

Qualitative Flow Cytometric Analysis of Malaysian Myelodysplastic Syndromes (MDS) Patients

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SUMMARY

Myelodysplastic syndromes (MDS) are a group of haematological malignancies categorized by ineffective hematopoiesis that result in dysplasia. Although morphological diagnosis is a traditional and standard technique that is used for the diagnosis of MDS, the heterogeneous blood and bone marrow characteristics of MDS patients can potentially obscure the right diagnosis. Thus, we have utilized flow cytometric immunophenotyping as a supportive mechanism to obtain a more accurate and faster method for detection of abnormal markers in MDS. Flow cytometry was used for analyzing bone marrow samples from newly diagnosed MDS patients to investigate the abnormal antigen expression patterns in granulocytic, monocytic, erythroid, lymphoid lineages and myeloid precursors. The results were compared with those obtained from cases that had Idiopathic Thrombocytopenic Purpura (ITP) as a control. The most common abnormality found in the granulocytic lineage was the decrease of CD10. Low expressions of CD13 were the most frequent abnormality in the monocytic lineage. The erythroid lineage was found to have low expression of CD235A+/CD71+, reduce of CD71 and decreased CD235a. In conclusion, this method is useful for confirming cases in which it is difficult to make a diagnosis by morphology.

KEY WORDS:

Myelodysplastic Syndromes, Flow cytometry, Immunophenotyping

INTRODUCTION

MDS is one of the main bone marrow (BM) disorders; occurring particularly in older people with incidences of approximately 3.5–4 per 100,000 person year¹. MDS encompasses a heterogeneous cluster of clonal hematopoietic malignancies which show ineffective hematopoiesis with an increased danger of change to acute myeloid leukaemia (AML). Development of MDS starts with abnormal clones which are characterized by morphological dysplasia and impaired differentiations. Ineffective haematopoiesis will lead to the cytopenic peripheral blood that forms all three blood cell lineages namely erythroid, granulocytic, and megakaryocytic².

The gold standard for diagnosis of MDS is morphological examination of blood film and BM. Morphological findings in MDS can differ highly and be complicated due to limitations in morphological analysis. For instance, different interpretations of BM features³ which are result from

suboptimal aspirate smears due to hemodilution, air-dried or badly stained smears, and/or an insufficient biopsy specimen⁴ prevent accurate diagnosis. In recent times, numerous approaches have been used to analyze pathological characteristics of BM in MDS. Analysis of the cell lineages and expression pattern of antigens will lead to discovery of abnormal co-expression and maturation asynchrony. This information could provide a characteristic model of the disease which will assist in accurate diagnosis of the malignancy and support the morphological analysis. In line with this, identification of MDS phenotype using flow cytometric immunophenotyping has greatly improved the diagnostic process, mainly as a consequence of increase of available monoclonal antibodies. Undoubtedly, immunophenotyping has become the foundation of many haematological related diagnoses in conjunction with supportive morphological and cytochemical analysis. The last decades have seen progress in flow cytometric immunophenotyping which has enhanced our capability to recognize various phenotypic abnormalities⁵. Although several studies have previously investigated the antigenic expression pattern in MDS patients, this is the first report of Ag expression pattern in the Malaysian population. Our flow cytometric immunophenotyping study in MDS patients depicts a common abnormality in granulocytic, monocytic and erythroid.

MATERIALS AND METHODS

We examined 30 patients with newly diagnosed MDS (as diagnosed by morphological assessment of the initial PB and BM sample according to the FAB criteria independently by two haematopathologist, without knowledge of the flow cytometric analysis, cytogenetic and FISH findings. For each individual case, routine haematoxylin and eosin (H&E) histologic section and well-prepared Wright-Giemsa-stained smears were evaluated⁶ whereby the samples were collected from February 2009 to November 2010 at Hospital Kuala Lumpur (HKL) after written informed consent from patients and ethical clearance by the Faculty of Medicine and Health Sciences, University Putra Malaysia (no: UPM/FPSK/PADS/T7-MJKEtikaPer/F01(LECT(JPAT)_MAC(10)02) and Hospital KL (HKL) (no: HKL /PAT/180/1). Patients group included: (4 patients) Refractory Anaemia (RA), (11 patients) Refractory Anaemia with Ringed Sideroblasts (RARS) and (15 patients) Refractory Anaemia with Excess Blasts (RAEB). There were 20 males and 10 females, median age 52 years (range 34 to 71). There were 9 Malays and 21 Chinese. The results obtained were compared with the BM samples of cases affected by

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disorders with no BM involvement, ITP⁷. 30 controls were used for statistical comparisons. The median age of this control group was 40 years (range 21 to 54) with 13 male and 17 female. There were 12 Malays and 18 Chinese. BM samples (approximately 500×10^3 cells/ test) were stained as per manufacturer instruction (BD Bioscience). The pattern recognition approach that we adopted in this study was the method suggested by van Lochem *et al* (2004)⁸. The monoclonal antibody-panel used in this study is listed in table 1.

The method for labelling the cells was carried out according to Li *et al.*, with some modifications according to the recommendation of the manufacturer to optimise the technique⁹. In brief, bone marrow samples were incubated for 15 min in the existence of 3 μ l of FITC, PE, PerCP-Cy5.5 and 2 μ l of APC conjugated antibodies at room temperature. Following Lysing of non-nucleated red cells with FACS Lysing solution (Becton Dickinson Biosciences, San Jose, CA, USA)-the cells were centrifuged (5 min at 300g) and resuspended in 500 μ l of FACS Flow (Becton Dickinson Biosciences, San Jose, CA, USA) for the flow cytometer analysis. Cell analysis was performed in FACSCanto (BD Biosciences, San Jose, CA, USA) immediately. For each combination of antibodies, a minimum total of 10,000 events were recorded. The data were analysed with FACSDiva Software V.6.1.1 (BD Biosciences, San Jose, CA, USA). Isotype controls for FITC Mouse IgG1, PE Mouse IgG1, PerCP/Cy5.5 Mouse IgG1 and APC Mouse IgG1 were used for non specific background staining.

Gating on CD45/SSC (Side Scatter) plot was used for choosing the population of interest. CD45 together with right angle light scatter was used to effectively identify the lymphocytes, monocytes, maturing myeloid cells and myeloblasts in marrow aspirates and serves as a reference for gating the different populations which are present in every tube¹⁰. The different subsets of cells that were recognised by this method were blast cells (CD45low/SSClow), lymphocytes (CD45high/SSClow), monocytes (CD45high/SSCintermediate), granulocytes (CD45high SSChigh)¹¹. The nucleated red cells were investigated as a (CD45low to negative/SSClow) population⁶.

A descriptive analysis was done for all variables studied. The student's t-test was used for statistical analysis of differences among groups. The Mann-Whitney U-test was employed for nonparametric variables. Statistical significance was distinct by a p value of 0.05 or less for all statistical tests. Actually, the assessment of antigenic low expression or aberrancy was done by comparing mean gated population fluorescence with that of control.

RESULTS

In flow cytometric analysis of granulocytic lineage 13 (43.33%) MDS cases showed decreased SSC (hypo granularity) in the granulocytic gate ($p=0.005$). Generally, the percentage of HLA-DR positive granulocytes was higher ($p=0.029$) in patients with MDS than controls as 6 (20.00%) cases showed presence of HLA-DR on granulocytes. Six cases (20.00%) showed a reduced expression of CD11b; however the difference was not statistically significant ($p=0.210$). Presence of CD34 was seen in 4 (13.33%) cases. Additionally, in mature

granulocytes low expression of CD10 was seen in 14 (46.66%) MDS cases. The percentage of the CD10⁺ mature granulocytes was much lower in MDS patients than in controls. Furthermore, the proportion of CD13 and CD33 were significantly lower in MDS cases ($p=0.000$) (figure 1).

Analysis of the monocytic lineage, showed 13 (3.33%) cases with minimal expressions of CD13 ($p=0.005$) compared to controls. Absence of CD33 was seen in 3 (10%) of MDS cases. Eight (26.66%) MDS patients showed decrease of CD14 expression ($p=0.337$) and 6 (20%) MDS cases showed CD34⁺ monocyte (CD14⁺/CD34⁺) ($p=0.037$). The percentage of HLA-DR⁺/CD11b⁺ monocytes was higher in MDS. Seven (23.33%) cases showed HLA-DR⁺/CD11b⁺ monocytes ($p=0.024$) (table 2).

Flow cytometric analysis of myeloid precursors showed enhanced HLA-DR⁺/CD11b⁺ in 13 (43.33%) MDS cases as compared to control. Eight (26.66%) MDS patients demonstrated lower percentage of CD13 and 4 cases (13.33%) with higher CD11b percentages.

Analysis of erythroid lineage by flow cytometry showed decrease of CD71 expression in 16 (53.33%) cases ($p=0.000$); in addition, there were 12 (40.00%) cases that showed the low expression of CD235a ($p=0.000$). Presence of CD235a⁺/CD71⁺ was lower in 18 (60%) of MDS cases (figure 2). There were no differences in antigen presentation on lymphoid lineages between MDS and controls.

Statistical significance was distinct by a p value of 0.05 for all statistical tests.

DISCUSSION

MDSs are a mixed cluster of myeloid neoplasia categorized by an aberrant maturation and differentiation of myeloid cells with a high danger of transformation to AML. Haematological and morphological findings can be unreliable and complicated in diagnosis of MDS cases¹⁰. The standard criteria for the MDS diagnosis are rely upon subjective morphological interpretation and demonstration of clonal cytogenetic abnormalities¹²⁻¹⁵. Recent studies have showed that flow cytometric immunophenotyping is less subjective and more reproducible in comparison to morphological assessment¹⁶. In recent times, a number of attempts have done to examine the immunophenotypic characteristics of BM in MDS^{11,17-26}.

Flow cytometric immunophenotyping of bone marrow aspirates resulted in detection of myeloid, erythroid, and megakaryocytic aberrancies in 30 cases with MDS (table 2). Previous studies showed that erythroid cells aberrancies such as low levels of CD71, low expression of CD235a and lower proportions of CD235a⁺/CD71⁺ in MDS patients. Importantly, the main immunophenotypic aberration noted was the extremely low CD71 expression on CD235a positive erythroid progenitors^{6,23}. In addition to current literature, our study also showed the low level of CD71 expression and low percentages of CD235a. It is worth noting that the low expression of CD235a⁺/CD71⁺ was the most frequent abnormality found in our MDS cases.

Table I: Monoclonal antibodies for flow cytometry analysis

Hematopoietic Compartment	Antibody Combination
Erythroid lineage	CD71/CD235a/CD45/CD10
Granulocytic lineage	HLA-DR/CD13/CD45/CD11b
Monocytic lineage	CD14/CD33/CD45/CD34
Lymphoid lineage	CD19/CD20/CD45/CD10

Table II: Immunophenotype abnormalities in haematopoietic cells of MDS cases

Flow Cytometric Abnormality	MDS % (n= 30)	P values
Granulocytes (no) (%)		
Abnormal Granularity	43.33 (13)	0.005
Presence of HLA-DR	20 (6)	0.029
Low expression of CD11b	20 (6)	0.210
Presence of CD34	13.33 (4)	0.218
Low expression of CD10	46.66 (14)	0.000
Monocytes:		
Abnormal CD11b or HLA-DR expression	23.33 (7)	0.024
Low expression of CD13	43.33 (13)	0.005
Low expression of CD33	10 (3)	0.099
Low expression of CD14	20 (6)	0.337
Presence of CD14/CD34	20 (6)	0.037
Erythroid Lineage:		
Low expression of CD71	53.33 (16)	0.000
Low expression of CD235a	40 (12)	0.000
Abnormal level of CD71 vs. CD235a	60 (18)	0.000

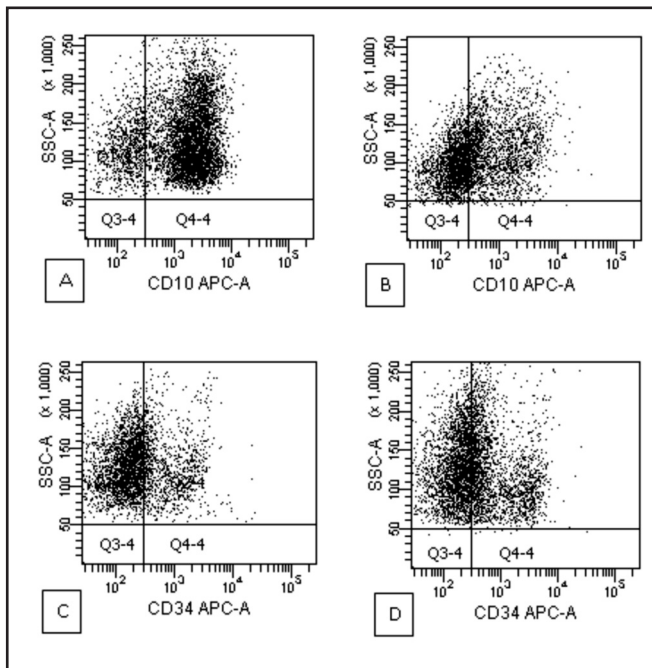


Fig. 1: Flow cytometric analysis of granulocytic population in MDS and control cases: CD10 expression on granulocytic population in control (A) and in MDS cases (B). Reduced expression of CD10 on granulocyte populations can be seen in MDS patients (p=0.000). Proportion of CD34+ granulocytes in control (C) and in MDS cases (D). Expression of CD34+ granulocytes was higher (p=0.218) in MDS cases as compared to control (Normally CD34 is appeared on the surface of immature cells but in MDS can be seen on mature cells).

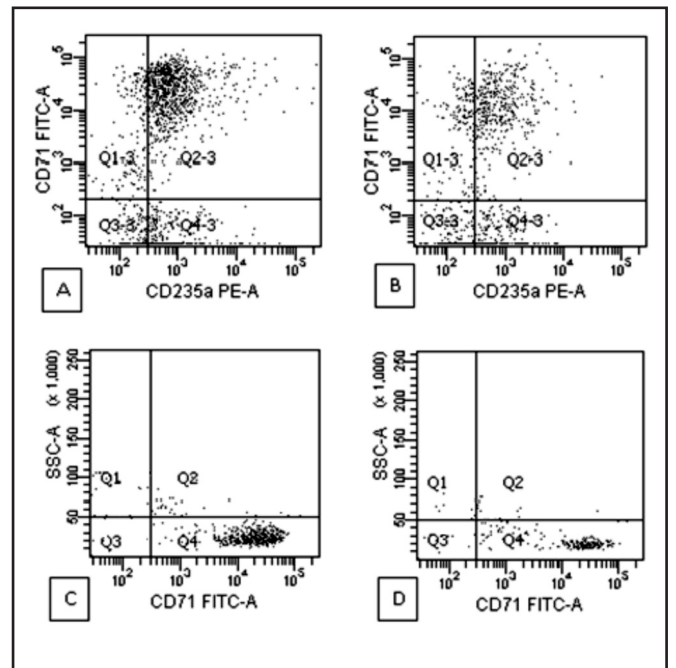


Fig. 2: Flow cytometric analysis of erythroid population in MDS and control cases: CD71 expression on CD235a+ cells in control (A) and MDS cases (B). Lower proportion of CD235a+ CD71+ nucleated red cells can be seen in MDS patients as compared to non-MDS (p=0.000). Proportion of CD71 on nucleated red cells in control (C) and in MDS cases (D). Lower expression of CD71 can be observed in MDS cases as compared to control (p=0.000).

Veltroni *et al.* reported that 78% of MDS patients had abnormalities either in myeloid or monocytic compartments²⁷. Abnormal granularity was one of the aberrancies observed in granulocytic lineages¹⁶ that was detected in about half of the cases in our study. Kussick and Stachurski observed a reduced CD33 and CD13 expression on the surface of all granulocytes and monocytes^{16,28}. In line with this, the low expression of CD13 and CD33 on granulocytes and monocytes was also detected in our study. However the decrease of CD33 and CD13 between the two groups of monocytes was not significantly different (table 2). In addition, a lower proportion of CD10⁺ granulocytes seen in our study is also strongly supported by previous studies^{5,11,16,24}. Decreased expression of CD11b on granulocytes^{5, 30} and monocytes²⁴ was another characteristic of MDS cases. Similarly, we found the decreased expression of CD11b on granulocytes as well yet the difference between two groups was not statistically significant.

In our study, as in previous literature, the low expression of CD14 on monocytes was observed^{16,24}. However the main abnormality of the monocytic lineage was reduce of CD13 expression. The aberrant presence of homogeneous HLA-DR and CD11b on the myeloid blasts is another abnormality found in MDS²⁸. The increase of mature CD markers such as CD11b was the similar to the survey by Kussick²⁸. The most common abnormality in myeloid precursors was the asynchronous expression of HLA-DR/CD11b. There were no differences in antigen presence on lymphoid lineages between MDS and controls.

CONCLUSION

In conclusion, although the combination of morphology and cytogenetic methods is sufficient to render a diagnosis in most MDS cases, yet it could be complicated when the cell morphology is indeterminate. In cases where morphology and cytogenetic analysis are not straight forward, flow cytometric immunophenotyping could be used as tool in establishing the diagnosis of MDS.

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