements (4,18,19). As shown in Cases 1 and 3, aberrant CD19 or CD56 expression on leukemic cells are key clues in monitoring MRD. Overexpression of myeloid associated antigens is a common manifestation of AML. For example, overexpression of CD34, HLA-DR, CD13 and CD33 was shown in Case 1 and enhanced CD13 and CD33 expression was demonstrated in Case 3. On the other hand, the absence of normal myeloid associated antigens may occur occasionally. CD34 and HLA-DR were aberrantly lost in Case 3. As mentioned above, the most important concept in detecting MRD is not just to rely on specific antigens but to view all antigenic abnormalities as a whole dysregulated pattern. All three cases with AML revealed distinct patterns of flow cytometric abnormalities. For example, Case 2 had acute promyelocytic leukemia. The diagnostic flow cytometric abnormalities showed a characteristic pattern of autofluorescence, hypergranularity, enhanced CD13, and CD33 and a lack of CD34 and HLA-DR, which was accompanied by aberrant loss of CD15 expression on the leukemic cells. This pattern of abnormality is so distinct that it allowed for the detection of a very rare population of MRD during subsequent follow-up. The potential benefit of detecting MRD during continuing chemotherapy is that it predicts the likelihood of subsequent relapse. It may therefore facilitate dose intensification and strategies such as stem cell transplantation, as shown in our patients.

Flow cytometry can also lead to a very rapid and specific diagnosis in cases that are difficult to evaluate with conventional morphology. Case 4 is such an example. Acute lymphoblastic leukemia was readily observed based on morphological findings. However, the presence of multiple intra-cytoplasmic vacuolizations raised concerns of Burkitt's leukemia. Unlike a time-consuming cytogenetics study, multidimensional flow cytometric analysis immediately confirmed this was precursor B-cell ALL and excluded the possibility of Burkitt's type leukemia because no clonal immunoglobulin light chain expression was found on the surface of the leukemic cells. This rapid and accurate diagnosis helped the clinical physician initiate appropriate treatment planning quickly. Again, after considering the abnormal antigen expression as a complete pattern, MRD was undetectable in this patient after induction chemotherapy.

Myelodysplastic syndromes are another complex and heterogeneous group of hematopoietic stem cell disorders that are sometimes difficult to evaluate morphologically. Flow cytometric analysis is especially useful in the evaluation of myelodysplasia. With the understanding of normal antigenic expression during hematopoietic development, the dysregulation of hematopoiesis in MDS could be characterized based on deviations from the normal patterns of antigenic expression demonstrated on multidimensional flow cytometry (3,20-23). Immunophenotypic abnormalities can be observed on myeloblasts and on maturing cells. Case 5 is an example in which MDS was recognized by the aberrant CD56 expression on blasts, monocytes and maturing myeloid elements. Wells et al also established a new flow cytometric scoring system in the analysis of myelodysplastic syndrome correlated with the results of the International Prognostic Scoring System (IPSS) and can predict outcome significantly (20).

The staging work-up for lymphoproliferative disorders is very important because treatment planning may differ for different stages of disease. Bone marrow examination is essential to define stage IV disease, which is conventionally evaluated by morphological interpretation. However, it may miss very rare instances of bone marrow involvement. In this situation, multidimensional flow cytometry can provide a rapid, sensitive and specific evaluation, as shown in Case 6. Although as few as 7.0% of lymphoma cells are involved in the bone marrow, flow cytometric analysis clearly revealed a group of clonal marginal zone B-cell lymphoma, which was difficult to view on conventional morphological examination.

In this article, with the demonstration of six unique cases, we showed that multidimensional flow cytometry is a powerful tool in the sensitivity and specificity in diagnosis, and for monitoring and characterization of different hematological disorders. By using the concept of a "pattern" for flow cytometry and recognizing any differences between normal and aberrant flow cytometric patterns, even very rare populations of abnormalities can be identified clearly. This may help to optimize subsequent treatment strategies, and will benefit the individual patient.

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